Kishan Gopal Ramawat
Jean-Michel Mérillon
Editors

Polysaccharides

Bioactivity and Biotechnology

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With 488 Figures and 154 Tables

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Preface

We are pleased to present a three volume treatise on "Polysaccharides: Bioactivity and Biotechnology." Polysaccharides are present throughout the plant kingdom and involved in various biological processes including defense for the plant and many useful applications for human welfare.

This work on polysaccharides is a reference work providing state-of-the-art knowledge composed by highly renowned scientists in their field. The book aims to present comprehensive, up-to-date, and well-established information about complex and astonishing structures, properties, and biotechnological applications of polysaccharides and their upcoming industrial applications. The book will be a valuable source on polysaccharides to those working in the field of industrial production and biotechnology development. Polysaccharides are a class of important biomacromolecules, with complex structures and various functional activities. Polysaccharides are extremely common and wide-spread in nature, with cellulose as the most common and abundant organic compound on the planet. It is believed that the second most common polysaccharide in the world after cellulose is chitin. Chitin is to shellfish what cellulose is to trees. This is a very timely compilation of recent developments about polysaccharides as no book is available in this format.

These polysaccharides find many uses: cf. for example the significant role of cellulose in the paper and textile industries, as a feedstock for the production of rayon (via the viscose process), cellulose acetate, celluloid, and nitrocellulose; as surgical threads (chitin), as sources of energy, dietary fibers, as a blood flow adjuvant; cosmetics, emulsion stabilizer, film former, binder, viscosity increasing agent, and skin conditioning agent; direct food additives in gums and chewing gum bases; and as vaccines. Many polysaccharides have been developed into useful products, including xanthan gum, dextran, welan gum, gellan gum, diutan gum, and pullulan.

This comprehensive and thoroughly up-to-date reference book presents the sources, identification, methods of analysis, biosynthesis, biotechnology, and applications of important polysaccharides likes starches, cellulose, chitin, gum, and microbial polysaccharides. Polysaccharides have received much attention recently because of the emergence of different biological activities, such as immunomodulatory, antibacterial, antimutagenic, radioprotective, anti-oxidative, anti-ulcer, antidepressant, antisepticemic, anticancer, and anti-inflammatory activities.

New industrial applications in pharmaceutical and medical sciences are being developed. Due to these properties and benefits, a vast body of data is being generated.

The various polysaccharides constitute important classes of biological polymers, which are dealt with in these volumes. The handbook is divided in several sections to encompass the entire spectrum of developments in this field: Part I. Biology and Biotechnology: occurrence, structure, distribution and biotechnology (bacterial polysaccharides, fungal polysaccharides, polysaccharides from lower plants, polysaccharides from higher plants including gums), biosynthesis of polysaccharides, applications in biotechnology, production by fermentation, biopolymers from marine prokaryotes, carrageenan, dextrans, polysaccharide nanocrystals, and modified gums in drug delivery. Part II. Food: structural (pectins, cellulose, xylans, gum, gumexudates, glycosamines), storage (starch, glycogens, fructans, minor polysaccharides), marine polysaccharides (alginate and brown sea weeds, carrageenan, and red sea weeds; agar, agarose, chitosan, and chitin derivatives), bacterial and synthetic polysaccharides (dextrans, cyclodextrins, gellans, xanthan, pullulan, etc.), polysaccharides from edible plant parts, and as functional foods. Part III. Methods: Extraction, identification, quantification, assays, and emerging technologies. Part IV. Bioactivity: applications in medicine, immunostimulant, polysaccharides from algal, aloe, brown seaweeds, fructus, ganoderma, medicinal mushrooms, scleroderma, and their biological activity. Most of the chapters are well illustrated, many of them in color, for the benefit of the readers. A comprehensive index section will assist in navigating through this book when searching for specific topics.

Well recognized international specialists in their respective fields of research have contributed these chapters. This book will be useful to everybody working in the field of botany, phytochemistry, pharmacy, drug delivery, molecular biology, forestry, biotechnology, and industrial/food and medical products. This work is arranged in 73 well illustrated chapters.

Because of the voluminous work for the treatise, this project was spread over almost 2 years, from concept to print. We would like to acknowledge cooperation, patience, and support of our contributors who have put their serious efforts to ensure the high scientific quality of this book with up-to-date information.

This work could not have been completed without active support of Springer team who took pains in streamlining the production process. We are particularly indebted to Drs. Tobias Wassermann, Sylvia Blago, and Stephanie Hügler for their continuous support from the very inception of the project. Our final and most heartfelt thanks we want to give to our wives Marie-Claude Mérillon and Manju Ramawat, who have supported us during the entire period of this book project.

March 2015

J. M. Mérillon
K. G. Ramawat

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About the Editors

Professor Dr. Kishan G. Ramawat is Former Professor and Head of the Botany Department, M.L. Sukhadia University, Udaipur, India, and can look back on longstanding research experience. He received his Ph.D. in Plant Biotechnology in 1978 from the University of Jodhpur, India, and afterwards joined the university as a faculty member. In 1991 he moved to the M.L. Sukhadia University in Udaipur as Associate Professor and became Professor in 2001. He served as the Head of the Department of Botany (2001–2004, 2010–2012), was in charge of the Department of Biotechnology (2003–2004), was a member of the task force on medicinal and aromatic plants of the Department of Biotechnology, Government of India, New Delhi (2002–2005), and coordinated UGC-DRS and DST-FIST programs (2002–2012).

Professor Ramawat had done his postdoctoral studies at the University of Tours, France, from 1983 to 1985, and later returned to Tours as visiting professor (1991). He also visited the University of Bordeaux 2, France, several times as visiting professor (1995, 1999, 2003, 2006, 2010), and in 2005 Poland in an academic exchange program (2005). Through these visits in France, Prof. Ramawat and Prof. Mérillon established a strong connection, which has resulted in productive collaborations and several book and reference work publications.

Professor Ramawat has published more than 170 well-cited peer-reviewed papers and articles, and edited several books and reference works on topics such as the biotechnology of medicinal plants, secondary metabolites, bioactive molecules, herbal drugs, and many other topics. His research was funded by several funding agencies.

In his research group, Prof. Ramawat has supervised doctoral thesis of 25 students. He is an active member of several academic bodies, associations, and editorial boards of journals.

Professeur Dr. Jean-Michel Mérillon Directeur de l'EA 3675/Faculté de Pharmacie/Université de Bordeaux is the "Directeur de l'EA 3675 (Groupe d'Etude des Substances Végétales à Activité Biologique + Polyphénols Biotech)" at the Faculté de Pharmacie, Université de Bordeaux, Institut des Sciences de la Vigne et du Vin, in Villenave d'Ornon, France. He received his M.Pharma. (1979) and Ph.D. (1984) from the University of Tours in France. He joined the University of Tours as assistant professor in 1981, and became associate professor in 1987. In 1993 he moved to the faculty of Pharmacy, University of Bordeaux, France, accepting a position as full professor. He is currently leading the "study group on biologically active plant substances" at the Institute of Vine and Wine Sciences, which comprises 25 scientists and research students. The group has been working on phenolic compounds from vine and wine for many years, mainly complex stilbenes and their involvement in health. Professor Mérillon has supervised the doctoral theses of 19 students. He is involved in developing teaching on plant biology, natural bioactive compounds, and biotechnology.

Professor Mérillon has published more than 145 research papers in internationally recognized journals, resulting in an H index of 35 (documents published between 1996 and 2015). He has co-edited books and reference works on secondary metabolites and biotechnology.

Throughout his career, Prof. Mérillon has traveled widely as a senior professor. Scientists from several countries have been and are working in his laboratory, and his research is supported by funding from the Aquitaine Regional Government, the Ministry of Higher Education and Research, and various private companies. In 2004, he founded the technology transfer unit "Polyphenols Biotech," providing support for R&D programs for SMEs and major groups from the cosmetic, pharmaceutical, agricultural, and health-nutrition sectors.

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Part I

Biology and Biotechnology

Plant Cell Wall Polysaccharides: Structure and Biosynthesis

1

Michael A. Held, Nan Jiang, Debarati Basu, Allan M. Showalter, and Ahmed Faik

Contents

Abstract

Plant cells are surrounded by cell walls consisting of complex networks of polysaccharides and glycoproteins. Cell walls play a vital role in a plant's development and its interactions with the environment. The biosynthesis of cell walls is fueled by carbon fixed by solar energy during photosynthesis. The amount of carbon fixed annually is estimated to ~2 × 10^{11} tonnes (Hall. Solar energy use through biology – past, present and future. Sol Energy 22:307–328, 1979). Thus, plant cell walls represent a valuable sustainable carbon source for human activities. Plants use complex mechanisms that require the coordinated action of hundreds of glycosyltransferases and other enzymes involved in sugar

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substrate interconversion to build cell wall polysaccharides. This chapter will focus on discussing current advances in the biosynthesis and structures of these wall polysaccharides, including cellulose, a variety of hemicellulosic polymers, pectins, and structural hydroxyproline-rich glycoproteins (extensins and arabinogalactan-proteins). These polymers are organized into complex but dynamic networks that are still the subject of extensive research. Much work is still needed to determine the functions of many glycosyltransferases involved in building these polymers. In addition, how plant cells manage to secrete and organize them into such complex networks remains a mystery. Thus, the future holds exciting discoveries in the field of plant cell wall polysaccharide biosynthesis.

Keywords
Polysaccharide • Glycosyltransferase • Cellulose • Hemicellulose • Pectin • Glycoprotein

1 Introduction

Plant cell wall polysaccharides hold great promise as raw material for food, feed, and biofuel production. For plants, cell walls are important for development and disease resistance, making them targets for improvements of postharvest storage and processing of fruits. Thus, understanding how plants synthesize and remodel their cell walls is an important and expanding area of research, particularly in the light of renewable energy. Plant cell walls are complex networks comprised of physically interacting polymers such as polysaccharides (pectin, hemicelluloses, cellulose), glycoproteins (i.e., extensins), and lignin. The modification of these physical interactions underlies cell expansion and plant growth and development. Considering the complexity of plant cell wall composition and structure, it has been difficult to develop a structural model. In 1973, Keegstra and Albersheim did extensive structural and compositional analyses of primary cell walls of sycamore cells and proposed a tentative model for the primary cell wall (Keegstra et al. 1973). In this model, cellulose and xyloglucan (XyG) interconnect through hydrogen bonds to form a network that is further connected to pectic polysaccharides through XyG. Treatment with endopolygalacturonase released peptides and pectic fragments, suggesting that pectic polysaccharides and the structural proteins are covalently linked (Keegstra et al. 1973). Later studies by Carpita and Gibeaut (1993) extended Keegstra's model and proposed a more dynamic model for the primary cell wall, in which three structurally independent but interacting subnetworks are present: a subnetwork of ordered cellulose microfibrils coated with and cross-linked by hemicellulosic polysaccharides, a second gel-like subnetwork made of pectic polymers acting as "matrix" polysaccharides that surround the first subnetwork, and a third subnetwork consisting of structural proteins that hold the polymers in place. For example, extensin may act as a template to direct orderly assembly of pectin and other polysaccharides during cell wall deposition (Qi et al. 1995). During plant

Table 1 Comparison of polymer contents (% w/w) between type I and type II primary cell walls
(According to Carpita and Gibeaut 1993; Carpita 1996; Carpita and McCann 2000)

Polymer	Type I wall	Type II wall
Cellulose	30–40	30–40
Pectin	~30	2–10
Proteins	10	2–10
Xyloglucan	20	Minor
Xylan	5	20–30
Mannan	3	5

growth, extensive remodeling and modifications of these subnetworks occur, which allow the cell wall to be flexible, extensible, and yet resistant to breakage and shearing.

Primary cell walls can be classified into two main types (Carpita and Gibeaut 1993): type I walls have XyG as the major cross-linking hemicellulose, while type II walls have xylans as the major cross-linking hemicellulose (~30 % w/w). In both types, cellulose (~30 % w/w) is the major load-bearing polymer. Pectin contents differ, as it represents ~30 % (w/w) in type I walls and only 2–10 % (w/w) in type II walls (Jarvis 1984; Carpita and Gibeaut 1993). While type I walls contain ~10 % (w/w) structural proteins, type II walls contain low amounts of these proteins (2–10 % w/w) (Chen and Varner 1985; Hong et al. 1989; Carpita and McCann 2000; Fincher 2009a). Table 1 summarizes the differences between type I and type II primary cell walls.

Synthesis of cell wall polysaccharides requires sophisticated, well-coordinated biosynthetic machinery involving specialized enzymes called glycosyltransferases (GTs). By definition, a GT is an enzyme that transfers a sugar moiety (in the case of plant cell wall, the transferred sugars are mainly monosaccharides) to a nucleophilic group (i.e., alcohol group on substrate acceptor) from an activated sugar phosphate (substrate donor), such as nucleotide diphosphate-sugar (NDP-sugar) (Keegstra and Raikhel 2001; Breton et al. 2006; Lairson et al. 2008). However, biosynthesis of polysaccharides is fundamentally different from nucleic acids (DNA and RNA) and proteins, as a template is not required to synthesize complex polysaccharides. Nucleic acids and proteins do require templates that dictate the specificity in the biosynthesis of various types of molecules. In the case of polysaccharides, the biosynthesis of the various types of polymers relies on the specificity of each GT. Thus, each glycosidic linkage in a polysaccharide would require a specific GT. Considering the structural complexity of a typical plant primary cell wall, several hundreds of different GTs would be needed for its synthesis. GTs that are able to transfer more than one monosaccharide onto the same substrate acceptor (hence building a long polymer) are considered processive enzymes and are called synthases. Synthases are usually responsible for the synthesis of polysaccharide backbones (usually made up of one or two types of monosaccharide). Most of the GTs are integral membrane proteins corresponding to about 1–2 % of the predicted genes in eukaryotic genomes (Lairson et al. 2008). As of August 2014, eukaryotic and prokaryotic GTs are categorized into 95 distinct families based on amino acid sequence similarity, according to the Carbohydrate-Active enZYmes (CAZy)

database (http://www.cazy.org/, Campbell et al. 1997; Cantarel et al. 2009; Lombard et al. 2014).

Catalytic domains of GTs are globular proteins and can be organized into two main types of structural folds, GT-A and GT-B, with N-terminal and C-terminal domains serving as binding sites for substrate donors or acceptors (Bourne and Henrissat 2001). The two domains are linked via a region that serves as the active site. In a GT-B fold, both domains are shaped as a Rossmann-like fold (β/α/β), with the acceptor binding site located at the N-terminal domain, while the C-terminal domain is the NDP-sugar binding site. In a GT-A fold, however, the two domains are structurally different, with the N-terminal domain having a Rossmann-like fold and serving as the donor binding site, while the C-terminal domain does not have a Rossmann-like fold and serves as the acceptor binding site. Regardless, GTs with either GT-A or GT-B folds can catalyze transfer reactions with either an inverting or a retaining mechanism (configuration of the C-1 bond of the NDP-sugar compared to the glycosidic linkage in reaction products). Only a few conserved residues in the donor binding sites and the catalytic sites determine the sugar-nucleotide donor specificity and the catalytic mechanism, respectively (Bourne and Henrissat 2001). Despite our knowledge of the chemical composition of plant cell wall polymers, most GTs involved in their biosynthesis remain undiscovered, and we lack a significant understanding of their biosynthetic mechanisms.

In this chapter, we summarize the current knowledge on the structure and biosynthesis of the main polymers in the walls of growing plant tissues, namely, cellulose, hemicellulose, pectin, and glycoprotein (most mature tissues have additional components, such as lignins and lignans that will not be covered in this chapter).

2 Structural Cell Wall Glucans

2.1 Cellulose Structure and Biosynthesis

Cellulose is the most abundant polysaccharide produced in nature and generally serves as the major scaffolding component for plant cell wall architecture. Cellulose is composed of linear, unsubstituted β-(1,4)-linked glucan chains (Fig. 1a). Individual glucan chains are synthesized at the plasma membrane by GTs, known as cellulose synthase catalytic subunits A (CESAs), which use UDP-glucose as activated sugar-donor substrates and Mg^{2+} cofactors (Pear et al. 1996; Delmer 1999). In plants, multiple, nonredundant CESA isoforms assemble into large multiprotein complexes referred to as cellulose synthase complexes (CSCs). CESAs add glucosyl units to the growing polymer to synthesize individual β-(1,4)-glucan chains. Growing glucans are extruded across the plasma membrane, where they assemble via van der Waals forces and hydrogen bonding into paracrystalline microfibrils (Doblin et al. 2002; Haigler et al. 2014).

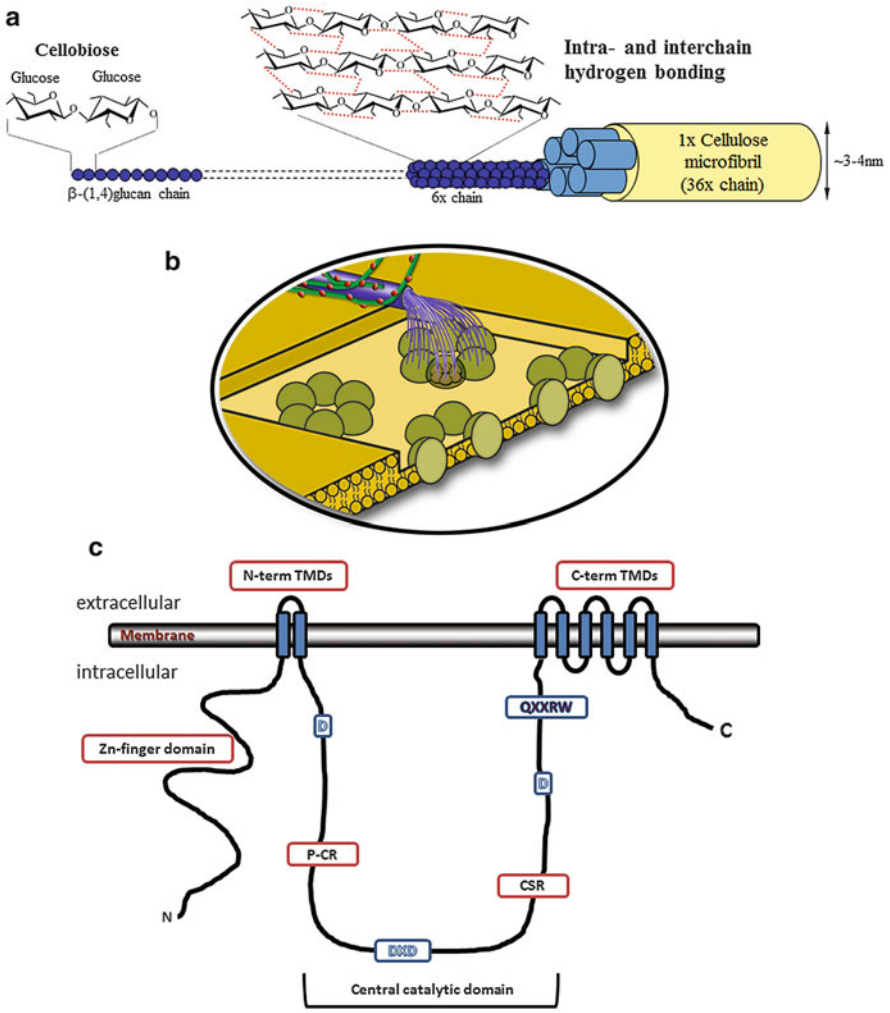

Fig. 1 Molecular features of cellulose, cellulose synthases (*CESAs*), and cellulose synthase complexes (*CSCs*). (**a**) Cellulose consists of paracrystalline microfibrils (*yellow*) containing as many as 36 individual β-(1,4)-linked glucan chains (*dark blue*). The fundamental monomer of each glucan chain is cellobiose. Extensive intra- and interchain hydrogen bonding (*red dashes*), along with van der Waals forces, promotes microfibril crystallinity. (**b**) CSCs form "rosette"-like particles (*green*) in the plasma membrane. Each lobe of the CSC contains multiple, nonredundant CESA isoforms which simultaneously synthesize β-(1,4)-glucan chains (*purple*). As the nascent glucans are synthesized and translocated outside the cell, they associate and form microfibrils which become tethered with hemicellulose (*green*/*red*). (**c**) The functional domains of CESA proteins include the Zn-finger domain, the N- and C-term TMDs, and the central catalytic domain (which possesses the plant-conserved region (*P-CR*), the class-specific region (*CSR*), and the conserved active site residues D, DxD, D, and QxxRW)

Crystallinity imparts structural rigidity and resistance to degradation. In the following sections, we will discuss cellulose biosynthesis, starting with the encoding genes and ending with the formation of a microfibril. The goals of these sections are to provide a general overview of the cellulose biosynthesis and structure and to highlight important research contributions instrumental to our understanding of the process. Many excellent reviews have been written, and readers are referred elsewhere for more detailed discussion of cellulose biosynthesis (Brown et al. 1996; Delmer 1999; Haigler et al. 2001; Williamson et al. 2002; Doblin et al. 2002; Somerville 2006; Mutwil et al. 2008; Taylor 2008; Crowell et al. 2010; Wightman and Turner 2010; Guerriero et al. 2010; Harris et al. 2010; Carpita 2011; Endler and Persson 2011; Lei et al. 2012; Slabaugh et al. 2014).

2.1.1 *CESA* Genes and Families

Genes encoding cellulose synthases were first identified in bacteria (Saxena et al. 1990; Wong et al. 1990). Attempts to use bacterial *CESA* coding sequences to find direct plant homologs were initially stymied due to the relatively low sequence similarity shared between them (Delmer 1999). A breakthrough came in the mid-1990s using gene expression profiling in cotton fibers. Cotton (*Gossypium hirsutum*) has served as a valuable model for cellulose biosynthesis and genomics, as mature fibers consist of ~90 % cellulose. Pear et al. (1996) identified two cDNA clones in cotton fibers that encoded members of the GT2 family and were loosely related to the bacterial CESA. According to the *C*arbohydrate *A*ctive En*z*ymes (CAZy) database (www.cazy.org), GT2 proteins are processive glycosyltransferases and are characterized by having conserved "D," "DxD," "D," and "QxxRW" amino acid motifs important for substrate binding and catalysis (Saxena et al. 1995; Campbell et al. 1997; Coutinho et al. 2003; Lombard et al. 2014). Other members of the GT2 family include chitin synthase (Glaser and Brown 1957; Sburlati and Cabib 1986) and hyaluronan synthase (DeAngelis et al. 1993; Jing and DeAngelis 2000; DeAngelis 1999; Tlapak-Simmons et al. 2004). Then designated *GhCelA1* and *GhCelA2* (later designated *GhCESA1* and *2*), Pear et al. (1996) showed that both genes were expressed highly during cotton fiber development and that their recombinant gene products could bind UDP-Glc substrate. Evidence for the direct involvement of *CESA* gene products in cellulose biosynthesis came later via numerous mutant analyses in *Arabidopsis thaliana* (hereafter called *Arabidopsis*) (Table 2). These reports were instrumental in determining the functional specificity of CESA isoforms in *Arabidopsis* (discussed further below). However, direct biochemical evidence of the activity of any CESA has not been achieved yet.

CESA genes are present in all plant genomes and are members of gene families (Richmond and Somerville 2000). For example, there are 10 *CesA* genes in the *Arabidopsis* genome, 10 in rice, 20 in maize, 10 in sorghum, 18 in poplar, and at least 7 in barley (Richmond and Somerville 2000; Appenzeller et al. 2004; Burton et al. 2004; Djerbi et al. 2005; Penning et al. 2009). Plant genomes also contain several clades of genes related to *CESA*s, designated *cellulose synthase-like* (*CSL*)

Table 2 List of important *CESA* mutants in *Arabidopsis*. Mutant alleles have been identified and mapped for nine of the ten *Arabidopsis CESA* genes

Gene	Locus	Mutant alleles	References
AtCesA1	At4g32410	*rsw1*	Arioli et al. 1998; Beeckman et al. 2002
AtCesA2	At4g39350	*cesa2-1; cesa2-2; Ath-A*	Mendu et al. 2011; Burn et al. 2002
AtCesA3	At5g05170	*ixr1; cev1; eli1; rsw5; thanatos*	Scheible et al. 2001; Ellis et al. 2002; Caño-Delgado et al. 2003; Wang et al. 2006; Daras et al. 2009
AtCesA4	At5g44030	*irx5*	Taylor et al. 2003
AtCesA5	At5g09870	*mum3; cesa5-1; cesa5-2*	Mendu et al. 2011; Harpaz-Saad et al. 2011; Sullivan et al. 2011
AtCesA6	At5g64740	*prc1; ixr2*	Desnos et al. 1996; Fagard et al. 2000; Desprez et al. 2002
AtCesA7	At5g17420	*irx3; fra5; mur 10*	Taylor et al. 1999; Zhong et al. 2003; Bosca et al. 2006
AtCesA8	At4g18780	*irx1; fra6; lew2*	Taylor et al. 2000; Zhong et al. 2003; Chen et al. 2005
AtCesA9	At2g21770	*cesa9-1; cesa9-2*	Persson et al. 2007; Stork et al. 2010
AtCesA10	At2g25540	nr	nr

nr none reported

genes. Together, *CESA* and *CSL* genes form a large gene "superfamily" (Richmond and Somerville 2000; Holland et al. 2000; Hazen et al. 2002; Suzuki et al. 2006; Fincher 2009a). To date, CSLs have mostly been associated with hemicellulose biosynthesis.

2.1.2 *CESA* Gene Expression

Plants synthesize two types of walls during cellular development: a thin flexible primary cell wall that is produced during cell growth and elongation and a thicker secondary wall that is deposited after cell elongation ends. Synthesis of both types of walls requires coordinated deposition of multiple cell wall polymers, including those for cellulose, pectin, hemicelluloses, and lignins. Coordination is initiated at the gene expression level. In *Arabidopsis*, for example, *CESA1*, *3*, and *6* (and *CESA2* to a lesser extent) are highly co-expressed in tissues making primary walls, while *CESA4*, *7*, and *8* are highly co-expressed in cells actively making secondary walls (Brown et al. 2005; Persson et al. 2005). Not only are the *CESA* genes highly co-expressed, but they are also highly co-expressed with genes thought to be associated with the CSCs and genes needed for the biosynthesis of other wall polymers, such as xylan and mannan (Brown et al. 2005; Persson et al. 2005). This phenomenon is not restricted to *Arabidopsis*. Similar sets of co-expressed primary and secondary wall *CESA*s have been identified in other plants, including barley (Burton et al. 2004), rice (Tanaka et al. 2003; Wang et al. 2010), maize (Appenzeller et al. 2004), and poplar (Joshi et al. 2004). Together, these findings show that there are two distinct gene expression programs

for making CSCs – one for primary wall biosynthesis and one for secondary wall biosynthesis – and each CSC is likely made up of at least three individual CESA isoforms.

Coordinated expression of *CESA* gene sets is very interesting, yet we know relatively little about what controls their expression. Advances have been made regarding the transcriptional regulation of secondary cell wall biosynthesis. Expression of genes associated with the secondary cell wall program appears to be regulated by broad targeting NAC domain (Kubo et al. 2005; Zhong et al. 2006; Zhong et al. 2007a) and MYB transcription factors (Zhong et al. 2007b; Goicoechea et al. 2005; Kim et al. 2013). These transcription factors (TFs) represent "master switches" that activate expression of large sets of genes (including other TFs) necessary for secondary cell wall polysaccharide and lignin biosynthesis. While much is known regarding these master switch TFs, diversity in wall architecture suggests a finer level of regulation. This may come at the posttranscriptional level in the form of small RNA regulation and/or at the posttranslational level in the form of reversible phosphorylation events (Held et al. 2008; Nühse et al. 2004; Taylor 2007; Chen et al. 2010).

2.1.3 CSC Assembly and Protein Components

CSCs are likely assembled in the Golgi apparatus and trafficked to the plasma membrane through the *trans*-Golgi and microtubule-associated cellulose synthase (MASC/SmaCC) compartments (Haigler and Brown 1986; Paredez et al. 2006; Crowell et al. 2009; Gutierrez et al. 2009; Wightman and Turner 2010). In the plasma membrane, glucan chain polymerization and assembly are thought to provide the energy that drives CSC movement (Diotallevi and Mulder 2007). The direction of CSC movement appears to be controlled by interactions with the underlying cortical microtubules (Paredez et al. 2006; Crowell et al. 2009; Gutierrez et al. 2009; Wightman and Turner 2010). CSCs, imaged using freeze fracture electron microscopy, appear on the surface of the plasma membrane as six-membered "rosette" structures of about 24 nm in diameter (Brown and Montezinos 1976; Mueller and Brown 1980; Giddings et al. 1980; Herth 1985). Immunolabeling has confirmed that CESA proteins are indeed found in CSCs (Kimura et al. 1999). Mutational analyses and co-immunoprecipitation studies have shown that at least three individual CESA isoforms are required to form an active CSC (Taylor et al. 2003; Gardiner et al. 2003; Tanaka et al. 2003; Persson et al. 2007). In *Arabidopsis*, the primary wall CSC is composed of CESA1, 3, and 6 (with CESA2, 5, and 9 being partially redundant with CESA6), while secondary wall CSCs are composed of CESA4, 7, and 8. Current consensus holds that each lobe of the CSC contains six CESA polypeptides (Doblin et al. 2002), with a CSC having a 6×6 symmetry. Assuming that each CESA is capable of synthesizing one β-(1,4)-glucan chain, this yields microfibrils that contain ~36 glucan chains per CSC (Fig. 1a, b), which compares well with the estimated diameter of larger microfibrils of ~3–4 nm (Ding and Himmel 2006; Ding et al. 2012; Haigler et al. 2014). However, some microfibrils are narrower (~2.4 nm; Kennedy et al. 2007), leading some to question the number of CESAs per CSC, the number

of CESAs needed to produce a single glucan chain, and the number of glucan chains per microfibril (Carpita 2011; Newman et al. 2013; Olek et al. 2014).

In addition to CESAs, other accessory proteins are associated with the CSC, including cellulose synthase interacting protein 1 (CSI1; Gu et al. 2010). CSI1 is a large armadillo repeat-containing (ARM) protein and is part of CSCs involved in synthesizing primary walls (Gu et al. 2010). ARM domains have been implicated in maintaining interactions with cytoskeletal components (Ozawa et al. 1989; Coates et al. 2006; Gebert et al. 2008). As such, CSI1 likely acts as a structural component of the CSC and may mediate interactions between CSCs and cortical microtubules (Gu and Somerville 2010). The second non-CESA protein shown to be associated with a CSC is a β-(1,4)-endoglucanase, called KORRIGAN (KOR). KOR has long been implicated in cellulose biosynthesis (Nicol et al. 1998; Lane et al. 2001; Sato et al. 2001) but only recently was shown to physically interact with the CSC (Vain et al. 2014). KOR is an essential component of the CSC, as mutation of *Kor* leads to cellulose deficiency (Nicol et al. 1998; Lane et al. 2001; Sato et al. 2001). Expression of *KOR* is strongly correlated with that of primary wall *CESA*s in *Arabidopsis* (Persson et al. 2005). The precise roles of KOR are still under investigation, but putative functions include the termination of glucan synthesis and glucan release, helping to assist glucan assembly/microfibril crystallization and relieving the strain incurred during glucan polymerization and/or microfibril formation. Additionally, it appears that KOR may also play a role in the trafficking of CSCs (Vain et al. 2014). Another important protein involved in cellulose biosynthesis is called COBRA (COB). COB is a GPI-anchored plasma membrane protein and is a member of a larger gene family composed of COB and COBRA-like proteins (COBLs) (Benfey et al. 1993; Roudier et al. 2002; Roudier et al. 2005). Both *COB* and *COBL* genes are important for proper cell expansion and cellulose biosynthesis (Schindelman et al. 2001; Li et al. 2003; Hochholdinger et al. 2008; Dai et al. 2011) and are highly co-expressed with primary and secondary wall *CESA*s (Persson et al. 2005), which may suggest a direct interaction with CSCs.

Several other proteins are associated with cellulose biosynthesis, including KOBITO (KOB1) (Pagant et al. 2002), SUCROSE SYNTHASE (SUSY) (Amor et al. 1995), POMPOM1/ELP1 (POM1) (Mouille et al. 2003; Zhong et al. 2002), RADIAL SWELLING3 (RSW3) (Burn et al. 2002), FRAGILE FIBER1 (FRA1) (Zhong et al. 2002), and FRAGILE FIBER2 (FRA2) (Burk et al. 2001; Burk and Ye 2002). Although both KOBITO and POM1 are localized to the plasma membrane, direct interaction of these proteins with a CSC or their precise molecular roles in cellulose biosynthesis has yet to be confirmed. RSW3, FRA1, and FRA2 likely impact CSC trafficking and interaction with cytoskeletal components.

2.1.4 Important Protein Domains of CESAs

Since their discovery, CESAs have been analyzed in terms of their functional domains (Pear et al. 1996; Vergara and Carpita 2001). Important CESA protein domains include the *N-terminal RING-like Zn-finger domain*, *N- and C-terminal transmembrane domains* (TMDs), and the *cytosolic catalytic domain*, which contains the *plant-conserved region* (P-CR), the *class-specific region* (CSR), and the

conserved GT2 catalytic motifs (Fig. 1c). Below is a brief discussion about the characteristics of each of these domains.

N-Terminal Zinc-Finger Domain

At the N-terminus of CESA proteins is a putative zinc-finger motif (Pear et al. 1996; Kurek et al. 2002). Zn-finger motifs are typically composed of tandem arrays of evenly spaced cysteine and/or histidine residues. RING Zn-finger domains are defined by the consensus sequence **CxxC**-$X_{9–39}$-**C-$X_{1–3}$-H-$X_{2–3}$-C/HxxC-**$X_{4–48}$-**CxxC**, where X can be any amino acid (Freemont 2000). Each RING domain can coordinate two Zn ions in a tetrahedral conformation mediated by the side chains of four Cys or three Cys and one His residues (Saurin et al. 1996). CESA proteins generally contain the consensus sequence **CxxCG**-x_{14}-**CxxC**-x_4-**CxxC**-x_7-**G**-x_3-**CxxC** (Fig. 1c). The function of the Zn-finger domain is not clear. Kurek et al. (2002) showed that bacterially expressed Zn-finger domains from cotton *GhCESA1* and *GhCESA2* were able to bind zinc and dimerize in an oxidatively dependent manner. A subsequent immunological study indicated that Zn-finger domains may also be important for mediating CESA protein turnover and estimated that the half-life of CESA protein is fairly short and on the order of ~30 min (Jacob-Wilk et al. 2006). While Zn-finger domains are commonly involved in protein-protein interactions (Saurin et al. 1996), these domains in CESAs do not appear to be necessary for interactions among secondary wall CESA proteins (Timmers et al. 2009).

Central Catalytic Domain

The central catalytic domain of CESAs lies between the N- and C-terminal TMDs and is composed of four "U" motifs, which contain conserved "D," "DxD," "D," and "QxxRW" motifs (Fig. 1c) important for UDP-glucose substrate binding and catalysis (Saxena et al. 1995; Vergara and Carpita 2001). These U motifs are thought to come together spatially to form a conserved GT-A fold (Saxena et al. 1995, 2001; Charnock and Davies 1999; Lairson et al. 2008; Morgan et al. 2013; Sethaphong et al. 2013; Slabaugh et al. 2014) and are shared by all CESAs and CSLs and other GT2s (Saxena et al. 1995). The central catalytic domain is the largest domain and lies on the cytosolic side of the plasma membrane (Bowling and Brown 2008; Carpita 2011). Recent structural studies have shown that this region forms a channel through which the nascent glucan chain is polymerized and guided through the plasma membrane (Morgan et al. 2013; Sethaphong et al. 2013).

Plant-Conserved Region (P-CR) and Class-Specific Region (CSR)

Within the central catalytic domain of plant CESAs are two important sub-domains known as the plant-conserved region (P-CR) and the class-specific region (CSR) (Fig. 1c) (Pear et al. 1996; Vergara and Carpita 2001). These regions are not found in their bacterial counterparts. The P-CR is a stretch of about 125 amino acids found between the conserved "D" and "DXD" motifs in plant CESAs (Pear et al. 1996). The CSR is highly conserved among CESA orthologs, suggesting that these

domains are important for assigning functional specificity among different CESA isoforms (Pear et al. 1996; Vergara and Carpita 2001). The assembly of CSCs seems to have evolved somewhere within the *Charophyta* branch of green algae (Roberts et al. 2002). Both the P-CR and CSR also seem to have arisen in *CESA* genes around the same time, suggesting these regions may be involved in rosette formation and/or maintenance. Recent structural investigations have also hinted at their roles in mediating CESA interactions within a CSC (Sethaphong et al. 2013; Olek et al. 2014).

Transmembrane Domains (TMDs)

CESAs generally contain eight predicted TMDs. Two membrane passes are toward the N-terminus (N-term TMDs), while six are grouped near to the C-terminus (C-term TMDs) (Fig. 1c). Together, these regions flank the central catalytic domain. The TMDs are thought to form a membrane-spanning pore through which nascent glucan chains are extruded (Morgan et al. 2013; Slabaugh et al. 2014). The TMDs represent a critical interface between the lipid bilayer of the plasma membrane and each CESA protein and are potentially involved in several functions, including entry into the secretory pathway, protein topology and trafficking, glucan translocation, and maintaining the organization and interactions of CESA subunits within a CSC.

2.1.5 Advances in CESA Structure and Catalytic Mechanisms

Recently, the crystal structure of a bacterial cellulose synthase (*Rs*BcsA) was determined (Morgan et al. 2013). The catalytic subunit consisted of eight TMDs, three interfacial helices that ran parallel to the face of the plasma membrane, and a cytosolic GT2 catalytic domain containing conserved D, D×D, TED, and QxxRW motifs. Six of the TMDs form 0.8 nm wide transmembrane pore, just wide enough to accommodate the extrusion of the nascent glucan (~0.7 nm) and long enough to hold a chain of ~10 glucosyl units in length. A model was suggested whereby glucosyl units are added one at a time to the nonreducing end of the growing polymer and simultaneously translocated across the membrane through the TMD pore. Glucosyl units are apparently free to rotate 180° from their neighbor in the catalytic site to produce a β-(1,4)-linked polymer.

An in silico model of the central catalytic domain of the cotton GhCESA1 protein (Gh506) was assembled (Sethaphong et al. 2013) using crystal structures of related GT2 proteins, including SpsA (Charnock and Davies 1999) and K4CP (Osawa et al. 2009). The Gh506 protein contained the 506 amino acids of central catalytic domain, which included both the P-CR and CSR domains, but lacked structural representation of the N-terminal Zn-finger domain and the N- and C-term TMDs. Structural co-alignment of Gh506 with the *Rs*BcsA catalytic domain showed striking similarities, supporting the validity of the predicted structure (Sethaphong et al. 2013; Morgan et al. 2013). This model positions both the P-CR and CSR (lacking in the *Rs*BcsA model) at the periphery of the catalytic domain, making them attractive candidates for mediating protein-protein interactions within a CSC. More recently, an additional model for a plant *CESA* was reported.

Using both 3D structure prediction and small-angle X-ray scattering, Olek et al. (2014) provided structural data for the catalytic domain of rice CESA8. While their data were similar to that of Sethaphong et al. (2013), specifically regarding the folding of the catalytic portion of the central catalytic domain, an alternate arrangement of both the P-CR and CSR domains was reported. Further, in vitro studies indicated that the CESA catalytic domains form dimers that synthesize a single glucan chain. Formation of catalytic dimers provides a convenient explanation for the formation of cellobiose, the fundamental monomer of a β-(1,4)-linked glucan (Fig. 1a). Future work aimed at identifying the composition and stoichiometry of rosettes formed in vivo will help elucidate the number of CESAs needed to produce a glucan chain.

2.1.6 Crystallization of Cellulose Microfibrils

Crystallization is the cornerstone of cellulose structure and function, but the actual process is not fully understood. Cellulose can exist in multiple forms or allomorphs. In nature, cellulose I predominates, whereby all glucan chains are aligned in a parallel orientation (as opposed to cellulose II, which has an antiparallel arrangement). Cellulose I is composed of two distinct crystalline phases, Iα and Iβ, which differ mainly by their hydrogen bonding patterns (Atalla and VanderHart 1984; Sugiyama et al. 1991). Higher plants are enriched in cellulose Iβ, whereas bacteria and algae are rich in cellulose Iα (Sugiyama et al. 1991). For our discussion here, let's consider a 6-lobed CSC where each lobe is simultaneously producing 6 glucan chains (36 glucan chains in total). As nascent glucans traverse the plasma membrane and exit the CSC, adjacent linear chains quickly associate by hydrogen bonding and begin to form paracrystalline microfibrils. In addition to hydrogen bond formation, factors that appear to affect crystallization include the rate of polymerization (Benziman et al. 1980), van der Waals interactions among glucan chains (Haigler et al. 2014), and the physical proximity of the emerging glucans (which is related to rosette architecture and protein composition).

2.2 Mixed-Linkage Glucan (MLG) Structure

MLG is another unbranched glucose polymer consisting of β-D-glucopyranosyl units linked by both (1,3)- and (1,4)-glycosidic bonds (Fig. 2). The MLG polymer has a unit structure consisting primarily of cellotriose (G3) and cellotetraose (G4) subunits at a ratio of 2.2–2.6:1 (Gibeaut and Carpita 1993; Fincher and Stone 2004; Fincher 2009b; Fig. 2). Single (1,3)-linkages are always flanked by at least two (1,4)-linkages in plant cell wall MLG. Longer cellodextrin subunits (5–20) are found in the polymer but in decreasingly smaller amounts, and the arrangement of these subunits is thought to be random within the MLG polymer (Staudte et al. 1983; Woodward et al. 1983; Buliga et al. 1986). The subunit composition of the MLG polymer has been investigated by digestion of the MLG polymer with (1,3),(1,4)-β-D-glucan-4-glucanohydrolase from *B. subtilis*. This enzyme specifically cleaves a β-(1,4)-glycosidic bond, only if it is preceded by a

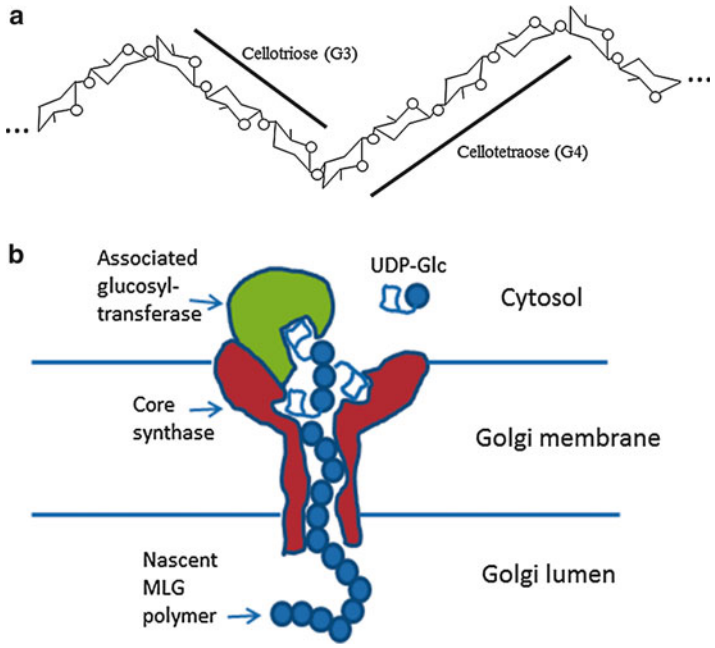

Fig. 2 Structural representation of mixed-linkage β-D-glucan (*MLG*) and the MLG synthase. (**a**) MLG is composed mostly of cellotriosyl (G3) and cellotetraosyl (G4) linked by single β-(1,3)-linkages, though longer cellodextrins are also observed. (**b**) The MLG synthase is thought to have three active sites and two GT components: a core synthase (*red*) to generate cellobiose and larger even-numbered cellodextrins and an associated GT (*green*) to add single glucosyl units from UDP-Glc to form odd-numbered subunits. Nascent polymer is extruded into the Golgi lumen prior to being packaged into vesicles for delivery to the cell wall. (Adapted from Buckeridge et al. 2004)

β-(1,3)-linkage, making this enzyme diagnostic for MLG (Anderson and Stone 1975). The oligomers released by digestion can be separated and quantified by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) relative to authentic barley MLG standards.

MLG is found in modest amounts in the cell walls of most cereal plant tissues, but it is heavily produced at specific developmental stages. The endosperm walls of barley caryopses are composed of over 70 % MLG (Fincher et al. 1986; Fincher 2009a). MLG is also heavily secreted in cells undergoing elongation growth (Gibeaut and Carpita 1991; Gibeaut et al. 2005; McCann et al. 2007). In elongating maize coleoptiles, maximum MLG deposition coincides with peak elongation rates (McCann et al. 2007). After elongation ceases, the MLG is digested by endogenous endoglucanases, from the middle lamellae inward, leaving trace amounts in the wall. MLG is thought to serve an architectural role in the walls of elongating cells by coating cellulose microfibrils. Due to their kinked molecular shape, they are thought to provide a gel-like matrix which facilitates wall elongation (Fincher and Stone 2004).

2.2.1 MLG Biosynthesis

MLG is synthesized in the Golgi apparatus by membrane-embedded GTs using UDP-Glc as a substrate and Mg^{2+} as a cofactor (Buckeridge et al. 1999; Gibeaut and Carpita 1993; Carpita and McCann 2010). Much of what is known about MLG biosynthesis comes from in vitro studies using enriched Golgi preparations from maize. MLG composition (e.g., the mole ratio of G3:G4) is influenced by substrate donor concentration (Buckeridge et al. 1999; Henry and Stone 1982). In vitro, high levels of UDP-Glc push the ratio of G3 to G4 to as much as 10:1, whereas lower substrate levels drop this ratio closer to 1:1 (Buckeridge et al. 1999). Importantly, the ratios of all odd-numbered cellodextrins to the next even-numbered cellodextrin mirror this phenomenon. Buckeridge et al. (2001) suggested that there are multiple sites of glucosyl transfer consisting of a core synthase that generates β-(1,4)-linked cellobiosyl units and an associated GT that adds single Glc residues to each cellobiose unit to form the fundamental cellotriose unit (Fig. 2). At high substrate concentrations, all three sites are filled and G3 formation is favored. At low substrate concentrations, the associated glucosyl transferase occasionally is empty and cannot complete the formation of cellotriose and higher-ordered odd-numbered oligomers. The core synthase remains full however and can iteratively polymerize cellobiose units to form G4 (and higher-ordered even-numbered subunits). Detergent extraction experiments support these findings (Urbanowicz et al. 2004). The associated GT was shown to be more susceptible to detergent extraction with CHAPS, as evidenced by a decrease in the G3:G4 ratio. This decrease was recovered by reconstituting the CHAPS extract into the in vitro reaction and diluting the detergent concentration back to tolerable levels (Urbanowicz et al. 2004). In the same work, the topology of the MLG synthase was also determined. Intact Golgi bodies treated with proteinase K lost the preference to form cellotriosyl residues, again judged by a decrease in the G3:G4 ratio (Urbanowicz et al. 2004). These data suggested that the even-numbered forming core synthase was more membrane protected than the associated odd-numbered forming glucosyltransferase (Urbanowicz et al. 2004).

There are many similarities between MLG and cellulose: (*i*) both are unsubstituted glucose polymers; (*ii*) both polymer synthases use UDP-Glc as substrate; and (*iii*) both require divalent cation cofactors. Thus, it was reasonable to suggest that the MLG synthase components might be related to cellulose synthase components (Richmond and Somerville 2000). MLG is a cereal-specific cell wall polymer, not produced by most other plant species. Indeed, a comparison of the cellulose synthase gene superfamilies of dicots with grasses showed that there were initially two (now three) clades of cellulose synthase-like genes that were unique to grasses. These clades were dubbed the *CSLF* and *CSLH* gene families, and it was hypothesized that these clades might encode proteins that formed all or part of the MLG synthase (Richmond and Somerville 2000; Hazen et al. 2002). Subsequent heterologous expression of either the rice *OsCSLF6* gene or the barley *CSLH* gene in *Arabidopsis* (a plant that does not naturally produce MLG in its cell walls) conferred the ability to produce small amounts of MLG (Burton et al. 2006; Doblin et al. 2009). Either gene, expressed on its own, was

sufficient to produce small amounts of MLG in *Arabidopsis* cell walls, suggesting that either CSLF or CSLH can act alone to catalyze MLG biosynthesis. Recombinant expression of CSLF4, 6, and 9 was also correlated with increased MLG contents in transgenic barley (Burton et al. 2011). MLG contents varied depending on the promoter used to drive transgene expression. Additionally, individual CSLF isoforms produced MLG polymers having different G3:G4 ratios, leading the authors to speculate that each CSLF isoform produces MLG having different fine structures (Burton et al. 2011). Several lines of evidence suggest that other cofactors and/or associated proteins are required for efficient MLG biosynthesis. First, MLG was produced at very low levels in *Arabidopsis* expressing the *OsCSLF6* gene (Burton et al. 2006). It was suggested that *Arabidopsis* may lack cofactors or associated proteins necessary for normal MLG biosynthesis (Burton et al. 2006). Second, transgenic expression of *HvCSLH* in *Arabidopsis* produced variable levels of laminaribiose (Doblin et al. 2009). Laminaribiose production suggested the presence of alternating β-(1,3)- and β-(1,4)-linkages in the MLG polymer, a structural feature not found in authentic cereal MLG. It was hypothesized that HvCSLH protein was likely misregulated in the heterologous background either due to changes in its membrane environment or due to suboptimal levels of associated factors (Doblin et al. 2009).

Since CSLF and CSLH each are involved in MLG biosynthesis, it has been suggested that the two may interact to form a catalytic heterodimer (Carpita 2011). Several lines of evidence argue against this possibility. First, the expression profiles are different for *CSLF* and *CSLH* during development and across tissue types (Burton et al. 2008; Doblin et al. 2009). If CSLF and CSLH participated in heterodimer formation, one might expect that they be expressed in the same tissues at the same developmental time point. However, gene expression data do not always correlate with protein abundance. Second, both the CSLF and CSLH proteins can catalyze MLG biosynthesis independently (Burton et al. 2006; Doblin et al. 2009; Vega-Sánchez et al. 2012). While CSLF and CSLH clearly synthesize MLG individually, one cannot rule out the possibility that the MLG synthase is a multiprotein complex consisting of CSLF and/or CSLH and additional protein components. Additionally, recent structural studies of cellulose synthases should shed light on both the mechanism of glucosyl transfer and the potential for complex assembly for the MLG synthase (Morgan et al. 2013; Sethaphong et al. 2013; Olek et al. 2014).

3 Structural and Reserve Cell Wall Hemicellulosic Polymers

3.1 Xylan Structure and Biosynthesis

Xylans are a group of heteropolymers that represent the major structural hemicellulosic polysaccharides in primary and secondary cell walls of commelinid monocots (including grasses) and the secondary cell walls of dicots (Ebingerrova et al. 2005). They represent the third most abundant polymer in the biosphere, after

cellulose and chitin. Within cell walls, xylan polymers contribute to cross-linking of cellulose microfibrils and lignin (Imamura et al. 1994; Balakshin et al. 2011) and to themselves through ferulic acid residues (Wende and Fry 1997; de O Buanafina 2009). Because of their extensive cross-linking, xylan polymers have a major impact on the integrity of cell wall and the recalcitrance of plant biomass to enzymatic digestion (Faik 2013). Importantly, a recent study identified a large proteoglycan complex called "Arabinoxylan Pectin Arabinogalactan Protein 1" (APAP1) in *Arabidopsis*, which provides direct evidence for the covalent linkage between xylans, pectins, and arabinogalactan-proteins (AGPs) and confirms the importance of xylans in maintaining the integrity of plant cell walls (Tan et al. 2013).

All xylans have a common backbone of β-(1,4)-linked D-Xyl residues. This backbone can be substituted at position C2 with glucuronic acid (GlcA) or 4-*O*-methyl-glucuronic acid (MeGlcA) and/or with arabinofuranose (Ara*f*) residues at position C2 and/or C3 (Fig. 3a). Depending on the nature of these substitutions, xylans can be categorized into three basic structural types (Izdorczyk and Biliaderis 1995; Carpita and McCann 2000; Ebingerrova et al. 2005; Faik 2010; Kulkarni et al. 2012): glucuronoxylan (GX), which has an average Xyl:MeGlcA ratio of 10:1 and is found mostly in hardwoods; neutral arabinoxylan (AX), which has an Ara:Xyl ratio varying from 1:2 to 1:1 and is found mainly in cereal endosperm walls; and glucuronoarabinoxylan (GAX), which contains GlcA, MeGlcA, and Ara*f* side chains at an average Xyl:Ara:GlcA ratio of 8:3:1 (Fig. 3a). GAX is found in both primary and secondary walls of vegetative tissues in grasses (Ebringerova and Heinze 2000; Faik 2010; Faik et al. 2013). Interestingly, GX from dicots has a complex tetrasaccharide, β-D-Xyl-(1,3)-α-L-Rha-(1,2)-α-D-GalA-(1,4)-D-Xyl called sequence 1 (Johansson and Samuelson 1977; Andersson et al. 1983; Pena et al. 2007) that seems to be absent in xylans from monocots (Ratnayake et al. 2013).

Xylan synthesis is complex than previously thought. Many GTs appear to be involved in their biosynthesis, but little is known about their biochemical functions. For simplicity, xylan synthesis can be divided into three steps: (*i*) backbone synthesis, including the synthesis of the reducing end oligosaccharide in xylans from dicots; (*ii*) addition of side chains; and (*iii*) modification of the polymer. However, these processes have not been demonstrated biochemically. The first genes involved in xylan biosynthesis were discovered through several genetic studies in *Arabidopsis irregular xylem* (*irx*) mutants (Turner and Somerville 1997; Brown et al. 2005). According to their xylan phenotypes, the mutated *Arabidopsis* genes can be categorized into two major gene sets. Gene set1 includes three pairs of genes, At2g37090/At1g27600 (called *irx9*/*irx9-L*), At4g36890/At5g67230 (called *irx14*/*irx14-L*), and At1g27440/At5g61840 (called *irx10*/*irx10-l*), while gene set2 includes four genes: At2g28110/At5g22940 (called *irx7*/*irx7-L* or *fra8*/*f8h*), At5g54690 (called *irx8* or *gaut12*), and At1g19300 (called *parvus* or *gatl1*). The characterization of *Arabidopsis* mutants in gene set1 revealed that these plants have shorter xylan backbones and double mutants (such as *irx10*/*irx10L* or *irx14*/*irx14-L*) have very little GX but still have the tetra-oligosaccharide, sequence 1 (Brown et al. 2009; Wu et al. 2009, 2010a). On the other hand, the characterization of

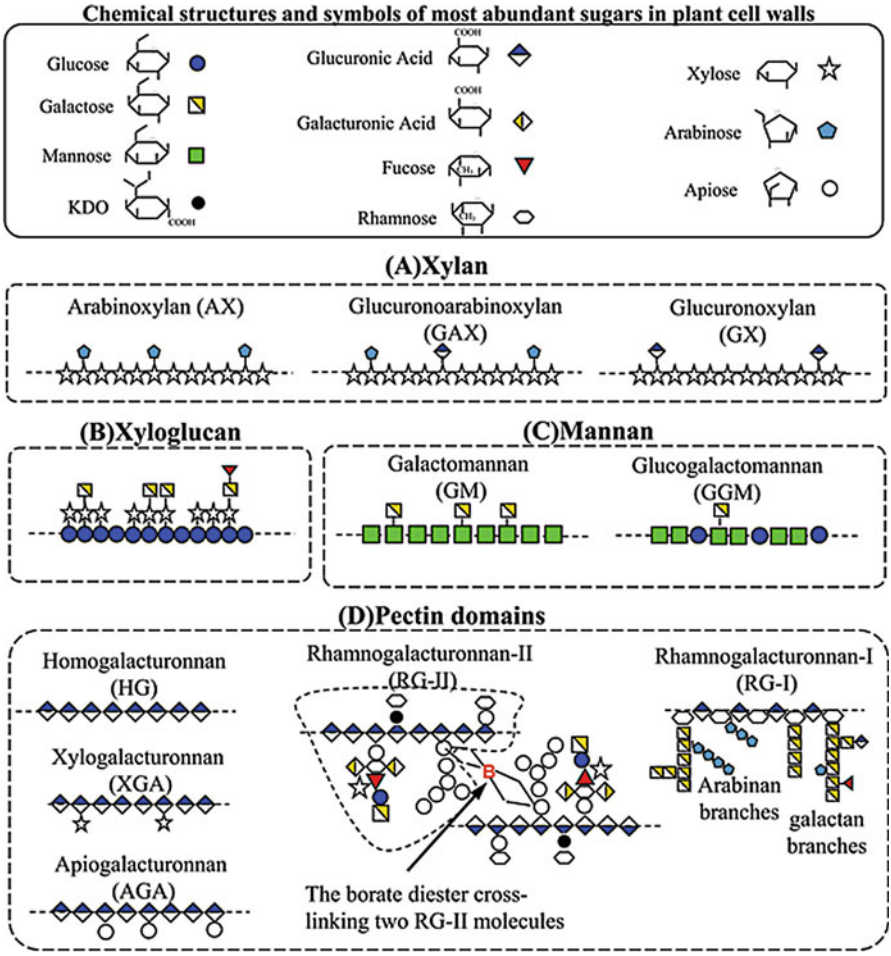

Fig. 3 Structures of hemicelluloses and pectins. *Top panel* shows the presentation of the structures and symbols of monosaccharide building blocks (monomers). Panels *A–D* represent simplified structures of xylan, xyloglucan, mannan, and pectin domains, respectively

Arabidopsis mutants in gene set2 showed that GX polymers from these plants have increased xylan backbone lengths and heterodispersity, but sequence 1 was absent in these polymers (Brown et al. 2007; Persson et al. 2007; Lee et al. 2009). Although biochemical function and the role of these proteins in xylan biosynthesis are still lacking, these observations support the following conclusions: *i*) IRX9 and IRX14 (both belong to GT43) and IRX10 (belongs to GT47) are involved in xylan backbone synthesis (Wu et al. 2010a; Chiniquy et al. 2013; Faik et al. 2013); (*ii*) IRX7/FRA8 and IRX7L/F8H (all belong to GT47) and IRX8/GAUT12 and PARVUS (both belong to GT8) are involved in the synthesis of sequence 1 in *Arabidopsis* (Pena et al. 2007); and (*iii*) sequence 1 may have a regulatory role in

GX biosynthesis in dicots. An earlier report by York and O'Neill (2008) proposed that sequence 1 could act as a primer or a terminator sequence for xylan biosynthesis in dicots. But more recently, Faik et al. (2013) proposed that sequence 1 might act as a driver of secretion of newly synthesized GX polymers to the cell surface, in addition to a terminator of the biosynthetic process. These authors hypothesized that sequence 1 is added to the newly synthesized GX in late Golgi compartments to terminate synthesis of GX polymers and allow their recruitment into secretory vesicles, which is consistent with GX phenotypes observed in mutant plants from gene set2.

Recent advances in xylan biosynthesis have shown that members of the GT43 and GT47 families are also involved in xylan backbone synthesis in grasses. For example, Zeng et al. (2008, 2010) demonstrated that GAX biosynthesis in wheat involves a synergetic/cooperative mechanism between three GT activities: a xylan glucuronosyltransferase (X-GlcAT), a xylan xylosyltransferase (X-XylT), and xylan arabinofuranosyltransferase (X-AraT). These authors used co-immunoprecipitation and proteomic analyses to demonstrate that enzymes involved in GAX biosynthesis form a protein complex containing members of the GT43, GT47, and GT75 families. Members of GT75 have been shown to possess mutase activity necessary for conversion of UDP-L-arabinopyranose (UDP-L-Ara*p*) to UDP-L-arabinofuranose (UDP-L-Ara*f*) (Konishi et al. 2007). So far, all genetic studies indicate that no cellulose synthase-like (*CSL*) genes are involved in the synthesis of the xylan backbone (Faik 2010; Carpita 2011; Doering et al. 2012; Rennie and Scheller 2014). The CSL subfamily is homologous to cellulose synthase catalytic subunit A (CESA) subfamily and both belong to the CAZy GT2 family. The CSLs have ten subgroups, CSLA through CSLJ (Richmond and Somerville 2000; Hazen et al. 2002; Farrokhi et al. 2006), and several of these CSLs are involved in hemicellulose biosynthesis, such as XyG (CSLC; Cocuron et al. 2007), mannan (CSLA; Dhugga et al. 2004; Liepman et al. 2005), and MLG (CSLF and CSLH; Burton et al. 2006; Doblin et al. 2009). Thus, xylan backbone synthesis may have a different mechanism compared to other β-glycans.

For xylan substitution, it is expected that at least four enzymes are needed to decorate the xylan backbone in dicots and monocots (Faik 2010): an α-(1,2) arabinofuranosyltransferase (α-(1,2)AraT), an α-(1,3)arabinofuranosyltransferase (α-(1,3)AraT), a β-(1,2)-xylosyltransferase (β-(1,2)XylT), and an α-(1,2)glucuronosyltransferase (α-(1,2)GlcAT). *Arabidopsis* members of the GT8 family, named Glucuronic Acid Substitution of Xylan (GUX1 and GUX2), are responsible for the addition of α-(1,2)-D-GlcA side chains onto the xylan backbone (Mortimer et al. 2010; Oikawa et al. 2010). Biochemical characterization of GUX1 and GUX2 activities showed that they strongly prefer xylohexaose as an acceptor, and the GlcA residue is almost exclusively added to the fifth Xyl residue from the nonreducing end (Rennie et al. 2012; Bromley et al. 2013). In the GT61 family, two wheat GT genes (*TaXAT1* and *TaXAT2*) and two rice genes (*OsXAT1* and *OsXAT2*) have been shown to mediate α-(1,3)arabinofuranosyl transfer onto xylan (Anders et al. 2012). Another gene from rice (*OsXAX1*) encodes a β-(1,2)XylT involved in extending Ara*f* with a Xyl residue to form β-D-Xyl-(1–2)-α-L-Ara*f* side chains

specific to AX from grasses (Chiniquy et al. 2012). However, the precise biochemical activities of XATs and XAX1 were not provided in these previous studies.

For xylan modifications, several genes have been identified in *Arabidopsis*, so far. A GX methyltransferase (*GXMT*), encoding a protein containing a domain of unknown function (DUF) 579, specifically transfers a methyl group from S-adenosylmethionine to the *O*-4 position of GlcA side chains of GX (Lee et al. 2012; Urbanowicz et al. 2012). The Trichome Birefringence-like (TBL) family member TBL29 contains a DUF231 domain and has been shown to transfer *O*-acetyl groups onto both *O*-2 and *O*-3 positions of xylose residues in the xylan backbone, hence affecting the solubility and digestibility of GX in plants mutated in these genes (At3g55990, At3g06550) (Manabe et al. 2013; Yuan et al. 2013; Xiong et al. 2013). Characterization of *Arabidopsis* mutants in *REDUCED WALL ACETYLATION* (*RWA1–4*) genes showed that these genes are required for xylan acetylation in the secondary cell wall (Lee et al. 2011). Members of a large family of acyltransferases named BAHD, a name derived from the first four biochemically characterized enzymes of this family (BEAT, benzylalcohol *O*-acetyltransferase; AHCT, anthocyanin *O*-hydroxycinnamoyltransferase; HCBT, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase; and DAT, deacetylvindoline 4-*O*-acetyltransferase) (Yang et al. 1997; Dudareva et al. 1998; St Pierre et al. 1998; Fujiwara et al. 1998), have been shown to possess transferase activities that are involved in the addition of ferulic and coumaric acid esters onto arabinofuranosyl residues of xylan (Piston et al. 2010; Bartley et al. 2013).

Despite advances in identifying xylan-biosynthetic genes, it remains unknown whether their proteins form a complex that can synthesize a xylan polymer in vitro. As is so often the case, the biochemistry is lagging behind the molecular genetic studies.

3.2 Xyloglucan Structure and Biosynthesis

Xyloglucan (XyG) represents a major group of hemicellulosic polymers found mainly in the primary cell walls of dicots (Carpita and Gibeaut 1993; Faik 2013). Within the cell wall, XyG interacts with cellulose microfibrils via hydrogen bonding to form a subnetwork that reinforces the load-bearing function of cellulose in the wall (Hayashi and Maclachlan 1984; Hayashi et al. 1987). All XyG polymers have a backbone of β-(1,4)-linked D-glucose (Glc) residues similar to that of cellulose but are branched to various degrees with α-(1,6)-linked Xyl residues (Fig. 3b). Up to 75 % of the β-glucosyl residues in the backbone can be substituted with Xyl residues in a regular manner (Hayashi 1989; Fry et al. 1993; Faik et al. 1997; Ray et al. 2004; Hoffman et al. 2005). Xyl residues can be further substituted with β-(1,2)-D-galactosyl (Gal) or α-L-fucosyl-β-(1,2)-D-Gal (Fuc-Gal) side chains (Fig. 3b) (Hoffman et al. 2005; Albersheim et al. 2010). Other minor monosaccharides are also found in the side chains in XyGs. For example, Xyl residues can be substituted by α-(1,2)Ara*f* or β-Ara*f*-(1,3)-α-Ara*f*-(1,2) side chains called S and T side chains, respectively (Table 3, York et al. 1996; Ray et al. 2004; Hoffman et al. 2005).

Table 3 lists a single-letter code as a simplified nomenclature of XyG side chains where G represents unsubstituted Glc residues and X designates Glc substituted with Xyl (Fry et al. 1993; Vincken et al. 1997; Buckeridge et al. 1997; Pena et al. 2008).

Furthermore, the Gal residue in the L side chain can be substituted by another α-(1,2)-L-Gal residue to generate the J side chain as in jojoba (Hantus et al. 1997). Pena et al. (2008) described XyGs from avascular and seedless vascular land plants (liverwort, mosses, and hornworts) that contain GalA β-(1,4)-linked that extends the Xyl side chains and branched Xyl side chains (named P and Q side chains, Table 3). They also reported the presence of α-L-Ara*p* attached to the C2 of Xyl (D side chain, Table 3) or attached to the Xyl that is branched with a β-(1,4)-Gal (M side chain) or attached to a Xyl bearing a β-(1,6)Gal-(1,6)-β-Gal-disaccharide (N side chain).

Depending on the degree of substitution of Glc residues in the backbone, XyGs can be grouped into at least three types: XXXG-type, where every three Glc residues out of four are branched with Xyl residues, and XXGGG- and XXGG-types, which have only two consecutive Glc residues substituted with Xyl residues. XXXG-type XyG is mostly found in the primary cell walls of dicots (O'Neill and York 2003), whereas XXGGG- and XXGG-types are found in Poaceae (monocots) and Solanaceae with low Gal content and little or no Fuc residues (Hayashi 1989; York et al. 1996; Gibeaut et al. 2005; Hoffman et al. 2005). Members of the Poaceae and Solanaceae have low amounts of XyG in their cell wall (1–5 % w/w), which is in sharp contrast with dicots, where XyG represents up to 25 % (Pauly et al. 2001; O'Neill and York 2003; Albersheim et al. 2010). Although it is believed that XyG is not fucosylated in grasses, studies in *Festuca arundinacea*, a monocot, showed the presence of fucosylated XyG (McDougall and Fry 1994). Since *Physcomitrella* (a bryophyte) produces a XyG of XXGGG-type (~40 % of Glc residues are substituted) and *Marchantia* (a liverwort) produces a XyG of XXGG-type (~50 % of Glc residues are branched), it is believed that these branching patterns appeared evolutionarily before the XXXG-type branching pattern. It is noteworthy that XyG has not been found in *charophytes* (Popper and Fry 2003; Moller et al. 2007; Popper 2008).

For simplicity, XyG biosynthesis can be divided into three steps: *i*) "xylosyl-Glc" backbone synthesis, (*ii*) extension of xylosyl side chains, and (*iii*) modification of XyG polymers. In *Arabidopsis*, this process would require at least seven specific GT activities: a β-(1,4)-glucan synthase to synthesize the XyG backbone; three α-(1,6)xylosyltransferases to transfer Xyl residues onto the three Glc residues of the β-glucan chain (e.g., for the three X side chains in XXXG-type XyG); two β-(1,2) galactosyltransferases to extend the two X side chains with Gal residues and generate XLLG and XXLG; and an α-(1,2)fucosyltransferase to transfer Fuc residues onto XLLG and XXLG to generate XLFG and XXFG subunits, respectively (Faik et al. 1997; del Bem and Vincentz 2010). Other transferases, such as acetyltransferases, are involved in the incorporation of acetyl groups onto Gal and Glc of XyG (Kiefer et al. 1989; Gille et al. 2011).

Table 3 Single letter code nomenclature for naming XyG side chain structures (Fry et al. 1993; Pena et al. 2008)

Code letter	Structure represented
G	| β-D-Glc |
X	| α-D-Xyl-(1,6)-β-D-Glc |
L	| β-D-Gal-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
F	| α-L-Fuc-(1,2)-β-D-Gal-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
S	| α-L-Ara*f*-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
D	| α-L-Ara*p*-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
T	| α-L-Ara*f*-(1,3)-α-L-Ara*f*-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
P	D-Gal β(1,4)| | β-D-GalA-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
Q	D-Gal β(1,4)| | β-D-Gal-(1,4)-β-D-GalA-(1,2)- α-D-Xyl-(1,6)-β-D-Glc |
M	D-Gal β(1,4)| | α-L-Ara*f*-(1,2)-α-D-Xyl-(1,6)-β-D-Glc |
N	D-Gal β(1,6)| D-Gal β(1,4)| | α-L-Ara*f*-(1,2)- α-D-Xyl-(1,6)-β-D-Glc |

The in vitro synthesis of "xylosyl-Glc" backbone of XyG requires the cooperative incorporation of Glc and Xyl residues from UDP-Glc and UDP-Xyl (Ray 1980; Gordon and Maclachlan 1989; Faik et al. 1997). Members of *Arabidopsis* cellulose synthase-like C (*CSLC*) subfamily, namely, *CSLC4* (At3g28180), *CSLC5* (At4g31590), and *CSLC6* (At3g07330), have been implicated in the synthesis of the β-glucan backbone of XyG (Cocuron et al. 2007). However, direct evidence of glucan synthase activity for any members of the CSLC family is still lacking. The characterization of AtCSLC4 indicated that it is Golgi localized, with its catalytic domain on the cytosolic side of the Golgi membrane (Davis et al. 2010). *AtCSLC* homologs exist in the genomes of plants from different species, including *bryophytes* and *lycopodiophytes* (Pena et al. 2008). *Arabidopsis* genes belonging to the GT34 family have been identified to encode XyG:α-(1,6)xylosyltransferases (XXTs) (Faik et al. 2002; Cavalier and Keegstra 2006; Vuttipongchaikij et al. 2012). This GT34 family contains seven *Arabidopsis* members, but only *AtXXT*1 (At3g62720), *AtXXT2* (At4g2500), and *AtXXT5* (At1g74380) were associated with XyG biosynthesis through biochemical characterization (Faik et al. 2002; Cavalier and Keegstra 2006) or genetic studies (Vuttipongchaikij et al. 2012). A recent study using bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation demonstrated that *Arabidopsis* XXT1, XXT2, XXT5, and CSLC4 proteins are able to form homo- and heterodimer complexes in Golgi membranes (Chou et al. 2012). Further, AtXXT1 and/or AtXXT2 have the capacity to incorporate the three Xyl residues onto a celloheptaose oligosaccharide (used as acceptor) in an in vitro assay (Cavalier and Keegstra 2006). Intriguingly, *xxt1*/*xxt2* double mutant plants in *Arabidopsis* have cell walls lacking detectable XyG without any growth defects (Cavalier et al. 2008). Considering the XyG content in *Arabidopsis* (up to 30 %w/w), one would expect the absence of XyG to have a major effect on the growth of these *Arabidopsis* mutants.

For GTs that extend X side chains of XyGs, *Arabidopsis* XyG fucosyltransferase (XyG-FUT) was the first GT to be identified in XyG biosynthesis (Perrin et al. 1999). These authors used a XyG-FUT enzyme assay (Camirand and Maclachlan 1986) to purify XyG-FUT activity from pea. Peptide sequence information was obtained from the pea protein preparation, which allowed the cloning of a homologous gene, called *AtFUT1* (At2g03220), from *Arabidopsis*. AtFUT1 belongs to the GT37 family and adds α-(1,2)fucosyl residues to the terminal galactosyl residue (e.g., L side chains closest to the reducing end in XXLG and XLLG subunits of XyG polymer). AtFUT1 does not act on oligosaccharides, but requires an acceptor with a minimum of three subunits (Faik et al. 2000; Shipp et al. 2008). *Arabidopsis* mutants in AtFUT1 (called *mur2*) produce XyG lacking Fuc, but with no visible phenotypes (Vanzin et al. 2002; Perrin et al. 2003), indicating that XyG fucosylation may not have an important role in plant growth. Thus, the role of fucosylated XyG in the physiology of plant growth is still unknown.

Screening of an EMS *Arabidopsis* mutant collection (called *mur* mutants) allowed for the identification of *MUR3* gene (At2g20370 from the GT47 family) that encodes a XyG galactosyltransferase (Madson et al. 2003). In an analysis of ten

Arabidopsis genes homologous to *MUR3*, a T-DNA insertion in the gene *GT18* (At5g62220) produced *Arabidopsis* plants with ~13 % less Gal in their cell walls, suggesting that this gene may also encode a XyG galactosyltransferase (Li et al. 2004). Although MUR3 can only transfer β-(1,2)-Gal onto the third Xyl residue in the repeating unit XXXG (Madson et al. 2003), the exact biochemical function of GT18 is unknown. One possible role is that GT18 is responsible for the addition of a Gal residue to the second X side chain in XyG subunit (i.e., XXXG), as the third X side chain has never been found decorated with a sugar in any XyG studied to date. More recently, an *Arabidopsis* gene from the GT47 family has been shown to catalyze the transfer of a galacturonic acid (GalA) onto Xyl residues, hence forming α-D-GalA-(1,2)-xylosyl side chains in the XyG. This type of side chain was found in XyG from *Arabidopsis* root hairs and is catalyzed by XyG-specific galacturonosyltransferase 1 (AtXUT1, At1g63450) (Pena et al. 2012).

Major post-biosynthetic modifications of XyG polymers include the addition of acetyl groups onto Gal residues in L and F side chains, as well as onto the Glc residues of the glucan backbone (Kiefer et al. 1989; Jia et al. 2005). *O*-acetylation of Gal residues in L side chains is catalyzed by two putative XyG-specific acetyltransferases: AtAXY4 (At1g70230) and AtXY4L (At3g28150) (Gille et al. 2011).

3.3 Mannan Structure and Biosynthesis

Mannans are a group of hemicellulosic polysaccharides with a β-(1,4)-glycan backbone that can consist either entirely of β-(1,4)-linked mannose (Man) residues or a combination of β-(1,4)-linked Glc and Man residues (Fig. 3c; Dhugga et al. 2004; Scheller and Ulvskov 2010). Both backbones can be substituted with α-(1,6)-linked Gal residues to produce galactomannan (GM) or galactoglucomannan (GGM, Fig. 3c; Moreira and Filho 2008). GM and GGM are minor components of primary cell walls but can represent one of the major polymers in secondary cell walls in dicot plants (Buckeridge et al. 2000b; Zhong and Ye 2009). GGM, on the other hand, is mainly found in the endosperm, as storage polysaccharides, of seeds in many plants, such as legumes and palms (Buckeridge et al. 2000a; Dhugga et al. 2004).

The first β-(1,4)-mannan synthase (ManS) was identified in guar seeds (Dhugga et al. 2004). ManS belongs to CSLA subfamily. Functional analysis of *Arabidopsis* members of the CSLA subfamily allowed the identification of CSLA2 (At5g22740), CSLA3 (At1g23480), CSLA7 (At2g35650), and CSLA9 (At5g03760) proteins as putative ManSs. Heterologous expression of these genes demonstrated their ManS activity in vitro (Liepman et al. 2005). Topology analysis of CSLA9 revealed that the protein has five transmembrane domains with the catalytic domain facing the Golgi lumen, which means GDP-mannose needs to be transported into the Golgi (Davis et al. 2010). More recently, a Golgi-localized mannan synthesis-related (MSR) gene was found to be highly and specifically expressed in fenugreek endosperm (Wang et al. 2013). In *Arabidopsis*, the

homologs MSR1 (At3g21190) and MSR2 (At1g51630) are highly co-expressed with the three ManSs, CSLA2, CSLA3, and CSLA9 (Wang et al. 2013), supporting a role, yet to be determined, in mannan synthesis (Wang et al. 2013).

Genetic studies in *Arabidopsis* revealed that mannan also plays an important role in embryo development and pollen tube growth, as an *Arabidopsis* mutant in the putative mannan synthase 7 gene (*AtCSLA7*) has an embryo lethal phenotype (Goubet et al. 2003).

4 Structure and Biosynthesis of Pectic Polymers

Pectin polymers form a gel-like matrix surrounding the "cellulose-hemicellulose" sub-network of the primary cell wall, as described in the model proposed by Carpita and Gibeaut (1993). These polymers are also found the in middle lamella (McClendon 1964; Yamauchi et al. 1986), the outer most layer of the primary cell wall that permits cell-to-cell contact and adhesion. Pectins play an important role in plant physiology, including defense, morphogenesis, and signaling (Ridley et al. 2001; Willats et al. 2001; Mohnen 2008; Peaucelle et al. 2012). Pectic polysaccharides are a source of oligogalacturonides (OGAs) released after pathogen attack, which can act as elicitors to trigger plant responses to pathogens (Lee et al. 1999; Galletti et al. 2008; Osorio et al. 2008).

Pectin polymers are a group of structurally complex polysaccharides characterized by their rich content in GalA (up to 70 %; Mohnen 2008; Atmodjo et al. 2013). This high proportion of GalA gives pectins a net negative charge. Three major groups of pectins are found in the wall: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and the substituted rhamnogalacturonan II (RG-II) (Fig. 3d; Mohnen 2008; Caffall and Mohnen 2009). The most abundant group of pectins (~65 % of total pectin) is HG, which is a linear homopolymer of α-(1,4)-linked GalA residues. Many of the GalA residues of HG are methyl esterified at the C6 carboxyl position and can be acetylated at the *O*-2 and *O*-3 positions (Fig. 3d; Mohnen 2008). The second most abundant pectic polymer is RG-I (~20–35 % of total pectin), which has a backbone of the repeating unit [4)-α-D-GalA-(1,2)-α-L-Rha-(1,]$_n$. Backbone Rha residues of RG-I are decorated with oligosaccharides, including L-Ara*f*-(1,5)-α-L-Ara*f*-(1,2)-α-L-Ara*f*-(1,3)-β-Gal-(1,4), β-Gal-(1,6)-β-Gal-(1,4), β-Gal-(1,6)-β-Gal-(1,4)-β-Gal-(1,4), and α-L-Fuc-(1,2)-β-Gal-(1,4)-β-Gal-(1,4)-Rha (Fig. 3d; McNeil et al. 1980; Lau et al. 1985). RG-II polymers represent only ~10 % of total pectin but are known for their structural complexity. They have a backbone of α-(1,4)-linked GalA residues similar to HG but are substituted with at least four types of oligosaccharides (called side chains A–D, Fig. 3d): side chain A (an octasaccharide) and side chain B (a nonasaccharide) are *O*-2 linked to GalA of the HG backbone, while side chains C and D (both disaccharides) are *O*-3 linked to the HG backbone (Ishii and Matsunaga 1996; O'Neill et al. 2001; Matsunaga et al. 2004). A small proportion of RG-II contains α-D-apiose (3-*C*-hydroxylmethyl-D-erythrose, a pentose) to form a polymer called apiogalacturonan (AGA) (Hart and Kindel 1970; Duff 1965;

Longland et al. 1989; McNeil et al. 1984). Apiose residues in RG-II serve as important cross-linking points with HG side chains (Stevenson et al. 1988) and borate ions to form cyclic diesters (Ishii et al. 1999; O'Neill et al. 1996). RG-II/borate cross-linking is critical for the structural integrity of the cell wall (O'Neill et al. 2001). A *Nicotiana plumbaginifolia* mutant, *nolacH18*, illustrates the importance of borate cross-linking for cell wall integrity, as this mutant is defective in organogenesis due to the loss of cell-cell adhesion (Iwai et al. 2002). HG can also be substituted with Xyl residues to form a type of pectin polymer called xylogalacturonan (XGA) (Ovodova et al. 2000; Zandleven et al. 2006, 2007). Covalent linkages via their backbones connect HG to RG-I, RG-II, and XGA (Nakamura et al. 2002; Zandleven et al. 2007; Round et al. 2010; Atmodjo et al. 2013). Figure 3d shows a schematic presentation of HG, RG-I, RG-II, AGA, and XGA domains of pectic polymers.

Pectic polysaccharides are synthesized in the Golgi lumen and transported to the surface of plant cells (Zhang and Staehelin 1992; Mohnen 2008). According to Mohnen (2008), at least 67 different transferases, including GTs, methyltransferases (MethylTs), and acetyltransferases (AcetylTs), are predicted to be required for pectin synthesis. Recently, Atmodjo et al. (2013) proposed two models for pectin synthesis. One model, called the consecutive GT model, postulates that a set of GTs are associated to synthesize the backbone and branches of pectic polysaccharides (including both HG and RG-I) by processively adding sugars from nucleotide-sugar donors onto acceptors. This process occurs through the *cis*-, *medial*-, and *trans*-Golgi stacks. The second model, called the domain synthesis model, hypothesizes that oligosaccharides are elongated to form HG or RG-I domains, which are transferred en bloc (by oligosaccharyltransferase) onto another growing domain. Such domain transfer might occur repeatedly until a full pectin polymer is formed (Atmodjo et al. 2013).

Several biosynthetic GTs involved in pectin synthesis have been identified, including an *Arabidopsis* HG:α-(1,4)galacturonosyltransferase (HG:GalAT), named as GALACTURONOSYLTRANSFERASE 1 (GAUT1, At3g61130) (Sterling et al. 2006). GAUT1 belongs to the GT8 family and was biochemically demonstrated to catalyze the transfer of GalA, from UDP-GalA, onto the nonreducing end of endogenous and exogenous HG acceptors (Sterling et al. 2006). More recently, Atmodjo et al. (2011) showed that GAUT1 and GAUT7 (At2g38650) form a functional protein complex in the Golgi apparatus.

RG-I biosynthesis, on the other hand, requires both GalAT and rhamnosyltransferase (RhaT) activities to generate the backbone, as well as multiple GalTs and AraTs for the synthesis of the side chains. To date, in vitro synthesis of the RG-I backbone has not been achieved (Atmodjo et al. 2013), pointing out the difficulty of studying pectin biosynthetic machinery.

For side chain additions onto pectin backbones, *Arabidopsis* GALACTAN SYNTHASE 1 (GALS1) of the GT92 family has been shown to act as a β-(1,4)-galactan:β-(1,4)-GalT that transfers successive β-(1,4)-galactosyl residues, from UDP-Gal, onto β-(1,4)-galactopentose oligosaccharides, forming β-(1,4)-galactan

side chains of RG-I (Liwanag et al. 2012). A protein complex of ARABINAN DEFICIENT 1 (ARAD1, At2g35100) and its homolog ARAD2 (At5g44930) were proposed as RG-I arabinan:α-(1,5)AraTs responsible for the synthesis of pectin arabinan side chains (Harholt et al. 2012). However, the precise biochemical function of these proteins remains to be demonstrated. RHAMNOGALACTURONAN XYLOSYLTRANSFERASE 1 (RGXT1, At4g01770) and RGXT2 (At4g01750), both from the CAZy GT77 family, were identified as α-(1,3)XylTs responsible for the addition of Xyl residues during RG-II synthesis (Fig. 3d; Egelund et al. 2006). Another XylT, At5g33290 from GT47 family (called XYLOGALACTURONAN DEFICIENT 1, XGD1), shown to catalyze the transfer of β-Xyl, from UDP-Xyl, onto the C3 position of GalA residues in HG (Jensen et al. 2008).

Pectin polymers can be heavily methyl esterified and also *O*-acetylated. Methyl groups are transferred from S-adenosyl-L-methionine (SAM) donors onto the C6 carboxylate groups of GalA residues in HG by enzymes called pectin HG: methyltransferase (HG:MT) (Goubet et al. 1998). *Arabidopsis QUA2* (At1g78240) and *QUA3* (At4g00740) and Cotton Golgi-Related 3 (*CGR3*, At5g65810) may encode such HG:MTs (Mouille et al. 2007; Held et al. 2011). Methyl esterification of the C6 carboxylic acid gives HG a more neutral charge, limits Ca^{+2} binding, and likely alters its physical properties. *O*-acetyl groups, on the other hand, are transferred onto different residues and at multiple positions, depending of the species (Gille and Pauly 2012). For example, sugar beet, potato, and spinach HG polymers contain *O*-acetyl groups at the C2 and/or C3 position of the GalA residues in the HG backbone (Keenan et al. 1985; Kouwijzer et al. 1996; Ishii 1997; Perrone et al. 2002). Depending on the tissues and/or species, the degree of *O*-acetylation of pectin varies from 0 % to 90 % (Schols and Voragen 1994; Voragen et al. 1998). Acetyl-CoA was shown to be the acetyl donor for pectin acetyltransferase enzymes, which are presumably located in the Golgi apparatus (Pauly and Scheller 2000). In the RG-I backbone, GalA residues can be *O*-acetylated at the C2 and/or C3 positions in bamboo, cotton, sycamore, potato, and spinach (Komalavilas and Mort 1989; Schols et al. 1990; Lerouge et al. 1993; Ishii 1997; Kouwijzer et al. 1996; Perrone et al. 2002). Furthermore, *O*-acetylation of the C3 of Rha residues was detected in okra, *Abelmoschus esculentus* (Senkhamparn et al. 2009). In addition, *O*-acetylation of 2-*O*-methyl-fucosyl residues of RG-II side chain was also identified in sycamore (Whitcombe et al. 1995; O'Neil et al. 2004). More recently, *Arabidopsis reduced wall acetylation 2* mutant plants (*rwa2*, corresponding to the At3g06550 gene) had reduced acetylation of pectin and non-pectic polysaccharides (Manabe et al. 2011). *O*-acetylation of pectins interferes with divalent cation binding to pectin, thus affecting the solubility and gelation properties of these polymers (Pippen et al. 1950; Rombouts and Thibault 1986; Kouwijzer et al. 1996; Ralet et al. 2003, 2005). Pectin *O*-acetylation also limits enzymatic breakdown (Renard and Jarvis 1999; Schols et al. 1990; Kauppinen et al. 1995). Thus, it is not surprising that plants regulate their growth through changes in the degree of methyl esterification and acetylation of pectin during the development.

5 Hydroxyproline-Rich Glycoproteins (HRGPs)

HRGPs, which include the extensins (EXTs) and arabinogalactan-proteins (AGPs), represent the third sub-network in plant cell walls. These glycoproteins represent some of the most abundant and highly modified proteins in plants. Much of the recent work on HRGPs has focused on the biosynthesis of their carbohydrate moieties and the functional contributions of these moieties.

5.1 Structure and Biosynthesis of Extensins (EXTs)

Extensins are a family of hydroxyproline-rich glycoproteins characterized by the repeating pentapeptide sequence Ser-Hyp-Hyp-Hyp-Hyp and by the presence of short oligoarabinosides attached to the continuous Hyp residues (Showalter 1993; Kieliszewski and Lamport 1994). Single Gal residues also are attached to some of the serine (Ser) residues in EXTs. These glycoproteins are rodlike proteins that exist in a polyproline II helix stabilized by the oligoarabinosides wrapping around the protein backbone (Van Holst and Varner 1984; Shpak et al. 2001). Some EXTs contain tyrosine residues that are modified to form intramolecular isodityrosine cross-links or intermolecular di-isodityrosine or pulcherosine cross-links (Brady and Fry 1997; Brady et al. 1998; Held et al. 2004). The intramolecular cross-links cause "kinks" in the rods, while the intermolecular cross-links result in the formation of extensin networks. Such cross-links are thought to be important in regulating plant growth and development and in forming a defensive barrier to plant pathogens. It should be noted that EXT-pectin cross-links also exist, but the extent and functional significance of these cross-links are unknown (Qi et al. 1995).

Biosynthesis of EXTs consists of several steps: (1) translation of EXT mRNAs, (2) signal peptide-mediated translocation of EXT into the rough endoplasmic reticulum, (3) posttranslational modification of peptidyl-proline (Pro) residues in the EXT core protein to form hydroxyproline (Hyp), (4) glycosylation of the EXTs in the endoplasmic reticulum (ER) and possibly in the Golgi, (5) secretion of EXTs to the cell surface and integration into cell wall, and (6) formation of intramolecular and intermolecular EXT cross-links in the cell wall. EXT glycosylation is the main focus here with Fig. 4 providing an overview of the various enzymes responsible for EXT glycosylation and Table 4 listing more detailed information about these GT enzymes and their genes.

A Ser galactosyltransferase gene (*SGT1*) was recently identified in *Chlamydomonas reinhardtii*, and homologous genes subsequently were identified in *Arabidopsis* and *Nicotiana tabacum* (Saito et al. 2014). The SGT1 enzyme is found mainly in the ER. Interestingly, SGT1 is not present in the CAZy database and was initially identified by biochemical purification of proteins having serine *O*-α-galactosyltransferase activity. In contrast, at least five distinct arabinosyltransferases referred to as hydroxyproline O-β-arabinosyltransferases (HPAT1–3), reduced residual arabinose (RRA1–3), xyloglucanase 113 (XEG113), EXT arabinose deficient (ExAD), and another unknown arabinosyltransferase are responsible

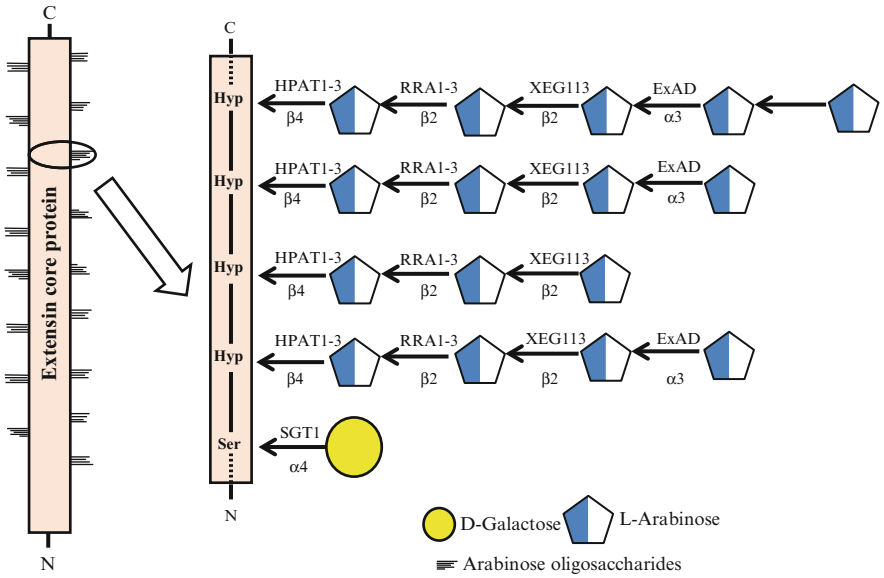

Fig. 4 Sites of action of glycosyltransferases acting on extensins are depicted within a representative extensin glycomodule sequence found in an extensin molecule

for sequential addition of 1–5 arabinosyl residues (making the oligoarabinoside) to continuous hydroxyproline residues (Velasquez et al. 2012; Saito et al. 2014). All these enzymes are thought to act in the Golgi. It should also be mentioned that the degree of EXT arabinosylation is variable, although the addition of 3 or 4 Ara residues per hydroxyproline is common. The first Ara is added directly to hydroxyproline residues in the protein backbone by the action of hydroxyproline *O*-β-arabinosyltransferase activity (HPAT1–3) (Ogawa-Ohnishi et al. 2013). At least three *Arabidopsis* genes possess this enzymatic activity, and they are related to, but not part of, the GT8 family in CAZy. The second Ara is added in a β-(1,2)-linkage by the action of one of the three RRA1–3 enzymes found in *Arabidopsis* (Egelund et al. 2007; Velasquez et al. 2011). These enzymes are members of the GT77 family and were identified from genetic mutants. The third Ara is also added in a β-(1,2)-linkage, but by the action of another GT, xyloglucanase 113 (XEG113) (Gille et al. 2009). This *Arabidopsis* enzyme is also a member of the GT77 family, and it was serendipitously identified by a novel mutant screen in which *Arabidopsis* cells were treated with a fungal endoglucanase. The fourth Ara is added in an α-(1,3)-linkage by the action of the EXT-arabinose-deficient (ExAD) enzyme (Petersen, personal communication). This enzyme is a member of the GT47 family and was identified by a genetic mutant screen similar to the one used to identify RRA1–3. An as yet unidentified GT enzyme adds the fifth Ara. It is important to keep in mind that not all EXTs exhibit hydroxyproline residues having all five Ara residues. Moreover, the anomeric nature of this fifth linkage is unknown.

Table 4 Information on the enzymes, genes, and mutants for extensin glycosylation

Enzyme abbreviation	Enzyme	GT family	Gene identifier	Mutants	Mutant phenotypes	References
SGT1	Serine *O*-α-galactosyltransferase	–	At3g01720	*sgt1* (SALK_059879C and SALK_054682)	Longer roots and larger leaves	Saito et al. 2014
HPAT1–3	Hydroxyproline *O*-β-arabinosyltransferase	GT8-like	At5g25265	*hpat1* (GABI_298B03), *hpat2* (SAIL_178_H04), *hpat3* (SALK_047668)	Pleiotropic phenotypes affecting growth and development	Ogawa-Ohnishi et al. 2013; Velasquez et al. 2011
			At2g25260			
			At5g13500			
RRA1–3	β-1,2-Arabinosyltransferase	GT77	At1g75120	*rra1* (SAIL_590_G09 and Garlic_76_G04)	Reduced root hair growth	Egelund et al. 2007
			At1g75110	*rra2* (Garlic_244_A03 and SAIL_70_D08)		Velasquez et al. 2011
			At1g19360	*rra3* (GABI_233B05)		
XEG113	β-1,2-Arabinosyltransferase	GT77	At2g35610	*xeg113-1* (SALK_066991)	Reduced root hair growth	Gille et al. 2009; Velasquez et al. 2011
				xeg113-3 (SALK_058092)		
EXAD	α-1,3-Arabinosyltransferase	GT47	–	–	–	Peterson, personal communication

Thus, at least five different GT enzymes are responsible for these five biochemically distinct arabinosyl linkages, even though two are in β-(1,2)-linkages. In other words, these various GTs are highly specific, and there are at least one or more enzymes for each sugar that is added. Moreover, since multiple genes encode at least two of these enzymes, this raises the question as to whether these genes and the enzymes they encode are redundant or nonredundant. Related to this question is whether these enzymes act on all EXT proteins or only on a subset of EXTs. Finally, another question is whether these enzymes act in a complex. To date, there is no evidence for the existence of such a complex, and the observation that EXT arabinosylation is variable may lend support to the absence of complex formation.

Genetic mutant analysis is being used to determine the functional contribution of Ser galactosylation and Hyp arabinosylation to EXT function in plants. A variety of mutant phenotypes have been observed to date (Table 4). For example, *sgt1* mutants have longer roots and larger leaves, while *hpa1–3* mutants have a variety of effects on growth and development, whereas the *rra1–3* and *xeg113* mutants demonstrate reduced root hair growth. Given the potential for redundancy at each of these glycosylation steps, it will be particularly important to examine biochemical phenotypes and correlate them to observed physiological/developmental phenotypes. Thus, increasingly more severe physiological and biochemical mutant phenotypes may be observed as redundant genes are systematically eliminated in a series of single, double, triple, etc. mutants.

5.2 Structure and Biosynthesis of Arabinogalactan-Proteins (AGPs)

AGPs are characterized by their abundance of Pro (which is modified to Hyp), alanine, serine, and threonine, by their extensive glycosylation of noncontiguous hydroxyproline residues with arabinose-/galactose-rich (AG) polysaccharide side chains, and by their ability to react with a chemical called Yariv reagent (Showalter 2001; Seifert and Roberts 2007). The AG polysaccharide side chains are composed of β-(1,3)-galactan backbones decorated with β-(1,6)-galactose side chains that are further decorated with α-L-Ara residues as well as other sugars, such as β-(methyl) glucuronic acid, α-rhamnose, and α-L-fucose, which are present in lesser amounts. Based on microscopic analyses and how the numerous Hyp-AG polysaccharides are predicted to protrude or wrap around the core protein, AGPs can exist as spheroidal or rodlike molecules. These structures are represented by the so-called wattle blossom and twisted hairy rope models of AGP structure. There are some reports that AGPs are capable of cross-linking either to one another or to other cell wall molecules, namely, pectin and arabinoxylan (Kjellbom et al. 1997; Tan et al. 2013). While definitive chemical evidence for AGP-AGP cross-linking is lacking, chemical evidence for AGP-pectin and AGP-pectin-arabinoxylan cross-linking is compelling. The extent and degree of such cross-linking reactions as well as the functional significance of such cross-linking await further investigation. It should also be noted that many AGPs encode a C-terminal glycosylphosphatidylinositol

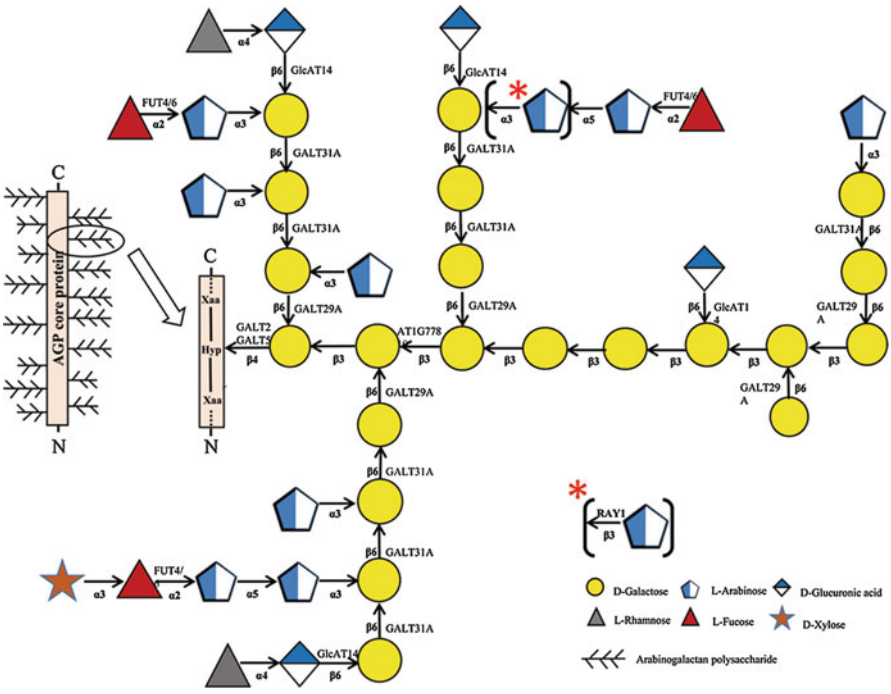

Fig. 5 Sites of action of known glycosyltransferases acting on AGPs are depicted within a representative AGP glycomodule sequence found within an AGP molecule

(GPI) anchor addition sequence, which results in a GPI anchor that serves to tether such AGPs to the outer leaflet of the plasma membrane while the rest of the molecule resides in the cell wall. Presumably, such AGPs are released from the plasma membrane to the wall by the action of specific phospholipases.

Biosynthesis of AGPs consists of several steps: (1) translation of mRNAs encoding the core protein of AGPs; (2) signal peptide-mediated translocation of AGP core proteins into the rough endoplasmic reticulum; (3) GPI anchoring of the C-terminus of the AGP core protein if specified; (4) posttranslational modification of Pro residues to form Hyp; (5) glycosylation of AGP core proteins, which likely begins in the ER but is more extensive in the Golgi; (6) secretion of AGPs to the cell wall or to the plasma membrane/cell wall (for GPI-anchored AGPs); and (7) glycosyl hydrolase-mediated hydrolysis of AGPs in the cell wall which is possible, as is the formation of AGP cross-links in the wall in some cases. AGP glycosylation is the main focus here with Fig. 5 providing an overview of the GT enzymes responsible for the various glycosylation reactions and Table 5 listing more detailed information about these GT enzymes and the genes that encode them.

Several of the GTs responsible for AGP glycosylation are known and cloned; however, many await discovery as previously reviewed (Tan et al. 2012; Knoch et al. 2014). Initial glycosylation of AGPs is catalyzed by a Hyp *O*-β-galactosyltransferase (GALT). At least two enzymes have such an activity in *Arabidopsis*, GALT2

Table 5 Information on the enzymes, genes, and mutants for AGP glycosylation

Enzyme abbreviation	Enzyme	GT family	Gene identifier	Mutants	Mutant phenotypes	References
GALT2	Hydroxyproline *O*-β-galactosyltransferase	GT31	At4g21060	*galt2-1* (SALK_117233)	Reduced root growth and root tip swelling under salt stress, reduced seed mucilage adherence	Basu et al. 2013
				galt2-2		
GALT5			At1g74800	(SALK_141126)		Basu et al. unpublished data
				galt5-1 (SALK_105404)		
				galt5-2 (SALK_115741)		
AT1G77810	β-1,3-Galactosyltransferase	GT31	At1g77810	Not reported	–	Qu et al. 2008
GALT31A	β-1,6-Galactosyltransferase	GT31	At1g32930	*galt31A* (FLAG_379B06)	Embryo lethal mutant	Geshi et al. 2013
GALT29A	β-1,6-Galactosyltransferase	GT29	At1g08280	Not reported	–	Dilokpimol et al. 2014
GlcAT14A	β-1,6-Glucuronosyltransferase	GT14	At5g39990	*glcat14a-1* (SALK_06433)	Enhanced cell elongation in seedlings	Knoch et al. 2013; Dilokpimol and Geshi 2014
GlcAT14B			At5g15050	*glcat14a-2* (SALK_043905)		
GlcAT14C			At2g37585			
FUT4	α-1,2-Fucosyltransferase	GT37	At2g15390	*fut4-1* (SAIL_284_B)	Reduced root growth under salt stress	Wu et al. 2010b; Liang et al. 2013; Tryfona et al. 2014
				fut4-2 (SALK_12530)		
FUT6	α-1,2-Fucosyltransferase	GT37	At1g14080	*fut6-1* (SALK_0783) and *fut6-2* (SALK_09950)	Reduced root growth under salt stress	Wu et al. 2010b; Liang et al. 2013; Tryfona et al. 2014
RAY1	β-Arabinofuranosyltransferase	GT77	At1g70630	*ray1-1* (SALK_053158) *ray1-2* (GABI_001C09)	Reduced root growth and reduced rosette size and inflorescence	Gille et al. 2013

and GALT5 (Basu et al. 2013). These two enzymes are members of a small family that includes six members (named GALT1–6) in the GT31 family. Interestingly, GALT1 does not use AGPs as substrates, but instead catalyzes the addition of Gal to Lewis a structures (Strasser et al. 2007). GALT3, 4, and 6 remain to be examined. GALT2 was localized to the ER and Golgi, whereas GALT5 was found only in the Golgi. Both enzymes were initially identified using bioinformatics, which predicted a GALT domain as well as a novel galectin domain, and were verified using heterologous expression coupled with an in vitro Hyp-GALT biochemical assay as well as by biochemical analysis of *galt2* and *galt5* genetic mutants.

Only one GALT (At1g77810) is reported to act in the synthesis of the β-(1,3)-galactan backbone (Qu et al. 2008). At1g77810 is a member of the GT31 family. This GT was expressed in COS cells and showed that Gal was added to a synthetic β-(1,3)-galactose disaccharide; however additional supporting evidence is needed. Moreover, it is likely that several other β-(1,3)-GALTs will ultimately be identified, particularly since the GT31 family is so large (20 members, each with a GALT domain, are predicted for *Arabidopsis*).

At least two β-(1,6)-GALTs act on AGPs, GALT31A and GALT29A. The two enzymes, however, have somewhat different activities and are members of two different CAZy GT families as their names indicate. GALT31A serves to elongate β-(1,6)-galactan chains, whereas GALT29A has this activity as well as another related activity, adding β-(1,6)-Gal to the β-(1,3)-galactan backbone (Geshi et al. 2013; Dilokpimol et al. 2014). In addition, these two enzymes are reported to interact with one another based on fluorescence resonance energy transfer (FRET) analysis. Whether other AGP GTs exist in such an enzyme complex remains to be determined.

Three β-glucuronosyltransferases (GlcAT), GlcAT14A, GlcAT14B, and GlcAT14C, are responsible for the addition of glucuronic acid (GlcA) residues to AGPs (Knoch et al. 2013; Dilokpimol and Geshi 2014). All three enzymes are members of the GT14 family and are reported to add GlcA to both β-(1,6)- and β-(1,3)-galactan chains. It should be noted that GlcA imparts a negative charge to AGPs that contain them and also serves as a potential site for calcium binding; both of these properties likely have functional and physiological ramifications.

Two *Arabidopsis* α-fucosyltransferases (FUTs), FUT4 (Atg2g15390) and FUT6 (At1g14080), serve to add α-(1,2)-fucose (Fuc) residues to AGPs (Wu et al. 2010b). However, these two enzymes have different substrate specificities. Both of these enzymes are members of the GT37 family. Interestingly, heterologous expression of *FUT4* in tobacco BY2 cells (which have AGPs lacking Fuc) seems to incorporate Fuc residues and facilitate the incorporation of xylose residues on AGPs (Wu et al. 2010b; Tryfona et al. 2014). FUT6 does not produce this extra-modification of AGP. However, it should be noted that not all AGPs contain Fuc.

Finally, only one arabinosyltransferase, Reduced Arabinose Yariv1 or RAY1 (At1g70630), is reported to act on AGPs. One thing that remains puzzling about this enzyme is that it catalyzes the addition of β-linked Ara residues, whereas all the Ara residues reported in AGPs over the years are α-linked.

In summary, these 11 different enzymes are responsible for glycosylation of AGPs; however, many GTs remain to be identified. Conventional wisdom would suggest that a different enzyme would be responsible for each different sugar linkage. Some notable enzymes remain to be identified, including α-arabinosyltransferases and α-rhamnosyltransferases. In three cases, multiple genes encode two or more of these enzymes, which raises the question as to whether these genes, and the enzymes which they encode, are redundant or nonredundant. Related to this question is whether these enzymes act on all AGPs or only on a subset of AGPs. Finally, another question is whether these enzymes act in a complex. To date, there is one piece of evidence suggesting such complexes exist (i.e., GALT31A-GALT29A).

Finally, genetic mutant analysis is being used to determine the functional contribution of AGP glycosylation. To date, there appear to be a variety of functions conferred, ranging from embryo lethality to conditional effects on root growth, cell elongation, and pollen tube growth. Consequently, the sugars are important, and perhaps this will be made clearer as redundant genes are knocked out in a single plant to reveal more striking phenotypes during normal growth and development.

6 Concluding Remarks

The last decade has brought great advances in the identification of glycosyltransferases involved in plant cell wall polysaccharide biosynthesis. While strong genetic evidence is now able to associate numerous GTs with specific polysaccharides, direct biochemical evidence confirming the activities of such GTs is just beginning to catch up. This is due in large part to the complexity of wall polysaccharide synthases. The need for multiple activities to simultaneously produce a single polymer represents a clear challenge for in vitro biochemical and cell biological studies. Many, but not all, polysaccharides appear to be synthesized by multiprotein complexes. Defining the protein components of GT synthase complexes and their synergistic mechanisms is at the forefront of current molecular studies. New insights into the 3D structures of GTs are setting the stage for future advances toward defining these protein complexes (Morgan et al. 2013; Sethaphong et al. 2013; Olek et al. 2014). These kinds of structural studies will also lead to a new level of mechanistic understanding. A detailed understanding of the biosynthetic mechanisms of plant cell wall GTs will be useful toward producing designer polysaccharides and engineering plants for biofuel, food, feed, and fiber applications.

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Cell Wall Evolution and Diversity

2

David S. Domozych

Contents

Abstract

Cell walls of green plants and other photosynthetic eukaryotes consist of complex polysaccharide-rich coverings that are central to cell growth, development, and defense. Most cell walls display a microarchitectural design consisting of a composite of fibrillar polysaccharides, such as cellulose, mannans, or xylans, tethered by other glycans (e.g., hemicelluloses) that are embedded in a matrix of polysaccharides and proteins. This design produces both remarkable strength and the capacity for controlled expansion. The biosynthesis and metabolism of the cell wall require a significant portion of the cell's genetic machinery and the activities of coordinated subcellular pathways that are, in turn, responsive to developmental and environmental signals. The evolution of cell walls was critical to the exploitation of, and survival in, Earth's diverse habitats.

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1 Introduction

"Life is sugarcoated." While this proclamation may reflect gross optimism in human affairs, it is remarkably accurate when describing the external surfaces of cells of most of the diverse biota that occupy modern Earth's ecosystems. External to the plasma membrane of most cells exists a diverse assortment of carbohydrate-rich materials that constitute the "extracellular matrix." These include the glycocalyx of animal cells, the slimes or mucilages that constitute the extracellular polymeric substance or EPS of biofilm-forming microorganisms, the intricately designed and layered scales of unicellular flagellates, and the highly complex mineralized coverings such as frustules of diatoms, to name just a few. However, the most common and arguably the most ecologically/economically important of life's extracellular matrices are the cell walls of plants and other photosynthetic eukaryotes.

The plant cell wall consists of a load-bearing structural framework of fibrillar polysaccharides that are tethered by other glycans. This framework is embedded in a matrix composed of neutral and charged polysaccharides, various proteins, water, and in some cases, polyphenolics like lignin (Sarkar et al. 2009; Fry 2000). Production of this complex microarchitectural design requires coordinated secretion and precise insertion of the constituent polymers into the cell wall. Subsequent biochemical modulation results in structural refinements that ultimately yields a covering of remarkable strength but with the capacity to expand during specific developmental stages and in response to environmental prompts. Underlying plant cell wall biosynthesis and metabolism is a large and complex molecular machinery that constitutes an estimated 10% or more of the nuclear genome of angiosperms (Popper et al. 2011; Vidaurre and Bonetta 2012; McCann and Rose 2010). The cell wall is of profound importance to the cell in regulating cellular expansion/morphogenesis, providing physical/chemical protection, contributing to adhesion, absorption, and transport of various molecules, and serving as a conduit in signal transduction. Cell wall materials also provide humans with food, building materials, fiber, textiles, paper, gels, and important base materials for the rapidly emerging biofuel industry (Keegstra 2010; Pauly and Keegstra 2010; Meng and Raugauskas 2014).

Cell walls though are not limited to plants. Many of the biochemical constituents of the plant cell wall are also found in many green algae, most notably in the late divergent taxa of the charophyte green algae or CGA (Sørensen et al. 2010, 2011; Domozych et al. 2012; Popper et al. 2011; Fangel et al. 2012). These algae are ancestral and most closely related to modern land plants. Previous interpretations of the phylogeny of the CGA based on molecular, biochemical, and cytological data

(Leliaert et al. 2012; Graham et al. 2009; Becker and Marin 2009) suggest that the ancestors of modern-day CGA were capable of making the successful transition to land because specific features of their physiology and biochemistry allowed for a degree of preadaptation to life on land. Sørensen et al. (2011) further proposed that the ability of the CGA to produce cell walls with specific polymer arrays was a critical aspect of this preadaptation for this terrestrial invasion. Interestingly, the general architectural design of a "fibril/matrix" model cell wall is also commonly found in many taxa of the chlorophycean green algae (e.g., chlorophytes, ulvophytes Leliaert et al. 2012), the red algae (Rhodophyta), and the brown algae (Phaeophyta). Though the specific polymeric compositions of both the fibrillar and matrix components in these taxa may vary, it seems quite clear that the design of a matrix-reinforced fibrillar composite (e.g., like reinforced concrete or fiberglass) was selected by a broad spectrum of algae as the most efficacious covering for survival in a wide range of habitats (e.g., terrestrial ecosystems, shallow wetlands, marine intertidal zones), each with specific challenges. Likewise, complex underlying genetic machineries were required and evolved for the biosynthesis and metabolism of the cell wall constituents. Emerging discoveries of specific cell wall-based genes, biosynthetic pathways, and regulatory controls derived from recently sequenced algal genomes and transcriptomes are now providing new insight into the evolution of cell walls and adaptations to life in specific habitats (Ulvskov et al. 2013; Michel et al. 2010). In this review, the diversity and evolution of cell walls will be examined based on the biochemistry, molecular biology, and development of their polysaccharides and other constituents. This review focuses on taxonomic groups with distinctive cell walls and well-developed multicellularity including the green plant line (land plants and green algae; Viridiplantae), red algae (Rhodophyta), and the brown algae (Phaeophyta). An overview is provided in Fig. 1. Brief descriptions of the extracellular matrices of unicellular taxa with polymers similar to, or evolutionary lineages leading to, the aforementioned organisms are also provided. Other photosynthetic eukaryotes that are typically included in the Protista and also produce extracellular coverings are not discussed here, but detailed descriptions may be obtained elsewhere (e.g., diatoms; Kröger and Poulsen 2008).

2 The Structural Framework of the Cell Wall: Cellulose, Mannans, and Xylans

Cellulose is a β1-4 glucan polymer that constitutes the most abundant biopolymer produced annually on the planet. It is the major fibrillar component of the cell walls of plants and many algal taxa (Popper et al. 2011). The cellulose biosynthetic machinery in photosynthetic eukaryotes is an evolutionary product of lateral gene transfer derived from an ancestral bacterial endosymbiont (Niklas 2004). Cellulose is typically found in insoluble, unbranched crystalline strands called microfibrils that result from both in inter- and intrapolymer hydrogen bonding of hydroxyl

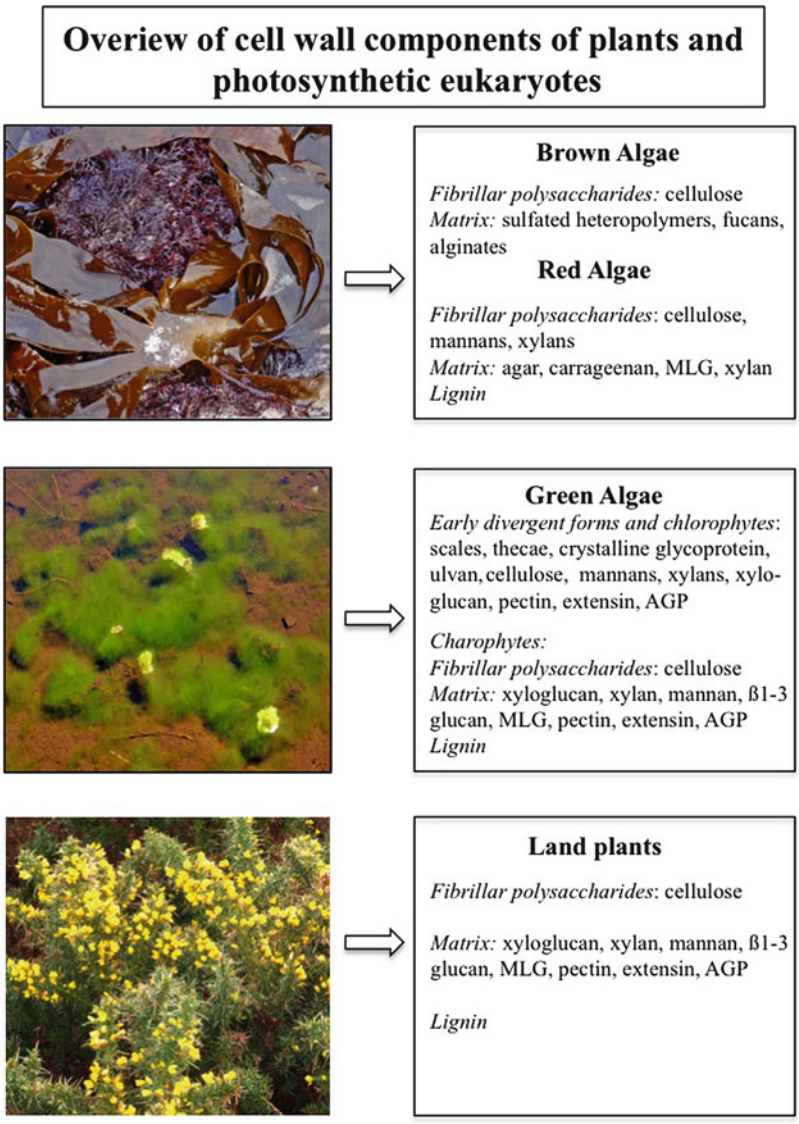

Fig. 1 Overview of the cell wall constituents of cell walls of plants, green algae, red algae, and brown algae

groups among adjacent β-1,4-glucan chains. Cellulose microfibrils are remarkably resistant to digestion and often possess tensile strength that is greater than some steels (Niklas 1992), i.e., outstanding characteristics for a polymer that serves as the load-bearing framework of the cell wall. The functional roles and dynamic actions

of cellulose are also influenced by the interconnected viscoelastic matrix of other polysaccharides and proteins that surrounds the microfibrils.

Much of our understanding of cellulose synthesis during wall development is derived from research on land plants. However, algae were some of the first organisms used in the initial identification of the cellulose biosynthetic machinery (Giddings et al. 1980; Roberts and Roberts 2007; Roberts et al. 2002). Cellulose microfibrils are typically produced in/on the plasma membrane and are then deposited or incorporated into the cell wall (McFarlane et al. 2014; Anderson et al. 2010; Lerouxel et al. 2006). More specifically, the enzyme complex responsible for its synthesis is the cellulose-synthesizing apparatus or CESA which has been classified in one supergene family that contains nine cellulose synthase-like (Csl) families and one cellulose synthase (CESA) family (Yin et al. 2009). CESA synthesis initiates in the Golgi apparatus (Gutierrez et al. 2009) and is then packaged in Golgi-derived vesicles that are transported to the plasma membrane. Here, CESA is incorporated into terminal complexes (TCs) that "weave" cellulose microfibrils (Somerville 2006; Guerriero et al. 2010). TCs function both in the polymerization of the glucan chains and the assembly of those chains into microfibrils (Roberts and Roberts 2007). Freeze fracture/etch processing followed by transmission electron microscopy (TEM)-based imaging has been critical in identifying TC structure and microfibril production. This technology has shown that in land plants and most of the late divergent taxa of the CGA, the TCs are arranged in hexameric rosettes of 25–30 nm. Each of the six components of the rosette contains six cellulose synthase units, each of which produces one β1-4 glucan chain. The 36 glucan chains produced by a single rosette associate via H-bonds to form a microfibril of approximately 2–4 nm in width and thickness. Verification of the role of TCs in the formation of cellulose was provided through labeling of TCs with a monoclonal antibody (mAb) specific for CESA using the green alga, *Micrasterias* (Nakishima et al. 2006). There is much variation in cellulose microfibril size. In many algal groups, microfibril size is much greater than that of land plants and in some red algae, microfibrils may measure from 25 nm to 68 nm (Tsekos 1999; Saxena and Brown 2005). Cellulose is probably produced on the inside of the plasma membrane and crosses to the outside via porin-based pores (Guerriero et al. 2010). The cellulose synthesis machinery also requires a pool of nucleotide sugars and is linked to activities of sucrose synthase, endo-β1-4 glucanases, and annexins (Baroja-Fernández et al. 2012; Davies 2014).

In most cellulose-producing algae, except for the CGA, variation also exists in the architecture of the TC. This, in turn, results in differences in size and arrangement of microfibrils (Tsekos 1999). For example, several chlorophycean green algae possess linear TCs that contain three rows of particles (Roberts and Roberts 2007) while in brown algae, single linear TCs with up to 100 nm subunits produce distinct ribbon-like microfibrils (Tsekos 1999). The red alga, *Erythrocladia subintegra*, produce single linear terminal complexes that measure 180 by 35 nm arranged in four rows of 30–140 particles while in the genera, *Radicilingua* and *Laurencia*, two rows of three particles each have been observed. However, these smaller complexes could represent microfibril-terminating synthesizing complexes.

In the xanthophytes, distinctly stacked or diagonally arranged linear TCs have been described (Mizuta and Brown 1992).

In land plants and many algae, cellulose microfibrils are typically laid down in layers or lamella, often as parts of a developmental phase. This most likely enhances the strength of the cell wall. That is, though cellulose microfibrils have great strength under tension but are relatively weak against shear and compressive forces, the multi-angled deposition of cellulose in lamella would allow the cell to resist forces both internal and external from various directions (Anderson et al. 2010). The orientation of cellulose microfibrils in the cell wall also correlates closely with the orientation of cortical microtubules found just underneath the plasma membrane (Taylor 2008). In land plants, this microfibril-microtubule orientation has been elegantly demonstrated using live-cell imaging of transformed cell lines (Paradez et al. 2006; Taylor 2008; Lerouxel et al. 2006; Crowell et al. 2011). Here, microtubules guide the trajectories of GFP-labeled TCs in the plasma membrane that, in turn, orient the deposition of microfibrils. However, the exact molecular mechanism behind the function of the microtubules is not yet fully defined. They may play roles in controlling the velocity of the cellulose synthesis machinery, determining the density of TCs in the plasma membrane, or targeting the insertion of CESA in the plasma membrane (Wolf and Greiner 2012; Gutierrez et al. 2009). However, other work on *Arabidopsis* demonstrated that cellulose deposition and microtubule orientation may sometimes be independent of each other (Wightman and Turner 2008). In addition, actin networks influence the rate of CESA vesicle delivery to the plasma membrane in land plants, most likely through its role in cytoplasmic streaming (Cai et al. 2011). Activity of the actin network may also control the amount of time CESA spends in the plasma membrane (Sampathkumar et al. 2013). Finally, recent work has shown that CESA is most likely recycled back into the cell by clathrin-mediated endocytosis and is possibly transported to the trans-Golgi network/early endosome (Bashline et al. 2013). This process may also require actin-mediated transport.

The interaction of cortical microtubule and actin cytoskeletal networks has also been explored in various algae. In the desmid, *Penium margaritaceum*, a band of cortical microtubules found in the central pre-division expansion zone is arranged in the same orientation as that of the innermost layer of cellulose microfibrils (Ochs et al. 2014). If the microtubules are altered using pharmacological agents (e.g., oryzalin), cellulose microfibril production is altered and the localized swelling of the cell occurs (Domozych et al. 2014b). This cortical microtubule band also serves a "marker" for expansion prior to cell division, i.e., similar to a pre-prophase band.

In red algae, cellulose synthase enzyme complexes are also most likely synthesized in the Golgi apparatus. The microfibrils that are ultimately produced at the plasma membrane are deposited in layers, but their orientation is variable. No specific correlation between cortical microtubule arrangement and microfibril positioning in the cell wall has yet been demonstrated, but it is likely that actin plays a key role in cellulose deposition (Tsekos 1999). Likewise, in some brown algae,

TC activity and microfibril production is controlled most likely by actin and not by microtubules (Bisgrove and Kropf 2001).

It is also important to note that many algal taxa produce microfibrillar wall materials that are not cellulosic (Popper et al. 2011). In coenocytic chlorophycean green algae, microfibrils may be made of β1-3 xylans, β1-4 mannans, and heteropolymeric fibrillar polysaccharides (Fernandez et al. 2010). In some red algae, microfibrillar β1-4 mannans and β1-3- or β1-4-linked xylans are produced (Craigie 1990; Rinaudo 2007).

Finally, cellulosic wall materials may also be part of other coverings that are not part of a typical cell wall. Thecate dinoflagellates (Pyrrophyta, Dinophyceae) produce an extracellular covering called the amphiesma. This complex structure consists of an outer continuous membrane, i.e., the plasma membrane, an outer plate, a single membrane-bound compartment that contains a cellulose-based theca, and an inner pellicle (Morrill and Loeblich 1983; Kwok and Wong 2003; Hohfeld and Melkonian 1992). The theca is most likely processed through the and Wong Golgi apparatus and an associated network of Golgi-derived vesicles. The amphiesma is often shed during development via a process called ecdysis (Pozdnyakov and Skarlato 2012). Much remains to be learned about the amphiesma and the cellulosic theca. However recently, Kwok and Wong (2010) demonstrated that cellulase activity was critical for coordination of cell cycle activity when amphiesma development occurs.

Our understanding of cellulose structure and synthesis in plants and algae has recently been enhanced by new technologies and the profound importance of this polysaccharide in the growing biofuel industry. For example, genomic sequencing of many algae is growing and the development of transformed cell lines expressing fluorescent protein-CESA complexes for cell wall studies of algae have recently made initial progress (Vannerum et al. 2010; Harris et al. 2010). New developments in atomic force microscopy imaging along with high-resolution light and electron microscopy imaging coupled with new probes will soon provide great insight into cellulose production (Brabham and DeBolt 2013). Similarly, chemical microarray screening employing large arrays of specific cellulose synthesis-affecting agents should soon provide insight into specific aspects of the biosynthetic machinery (Zabotina et al. 2008). All of these should soon resolve many questions about the biology and evolution of cellulose and other fibrillar wall components in plants and algae.

3 Matrix Polysaccharides

While cellulose and other fibrillar polysaccharides form the structural framework of the cell wall, other polysaccharides form a cross-connecting matrix that is critical for wall infrastructure, development, and functioning. Significant insight has been obtained concerning specific matrix polymers in flowering plants and gel-forming polysaccharides of red and brown algae (e.g., agar, carrageenan, alginates) over the past two decades. Only recently though have investigations into the chemistry and

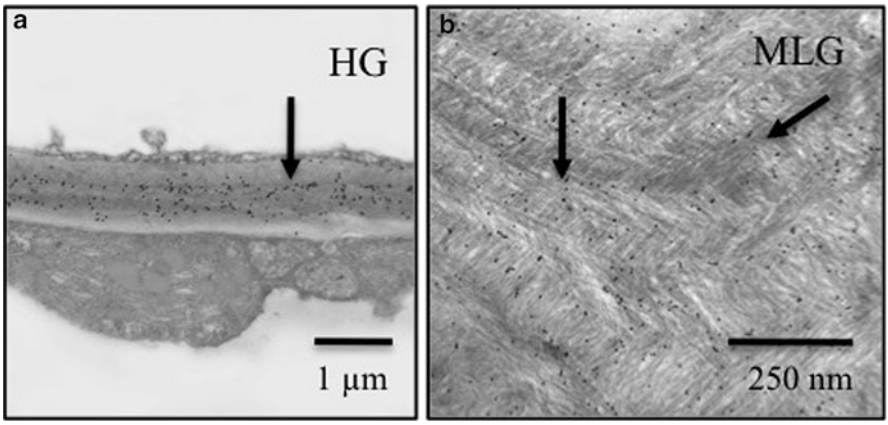

Fig. 2 TEM immunogold imaging of the cell wall of the early divergent vascular plant, *Equisetum arvense*. (**a**) Highlights the presence of homogalacturonan (*HG*) throughout the cell wall (*arrows*) using the monoclonal antibody, JIM5. (**b**) Shows MLG interspersed between layers of cellulose microfibrils (*arrows*) using the monoclonal antibody, BS-400-2

evolution of the diverse matrix polymers in early divergent green plants (i.e., algae and bryophytes) and other algal taxa begun (Franková and Fry 2013; Popper and Fry 2004, 2003).

3.1 Hemicelluloses

Hemicelluloses constitute a diverse group of polysaccharides that associate with/tether cellulose microfibrils and create a cohesive network in plant cell wall architecture. They contain a β1-4-linked glycan backbone that is substituted with varying glycan side chains. Hemicelluloses include xyloglucans, xylans, mannans, and mixed-linkage glucans (MLGs; β1-3,1-4 glucans) and β1-3 glucans. Details of their structural diversity and distribution in various taxa may be found in recent literature (Scheller and Ulvskov 2010; York and O'Neill 2008; Faik 2010; Burton and Fincher 2009; Piršelová and Matušíková 2013). Recent studies have demonstrated that hemicellulosic polymers are made in the Golgi apparatus via a variety of glycosyltransferases and are then transported to the plasma membrane for secretion to the cell wall (Driouich et al. 2012; Pauly et al. 2013). Once in the apoplast, they form associations with cellulose microfibrils. For example, in the early divergent vascular plant *Equisetum arvense*, MLGs are found interspersed with layers of cellulose microfibrils (Fig. 2). Additionally, hemicelluloses may be remodeled by endotransglycosylases (e.g., XET) and other enzymes that modify their side chains (Franková and Fry 2013). Hemicelluloses have a variety of functions including contributing to the control of expansion and differentiation of the plant cell wall (Hayashi and Kaida 2011).

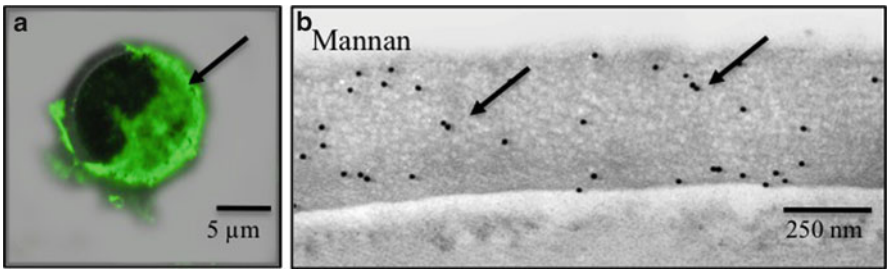

Fig. 3 (**a**) Confocal laser scanning microscope image of mannan (*arrow*) in the outer wall of the resting cyst of the chlorophyte, *Haematococcus pluvialis*. This cell was labeled with the monoclonal antibody, LM21. (**b**) TEM immunogold labeling of the mannan in the cell wall of *Haematococcus* (*arrows*) using LM21

Recently, hemicellulosic polysaccharides have been identified in several green algal groups, most notably the late divergent taxa of the CGA (e.g., mannans, xyloglucans, xylans, β1-3 glucans, and MLG; Sørensen et al. 2011). Likewise, in the chlorophyte flagellate, *Haematococcus pluvialis*, mannan is a major component of the outer layer of the cell wall of the red cyst phase (Fig. 3). When the cell enters into its flagellated green phase, the mannan is sloughed off and a new crystalline glycoprotein cell wall is deposited. In ulvophycean green algae, highly complex xylogalactoarabinans, glucuroxylorhamnans, and rhamnoxylogalactogalacturonans have been identified (Ray and Lahaye 1995). It is important to note that to date, only a few detailed investigations of hemicellulosic polysaccharides in the diverse array of green algal taxa have been undertaken. Only when sufficient data is available will insight into evolutionary trends in cell wall biochemistry be possible. A similar story may be told about the cell walls of other algal groups where significant biochemical complexity has also been noted. For example, fuco-glucuron-xylans have been found in brown algae while glucomannans and sulfated MLGs have been identified in red algae (Kloareg and Quatrano 1988; Popper et al. 2011). Currently, very little is known about the biosynthesis or metabolism of these polysaccharides.

4 Pectins

Pectins represent a group of galacturonic acid (GalA)-containing polysaccharides that are found in the cell walls of many plants and several alga taxa (Popper et al. 2011). Pectins have many functions in the wall including regulation of turgor-driven expansion, cell-cell adhesion (e.g., middle lamella of multicellular plants), absorption, and defense (Wolf and Greiner 2012; Caffall and Mohnen 2009; Yapo 2011). The most common type of pectin is the homogalacturonan (HG) which consists of alpha-1,4,-linked GalA. HG may be methyl- or acetyl-esterified at the C-6 or C-2 position, respectively. These high methyl-esterified HGs are produced in the Golgi apparatus and carried by vesicles to the PM for release. Once in the wall/apoplast, HGs can be enzymatically de-esterified, most commonly by pectin

methylesterase (PME). The release of the methyl group exposes the negative charge on the C-6 of the GalA residues which then allows for binding with cations and, more importantly, cross-linking with adjacent HG chains via divalent cations like calcium (Ca^{2+}). This cross-linking takes place in areas of ten or more de-esterified GalA residues. Cross-linking results in the formation of a stable gel, a property that is strain stiffening which in turn affects the physical properties of the wall (Braybrook et al. 2012). Pectins also consist of other GalA-containing polysaccharides, some of which represent the most complex biopolymers on the planet (Cosgrove 2005). These included substituted pectins like xylogalacturonan, apiogalacturonan, and the complex rhamnogalacturonan-II (RG-II) found to date only in land plants. Rhamnogalacturonan-I or RG-I is another type of pectin that consists of a disaccharide repeat of alpha-1,4-GalA-alpha-1,2-L-Rha. The Rha residues often contain branches of β-1,4-galactan, branched arabinans, and other arabinogalactan side chains (Harholt et al. 2010). Recent analyses of the cell walls of the CGA have shown that HG is a common cell wall constituent (Sørensen et al. 2011). Likewise, RG-I-like epitopes also appear in the cell walls of some green algae (Domozych et al. 2014a).

In higher plants, pectin synthesis initiates in the Golgi apparatus, and pectins are carried to the cell surface or cell plate by Golgi-derived vesicles (Caffall and Mohnen 2009). Recently, Atmodjo et al. (2013) described a hypothetical model for pectin synthesis called the domain synthesis model. Here, an oligosaccharide or polysaccharide primer is first synthesized. The primers are then elongated by elongation enzymes to make particular pectin domains like HG or RG-I. The domains are transferred en bloc onto the growing polysaccharide in the apoplast/cell wall. At least 16 gene families have been identified for pectin biosynthesis and modulation in late divergent land plants (e.g., angiosperms). Genes from these families have also been identified in both bryophytes and CGA (McCarthy et al. 2014).

In algae, the study of pectins and their secretion has focused the CGA especially desmids (Zygnematales) and taxa of the Charales (e.g., *Chara*). *Micrasterias*, a desmid that has been widely used as a model system for cell morphogenesis studies (Lutz-Meindl and Brosch-Salomon 2000; Meindl 1993), possesses a primary cell wall that contains HG with medium levels of methyl esterification (Eder and Lutz-Meindl 2008). The outer portion of this wall apparently has lower levels of methyl-esterified HG than the inner portion. The desmids, *Netrium digitus* (Eder and Lutz-Meindl 2010) and *Closterium acerosum* (Baylson et al. 2001), also possess HG in their cell walls, most commonly noted at expansion loci of the cell. Perhaps the most prominent example of pectin in the green algae is found in the simple cylindrical desmid, *Penium margaritaceum* (Fig. 4), This alga only produces a primary cell wall that contains a distinct Ca^{2+}-HG "lattice" in the outer wall layer (Domozych et al. 2007a, 2009), The pectin is embedded in an inner layer of cellulose microfibrils, and the interface between these two polymeric domains, the median layer, contains HG and most likely, RG-I. The large *Chara corallina* is distinguished by having some of the largest plant cells (e.g., intermodal cell). The cell walls of its vegetative thallus contain cellulose microfibrils that are cross-linked by HG that is cross-linked by Ca^{2+} (Proseus and Boyer 2007, 2008, 2012), HG and RG-I epitopes

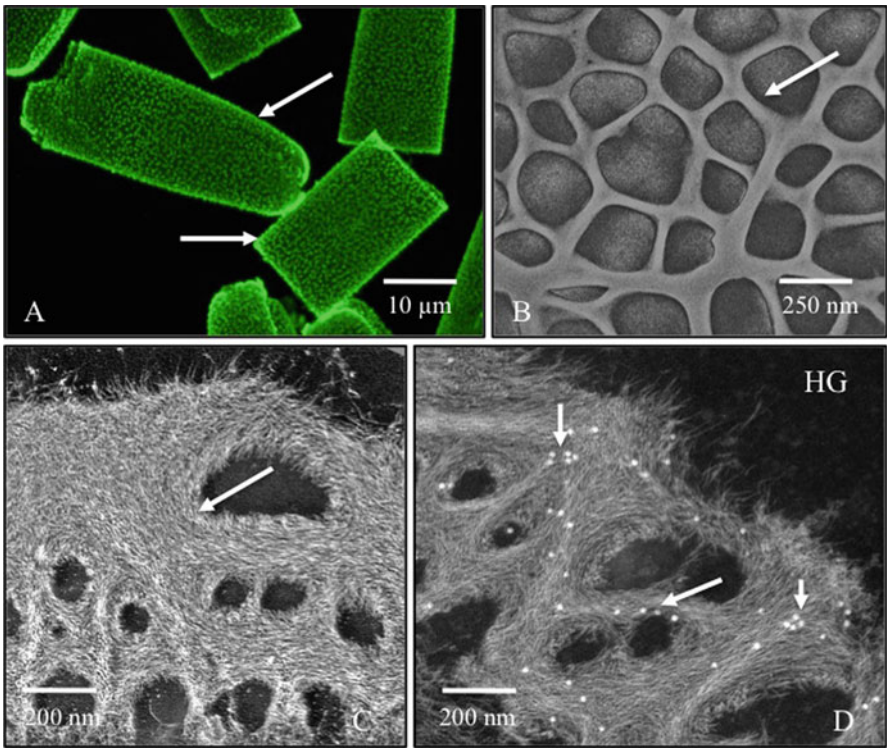

Fig. 4 (**a**) Confocal laser scanning microscope image of the HG (*arrow*) of isolated cell walls of the CGA, *Penium margaritaceum*, using the monoclonal antibody JIM5. (**b**) Shadow-cast TEM image of the outer pectin-rich wall layer of *Penium*. A distinct lattice-like covering is apparent (*arrow*). (**c**) TEM image of a partially decalcified cell wall of *Penium*. (Note that the outer layer (*arrow*) consists of tightly bound fibrils (*arrow*)). (**d**) These fibrils consist of HG as labeled (*arrows*) by the monoclonal antibody, JIM5

have also been identified in other CGA (Sorensen et al. 2011), but detailed studies of them in specific taxa have not yet begun.

The last two and a half decades have seen growing amounts of research in the synthesis and secretion of pectins in green algae. In *Micrasterias*, pectins are initially synthesized in the Golgi apparatus and are transported to the cell surface via "dark" vesicles that emerge from the peripheries of the medial-to-trans loci of a Golgi body (Lutz-Meindl and Brosch-Salomon 2000; Holzinger 2000). Interestingly, it is believed that these vesicles also carry cell arabinogalactan proteins (AGPs). The pectins are transported to expansion zones where they become part of the growing primary cell wall. In a study employing electron energy loss spectroscopy (or EELS) of thin sections of HG-carrying vesicles, notable levels of Ca^{2+} were also detected. This led to the suggestion of a regulatory role of Ca^{2+} in the gelation qualities of the HG prior to secretion (Eder and Brosch-Salomon 2008). The delivery of the HG vesicles to specific sites is mediated by actin (Pflugl-Haill et al. 2000) and a Rab GTPase,

MdRABE1, may also be involved in spatial targeting of vesicles to particular sites on the cell surface (Vannerum et al. 2012). In the desmid, *Closterium acerosum*, osmotically generated Hechtian strands may mark sites at the plasma membrane where vesicles fuse (Domozych et al. 2003). Once secreted, the pectin-modulating enzyme, PME, most likely remodels the pectin. PME activity was also described in the desmid, *Netrium* (Eder and Lutz-Meindl 2010).

In *Penium margaritaceum*, considerable cell wall expansion occurs prior to cell division at a narrow band at the central isthmus. The inner cellulose layer is first produced in a narrow 1 μm band. High-esterified HG is added here (Domozych et al. 2009, 2011 where it apparently de-esterified as it is pushed toward the polar zones. This allows for Ca^{2+} complexing and the formation of the outer HG lattice. After cell division, residual wall expansion occurs at the polar tip of expanding daughter semicells. These events can be easily monitored and quantified using live-cell labeling with mAbs with specificity toward various HG epitopes (Domozych et al. 2011). In *Penium* though, the expansion process is not always symmetrical as size and positioning of old and new cell wall may not equivalent. The expansion zone at the isthmus is highlighted by a cortical band of microtubules and microfilaments positioned around the nucleus. Interestingly, the positioning of the microtubules in this band matches the orientation of the cellulose microfibrils in the inner wall at this zone. After cell division when daughter nuclei are separated and positioned in the center of the new daughter semicells, the cortical microtubule/microfilament bands reform around each nuclei. Deviations in the repositioning of the nuclei and the surrounding cortical cytoskeletal elements may cause asymmetry in the wall development.

Pectin secretion and incorporation into the cell wall has also been well studied in charalean taxa of the CGA specifically *Chara corallina* (Proseus and Boyer 2007) where a novel pectin cycle was proposed. Turgor pressure is the driving force generated from the cell interior that fuels expansion. Newly secreted pectin added to the cell wall removes Ca^{2+} from the cell wall in localized regions. This loosens the tension in the wall and causes wall expansion. Ca^{2+} then enters the wall to reestablish cross-links that enhance the tensile strength of the cell wall.

5 The Matrix Polymers of Brown Algae

Modern brown algae (Phaeophyceae) constitute a large and diverse assemblage of primarily marine taxa displaying complex multicellular forms and in some cases, growing to 50 m or longer (e.g., *Macrocystis*). They also exhibit highly complex reproductive cycles and postfertilization developmental strategies initiating with polar expansion of the zygote (Graham et al. 2009). The cell walls of brown algae are distinct in that they contain a small amount of cellulose (e.g., 1–8 %) embedded in a thick matrix (Michel et al. 2010) primarily composed of anionic polysaccharides including alginates and fucoidans (Kloareg and Quatrano 1988). Early biochemical analyses of these and other wall constituents and their biosynthetic mechanisms have recently been complemented by molecular studies especially after the sequencing of the genome of the model taxon, *Ectocarpus siliculosus*

(Coelho et al. 2012; Cock et al. 2010; Le Bail et al. 2011). Alginate polymers primarily consist of blocks containing two uronic acids, β-1,4-D-mannuronic acid and alpha-1,4-L-guluronic acid that complex divalent cations to form gels (Michel et al. 2010). They may also bind to phlorotannins that consist of halogenated (e.g., iodine) and/or sulfated phenolic compounds (Berglin et al. 2004; Verhaeghe et al. 2008). Alginates tether cellulose microfibrils and are critical for maintaining wall architecture. Evolutionarily derived from lateral gene transfer of an *Actinobacterium*, alginates were critical to the development of the multicellular condition and inclusive morphological complexity of brown algae.

Alginates are synthesized in the Golgi apparatus and are transported in GA-derived vesicles to cell wall expansion sites at the cell surface (Schoenwaelder and Wiencke 2000; Callow et al. 1978; Nagasato et al. 2010; Nagasato and Motomura 2009). During cytokinesis, alginates are found in electron-dense vesicles at the growing septum, and vesicle movement appears to be closely associated with actin dynamics. In *Dictyota*, alginate processing is also involved in the formation of plasmodesmata (Terauchi et al. 2012). Recent investigations into the molecular genetics of alginate biosynthesis have shown that the guluronic acid residues of alginates are remodeled during development and in response to environmental conditions by mannuronate C5-epimerases (Michel et al. 2010).

Fucoidans of brown algae are alpha-L-fucose-rich polysaccharides that display considerable structural diversity. Some fucoidans are highly sulfated homofucans while others are highly substituted with xylose, galactose, mannose, and glucuronic acid (Popper et al. 2011; Jeon et al. 2011). Sulfation is a distinguishing characteristic of many marine algal polysaccharides and is associated with adhesion. Fucoidans are also produced in the GA, packaged in vesicles and delivered to wall growth zones via actin-mediated transport. In *Silvetia*, fucoidans are the dominant polysaccharide in the cytokinetic region (Nagasato et al. 2010).

Brown algae display a variety of expansion patterns including both tip-based and diffuse growth mechanisms (Bisgrove and Kropf 2001). This allows for the formation of diverse morphologies including simple filaments, flattened blades, and large cones. During expansion and morphogenesis, it is thought that interactions of transmembrane proteins with components of the extracellular matrix are key to development (Le Bail et al. 2011; Fowler et al. 2004). Additionally, tip-based growth requires specific coordination of cortical Ca^{2+} gradients, the targeted fusion of vesicles at the expansion tip, spatial regulation of the secretion of cell wall components, and regulation of actin cytoskeleton dynamics. In the well-studied *Fucus distichus*, polar growth is regulated by Rho GTPases that are associated with actin assembly at tip and vesicle secretion (Katsaros et al. 2003).

6 The Matrix of Red Algae

Red algae produce matrices rich in sulfated polysaccharides (Painter 1983; Popper et al. 2011; Kloareg and Quatrano 1988; Rinaudo 2007; Craigie 1990). The most common are sulfated galactans (sulfation percentages of 20–38 %) containing a

backbone of repeating galactose (Gal) and 3,6-anhydrogalactose-linked β1-4 and β1-3, respectively. These polysaccharides are exemplified by the commercially important agars and carrageenans. The geographic positioning of the various galactans in the red algal thallus may be critical for calcification process common to many red algae (Martone et al. 2010). Likewise, competent secretion of matrix polysaccharides of the cell wall may be critical for maintenance of anterior-posterior axis of the thallus including the production of monospores (Li et al. 2008). Matrix polymers are processed in the Golgi apparatus and unique mucilage sacs that are associated with the endoplasmic reticulum (Ramus 1972; Tsekos 1981).

6.1 Arabinogalactan Proteins

In addition to polysaccharides, the matrix of cell walls may also contain an assortment of nonenzymatic and enzymatic proteins; arabinogalactan proteins or AGPs are hydroxyproline-rich glycoproteins (HRGPs) found on plant cell surfaces, specifically the cell wall and surrounding loci (Showalter 2001; Nguema-Ona et al. 2012). AGPs have diverse functions including playing important roles in growth, development, and adhesion (Ellis et al. 2010). These nonenzymatic proteins are highly glycosylated with the carbohydrate moiety often constituting 90 % or more of the mass of the molecule. The protein component of an AGP is characterized by alanine-hydroxyproline (Ala-Hyp) repeat motif interspersed with serine (Ser). *O*-glycosylation of specific amino acid residues is common with either short arabinose oligosaccharides or large β1-3-linked galactans containing 1,6-linked side chains of Gal, arabinose, rhamnose, and glucuronic acid. Repeating Ser $(Hyp)_4$ units in the protein are sites of *O*-glycosylation with the short arabinose side chains while the larger arabinogalactosyl side chains are more commonly situated on Ser, Ala, valine (Val), or threonine (Thr). Some AGPs have glycosyl-phosphatidylinositol (GPI) for anchorage in the plasma membrane. Recently, Tan et al. (2013) showed that in *Arabidopsis*, AGP is connected to HG/RG-I pectins and hemicellulosic arabinoxylan. This, in turn, suggests that wall polymers form large macromolecular complexes of interacting polymers. AGPs have been found in all land plant groups (Nguema-Ona et al. 2012; Lee et al. 2005), and recently, AGP epitopes have been identified in green algae including the CGA (Sørensen et al. 2011). More specifically, AGP-like epitopes were identified in the cell wall of the CGA including *Closterium acerosum* (Baylson et al. 2001), pore complexes of *Pleurotaenium trabecula* (Domozych et al. 2007b), and in the primary cell wall and pores of the secondary cell wall of *Micrasterias* (Eder et al. 2008). In *Micrasterias*, AGPs are first noted in Golgi bodies and then in primary wall vesicles and a group of 160–300 nm mucilage vesicles before release to the cell wall. AGPs have also been identified in the chlorophyte, *Oedogonium* (Estevez et al. 2008). Green algal AGPs appear to have some distinct differences to those found in land plants. For example, green algal AGPs have not been shown to bind to, or be

affected by, β-Yariv reagent, an agent commonly used for isolating and quantifying to land plant AGPs (Willats and Knox 1996).

6.2 Extensin

Extensins represent a second group of HRGPs found in plants and some algae. They possess a characteristic Ser-$(Hyp)_4$ repeat motif and are also significantly *O*-glycosylated (Lamport et al. 2011; Wilson and Fry 2006). In plants, they are synthesized in the Golgi apparatus and are transported to the cell surface by Golgi-derived vesicles. Once secreted, they self-assemble to form a scaffolding in the cell wall and function in maintaining wall architecture, increasing tensile strength of wall, cell plate formation, and defense responses (Velasquez et al. 2011). In green algae, extensin-like proteins have been found in CGA (Sørensen et al. 2011) and, most prominently, in the volvocalean taxa of the Chlorophyceae (Ertl et al. 1992; Roberts et al. 1985; Roberts 1974).

The cell wall of the unicellular flagellated chlorophyte, *Chlamydomonas reinhardtii*, contains 25–30 different extensin-like HRGPs (Sumper and Hallmann 1998) that are arranged in interlocking fibrillar and granular elements (Voight and Frank 2003) to form a "crystalline cell wall." These HRGPs are also components of gametic membranes and sexual agglutinins (Imam et al. 1985; Woessner and Goodenough 1994). In the colonial flagellate, *Volvox*, HRGPs make up rod-shaped modules and are combined with hydroxyproline-free domains to form the complex ECM including the colonial matrices (Kirk et al. 1986). These extensin-like HRGPs self-assemble into their wall/matrix upon secretion. During development of the glycoprotein cell wall of *Chlamydomonas*, the first elements to emerge during wall synthesis are long fibers that constitute the innermost and outermost wall layers. The other wall layers assemble later within this primary matrix (Goodenough and Heuser 1988). Prolyl-4-hydroxylase is an important enzyme in modeling of these HRGPs (Keskiaho et al. 2007). Disruption of its activity through knockdown genetics leads to highly abnormal cell walls lacking the typical multilayered ultrastructure (Goodenough and Heuser 1988). The cell biology of cell wall development has been studied in the volvocalean unicell, *Gloeomonas kupfferi*. Here, the inner wall layer is formed in the Golgi apparatus and transported initially to the contractile vacuole. It is then released from the vacuole to the anterior cell surface where it initiates self-assembly of the glycoprotein wall (Domozych and Dairman 1993).

Cell wall proteins like extensin have also been identified in other algal taxa. In the green alga, *Botryococcus braunii*, an alga of considerable importance in the biofuel industry, the colonial habit is maintained by a retaining thick wall/sheath (Weiss et al. 2012). This ECM consists of β1-3 and β1-4 glucans, a distinct polysaccharide-containing arabinose, galactose, and a small amount of deoxyhexoses as well as an extensin-like protein. Extensin-like proteins have also been found in chlorophyte green algae such as *Codium* and *Oedogonium* (Estevez et al. 2008, 2009.

7 Other Cell Wall Features: Lignins and Mineralization

The cell walls of plants and photosynthetic eukaryotes may also contain significant amounts of constituents other than carbohydrates and proteins. Their presence is often associated with specific phases of wall development or with specific taxa. These include:

Lignin constitutes a complex group of aromatic heteropolymers that are products of oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (da Costa et al. 2014; van Holme et al. 2010: Boerjan et al. 2003; Ralph et al. 2004) including p-hydroxyphenyl, guaiacyl, and syringyl subunits (Bonawitz and Chapple 2010) Lignin is most commonly found in the secondary cell walls of land plants but may also be produced in response to wounding, stress, or infection (Caño-Delgado et al. 2003; Tronchet et al. 2010). Lignin and its specific binding pattern to cellulose contribute significantly to cell wall rigidity and resistance to degradation (Ding et al. 2012; Vanholme et al. 2010). Lignin-like polymers have recently been identified in late divergent taxa of CGA (Sørensen et al. 2011) and in the red alga, *Calliarthron cheilosporioides*, an organism found in high-energy surf habitats of marine intertidal zones (Martone et al. 2010).

Significant mineral complexing is characteristic of the cell walls of many plants and algae. For example, in the early divergent vascular plant, *Equisetum*, silica binds to specific wall polymers including 1–3 glucan or callose. It is believed that silica enhances rigidity and defense against biotic and abiotic stresses (Law and Exley 2011; Currie and Perry 2009). Ca^{2+} is also a common component of cell walls especially in its cross-linking with pectins (Hepler 2005; see Sect. 4). Significant calcification though that leads to a highly mineralized cell wall is commonly found in charophytes like *Chara* (Proseus and Boyer 2008) and in red algae, especially many taxa found in coral reefs (Craigie 1990; Kloareg and Quatrano 1988). However, one of the most spectacular calcification processes in an ECM of a photosynthetic eukaryote occurs in haptophytes or prymnesiophytes (Graham et al. 2009). The covering of many taxa within this algal group is characterized not by a cell wall but by a coccosphere that is comprised of multiple plate-like coccoliths (Marsh 1999). A coccolith consists of an organic base plate to which calcite is complexed Mackinder et al. 2010). Coccolith production has major ecological significance as it is responsible for up to 89 % of the total burial flux of calcium carbonate to ocean sediments (Mertens et al. 2009). The production of the coccolith in some taxa (e.g., *Emiliania*) initiates in the Golgi apparatus and a Golgi-derived coccolith vesicle (CV). The distal portion of this vesicle consists of a distinct reticular body that is comprised of a continuously changing membranous mass containing organic material derived from the Golgi body and endoplasmic reticulum. The reticular body becomes closely associated with the CV from the time of crystal nucleation to secretion (Kayano et al. 2010; Marsh 1999; Taylor et al. 2007; Brownlee and Taylor 2004). It is thought that these features provide an important regulatory role of the reticular body in supplying the organic and inorganic materials required for calcification (Drescher et al. 2012). Likewise, the reticular body might provide a large surface area that could accommodate vast

numbers of Ca^{2+}- and perhaps bicarbonate ion pumps. This would provide a supersaturated solution of cations during calcite nucleation and growth. V- and P-type of Ca^{2+}-stimulated ATPases are also involved in the coccolith biosynthesis (Corstjens and González 2004: Corstjens et al. 2001; Araki and Gonzales 1998). Nucleation and growth of the calcite crystals are also controlled by an organic base plate scale in association with acidic polysaccharides and proteins (Hirokawa et al. 2005; Kayano et al. 2010; Shroeder et al. 2005). It is also been shown that both microtubules and microfilaments are involved in coccolith formation (Langer et al. 2010).

8 "Ancestral Precursors" of the Cell Wall?: Scales and Fused Scales

Many early divergent green algal flagellates (e.g., prasinophytes; Leliaert et al. 2012) are covered with a single layer or multiple layers of scales that sometimes number in the thousands (Moestrup and Walne 1979). Scales are composed of 90 % polysaccharide that contains the unusual 2-keto-sugar acids, Kdo (3-deoxy-2-manno-octulosonic acid), 5-*O*-methyl Kdo, and 3-deoxy-2-lyxo-heptulosaric acid (Becker et al. 1994). Additionally, scales may be associated with small amounts of protein that may be involved in adhesion of scales to the cell surface (Becker et al. 1996). Scales are also found in the early divergent clade of the CGA, the Mesostigmatophyceae, represented by *Mesostigma viride*. Here, an outermost layer of scales, typically numbering 800 per cell, overlays an inner layer of small scales. These outer scales manifest in a spectacular "basket" shape and contain glucose, 3-deoxy-lyxo-2-heptulosaric acid, and two protein components (Domozych et al. 1991). In prasinophytes and *Mesostigma*, scale synthesis occurs in the Golgi apparatus. Individual scale types may be observed being formed and maturing in Golgi cisternae. The scales are segregated into vesicles and are carried to a vacuole-like scale reservoir where they are arranged and then deposited onto the cell and/or flagellar surfaces (Moestrup and Walne, 1979; Domozych et al. 1991).

The green algal flagellates, *Tetraselmis* and *Scherffelia*, possess a cell wall-like covering, called a theca, that is derived ontogenetically from fused scales (Domozych et al. 1981; McFadden and Melkonian 1986). The theca contains no cellulose but consists of a polysaccharide containing 50 % Kdo and 15 % GalA (Vierkotten et al. 2004; Becker et al. 1995). Scale-like thecal precursors are synthesized in the Golgi apparatus and are transported to the anterior region of the cell by Golgi-derived vesicles. Here, they are released to the surface where they coalesce to form the theca. During this stage, pre-theca components are converted to complete theca by the addition of long Kdo chains substituted with sulfated GalA disaccharides that are cross-linked by Ca^{2+} (Becker et al. 1994). For scaly and thecate green algae, virtually nothing is known about the molecular genetics of the biosynthesis of the extracellular coverings.

9 Conclusions

Studies of the cell wall are currently in an exciting phase as most structural, chemical, developmental, and evolutionary features of the wall remain to be discovered. The recent emergence of new and powerful analytical tools and their application in cell wall studies are rapidly providing critical data in the deciphering of the infrastructure and dynamics of the cell wall. These include genomic and transcriptomic sequencing and analyses of a greater taxonomic array of organisms, high-throughput screening of wall polymers using the expanding library of specific monoclonal antibodies and carbohydrate-binding modules, and the dissection of the nanostructure of the cell wall with new high-resolution microscopy technologies including immuno-based probing, electron tomography, and atomic force microscopy. This new research has resulted in valuable new insight into the complex glycomics of the wall, the highly coordinated genetic machinery required for wall synthesis and metabolism that interacts with precise developmental phases and in response to environmental stress, and the interactions of constituent polysaccharides, proteins, and other constituents. This new era of research holds much promise for the elucidation of the evolution and diversity of the cell wall and its application to economic and environmental activities.

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Bacterial Polysaccharides: An Overview

3

Swati Misra, Varsha Sharma, and Ashok Kumar Srivastava

Contents

Abstract

Bacterial cell wall and membrane are associated with a variety of glycoconjugates and polysaccharides which aids in structural formation as well as performing various functions in the bacterial cell. In gram-negative bacteria, peptidoglycan is majorly present in the periplasmic space and it provides mechanical strength as well as shape to the cell. In some cases, the periplasm contains membrane-derived oligosaccharides (MDOs), which are involved in osmoregulation. The outer membrane mainly contains lipopolysaccharides (LPSs) that bind to divalent cations or chelators for structure stabilization and to increase outer membrane permeability. This LPS contains lipid A, also known as endotoxin, which has shown a powerful biological effect in mammals such as fever, septic shock, multiple organ failure, and mortality. The mucoid (slime-producing) strains contain capsular polysaccharide which

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aids as virulence factor. The gram-positive bacteria lack an outer membrane and have a much thicker peptidoglycan layer along with a specialized polysaccharide known as teichoic acid. It provides cell wall integrity through complex formation with cations and also assists in cell growth regulation. The present report attempts to provide an overview of bacterial polysaccharide structure, occurrence, and their important functions, along with the biosynthesis and major inhibitors to block biosynthetic pathways.

Keywords

Peptidoglycan • Teichoic acid • Bacterial glycoproteins • Membrane-derived oligosaccharides • Lipopolysaccharides • Capsular polysaccharide

Abbreviations

ABC	ATP-binding cassette
ACP	Acyl carrier protein
CPS	Capsular polysaccharide
Gal	Galactose
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GT	Glycosyltransferase
HMM-PBPs	High molecular mass PBPs
LAL assay	Limulus amebocyte lysate assay
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
LTA polymers	Lipoteichoic acid polymers
LDAP	L-Diaminopimelic acid
Man	Mannose
MDOs	Membrane-derived oligosaccharides
MurNaC	*N*-Acetylmuramic acid
NBD	Nucleotide-binding domain
OPGs	Osmoregulated periplasmic glucans
PBPs	Penicillin-binding proteins
PG	Lysophosphatidylglycerol
WTAs	Wall TAs

1 Introduction

In nature, mostly the carbohydrates exist as polymers rather than monomers. In this context, polysaccharides are believed to be the most abundant polymeric carbohydrate. These polymers consist of ten or more simple sugar units and composed of thousands or hundreds of thousands of simple sugars which are bonded together by glycosidic bonds so as to have molecular masses as high as 100 million atomic mass units. The main function of the polysaccharide is determined by the monomers it

contains or the manner in which they are linked together. Polysaccharides present in plants, animals, and bacteria are majorly responsible for their structural and functional roles. The most common polysaccharides are cellulose in plants and glycogen in animals. They act as storage polysaccharides while certain others provide the structural support and are known as structural polysaccharides. Among the structural polysaccharides is chitin which is generally present in invertebrate animals.

On the basis of morphological localization, bacterial polysaccharides could be divided into groups: intracellular polysaccharides which are located inside or as part of the cytoplasmic membrane or cell wall such as peptidoglycan, periplasmic glucans, lipopolysaccharide (LPS), lipooligosaccharide (LOS), and capsular polysaccharides which form a structural part of the cell wall (Chawla et al. 2009) or the extracellular polysaccharides also known as exopolysaccharides consisting of branched, repeating units of sugars or sugar derivatives. Extracellular polysaccharides are further classified as: homopolysaccharides (cellulose, dextran, pullulan, curdlan) and heteropolysaccharides (gellan, xanthan) on the basis of the sugar or its derivative a particular molecule contains. Bacteria and many other microbes including fungi and algae often secrete diverse polysaccharides as an evolutionary adaptation to help them adhere to the surface as part of the cell wall component or storage units or virulence factors and also to prevent them from drying out. Exopolysaccharides have shown wide industrial applications mainly in food and pharmaceutical products and to certain extent in textile, paper, and cosmetics, as gelling agents and medicines for wound dressings (Sutherland 1998). In context to exopolysaccharides, accumulation of polyhydroxybutyrate (PHB) has been extensively studied. This is a kind of polyesters which is synthesized by a number of microorganisms as energy reserve materials under unfavorable conditions, i.e., in the limitation of some essential nutrients under an excess availability of carbon source (Lee 1996; Khanna and Srivastava 2005). The properties of pure PHB are comparable to the commonly used petroleum-derived bulk plastics, e.g., polypropylene. It is a unique natural biopolymer, which exhibits three exceptional features (Hrabak 1992): (i) thermoplastic process ability, (ii) 100 % resistance to water and moisture, and (iii) 100 % biodegradability. It could therefore be used for applications similar to those of common plastics and would fit well into new waste disposal and management strategies. While working with PHB production, it was observed that the fermented broth containing 125 gl^{-1} of sucrose had resulted in 22.65 gl^{-1} of PHB from 29.7 gl^{-1} of dry cell weight (DCW) in 38 h at 33 °C, 200 rpm by *A. australica* using constant feed rate (100 ml/h for 15 h) in fed-batch cultivation (Gahlawat and Srivastava 2013). Recently, an attempt has been made on the usage of renewable resources such as glycerol (biodiesel by-product) in order to make the process sustainable and cost effective and ensure renewabilities. The microorganisms investigated in the present study using these categories of substrates are *Alcaligenes latus*, *Ralstonia eutropha*, and *Bacillus* sp. (data not shown). The biopolymer produced by *Bacillus* sp. will be suitable for medical use, and to develop an efficient production process, studies are continuing in author's laboratory.

In the light of the current state of the art on bacterial intracellular polysaccharides, the bacterial cell wall deserves special attention as it is important for maintaining the cell viability by protecting the cell protoplast from mechanical damage either through osmotic rupture or lysis. The bacterial cell wall is composed of various unique components which are not present in nature; it is also believed to be the most important site for the attack by antibiotics. The cell wall provides ligands for the adherence and also the receptor sites for drugs or viruses. They provide immunological distinction among the bacterial strains (Todar 2011).

Realizing the important features of bacterial cell wall, this communication attempts to elucidate varied bacterial intracellular polysaccharide characteristics which are generally present in the cell wall or in the cellular membrane, their structure, occurrence, importance, or the functional role played in the cell, and its applications for human welfare and future prospects.

2 Peptidoglycan

Peptidoglycan (murein) is a polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of bacteria (except Archaea), which forms the cell wall. The peptidoglycan in most of the gram-negative bacteria consists of alternating residues of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNaC). *N*-acetylmuramic acid is attached covalently to short tetrapeptide chains which are composed of L-Ala, unusual D-amino acids (D-Glu and D-Ala), and L-diaminopimelic acid (LDAP). These unusual D-amino acids do not generally occur in proteins and therefore help to protect against the attacks by most peptidases. The tetrapeptide side chains could also be linked to one another by an interpeptide bond between a free amino group on diaminopimelic acid (DAP) of one chain and free carboxy group on nearby D-Ala on the other tetrapeptide side chain of *N*-acetylmuramic acid (Fig. 1).

The assembly of peptidoglycan on the outside of the plasma membrane is mediated by a group of periplasmic enzymes, which are transglycosylases, transpeptidases, and carboxypeptidases. These enzymes are designated as penicillin-binding proteins

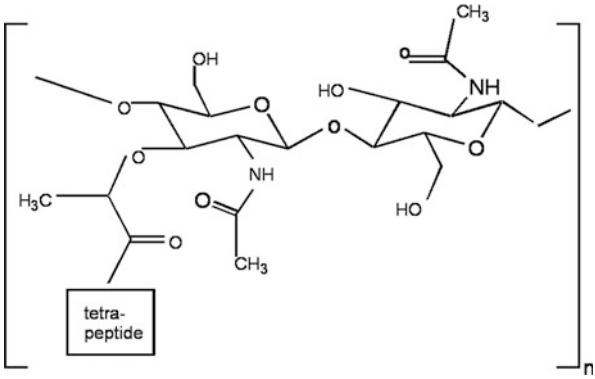

Fig. 1 Structure of peptidoglycan

(PBPs) being specific targets of the β-lactam antibiotics and are involved in the late stages of peptidoglycan synthesis which aids in the formation of a 3D mesh-like layer and a strong and rigid cell wall. Penicillin-binding proteins (PBPs) vary from species to species in number, size, amount, and affinity for β-lactam antibiotics. The high molecular mass PBPs (HMM-PBPs) mainly consist of two domain proteins that either belong to class A or class B, depending on the structure and the catalytic activity of their N-terminal domain (Heijenoort 2001). The C-terminal domain of both classes is responsible for transpeptidation activity, and β-lactam antibiotics covalently bind to its catalytic center. In class A HMM-PBPs, the N-terminal domain is responsible for their transglycosyltransferase activity, while, in the case of class B, the N terminal is involved in the interactions with other membrane proteins (Marrec-Fairley et al. 2000). It is well understood that class A HMM-PBPs are bifunctional enzymes capable of catalyzing both transglycosylation and transpeptidation.

The peptidoglycan layer is substantially thicker in the gram-positive bacteria (20–80 nm) as compared to gram-negative bacteria (7–8 nm) with the attachment of S-layer. Gram-positive bacteria are more sensitive to penicillin than gram-negative bacteria because the peptidoglycan is not protected by an outer membrane and is a more abundant molecule. In gram-positive bacteria, there are numerous different peptide arrangements among peptidoglycans. The best-studied murein is of *Staphylococcus aureus* wherein in place of diaminopimelic acid (DAP) as present in *E. coli* is diamino acid or L-lysine in position 3 of the tetrapeptide side chain, and the interpeptide bridge of amino acids connects a free amino group on lysine to a free carboxy group on D-Ala of a nearby tetrapeptide side chain. This arrangement allows a more frequent cross bonding between nearby tetrapeptide side chains. The free amino group of L-lysine is substituted with a glycine pentapeptide which then becomes an interpeptide bridge forming a link with a carboxy group from D-Ala in the adjacent tetrapeptide side chain as in the case of *S. aureus*. There are at least eight different types of peptidoglycan which exist in gram-positive bacteria and differ from species to species mainly with respect to the amino acids present in the third position of the tetrapeptide side chain and in the amino acid composition of the interpeptide bridge. The assembly of interpeptide bridge in gram-positive murein is inhibited by beta-lactam antibiotics (penicillin) in the same manner as the interpeptide bond in gram-negative murein.

Peptidoglycan forms 90 % of the dry weight of gram-positive bacteria but only 10 % for gram-negative strains. The cross-linked structure confers mechanical strength to cells and its desired shape in order to withstand internal osmotic pressure. It has sufficient plasticity to allow cell growth (elongation) and division (septation).

2.1 Biosynthesis of Peptidoglycan

The biosynthesis of peptidoglycan has been reported by various researchers in different microorganisms for both gram-positive and gram-negative bacteria (Matsuhashi 1994; Van Heijenoort 1998).

The peptidoglycan monomers are synthesized in the cytosol and are then attached to a membrane carrier bactoprenol. Bactoprenol transports peptidoglycan monomers across the cell membrane where they are inserted into the existing peptidoglycan. In the first step of the peptidoglycan synthesis, the glutamine, which is an amino acid, donates an amino group to sugar, fructose 6-phosphate. This converts fructose 6-phosphate into glucosamine-6-phosphate. In step two, an acetyl group is transferred from acetyl CoA to the amino group on the glucosamine-6-phosphate, creating *N*-acetylglucosamine-6-phosphate. In step three of the synthesis process, the *N*-acetylglucosamine-6-phosphate is isomerized, which will change *N*-acetylglucosamine-6-phosphate to *N*-acetylglucosamine-1-phosphate. In step four, the *N*-acetylglucosamine-1-phosphate, which is now a monophosphate, attacks the uridine triphosphate (UTP), which is a pyrimidine nucleotide and has the ability to act as an energy source. In this particular reaction, after the monophosphate has attacked the UTP, an inorganic pyrophosphate is released and is replaced by the monophosphate, creating UDP-*N*-acetylglucosamine. (When UDP is used as an energy source, it gives off an inorganic phosphate.) This initial stage is used to develop the precursor for the NAG in peptidoglycan. In step five, some of the UDP-*N*-acetylglucosamine (UDP-GlcNAc) is converted to UDP-MurNAc (UDP-*N*-acetylmuramic acid) by the addition of a lactyl group to the glucosamine. Also in this reaction, the C3 hydroxyl group will remove a phosphate from the alpha carbon of phosphoenolpyruvate. This creates what is called an enol derivative that will be reduced to a "lactyl moiety" by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) in step six. In step seven, the UDP-MurNAc is converted to UDP-MurNAc pentapeptide by the addition of five amino acids, usually including the dipeptide D-alanyl-D-alanine. Each of these reactions requires involvement of the energy source Adenosine triphosphate (ATP). The above reactions are referred to as stage one. Stage two occurs in the cytoplasmic membrane. It is in the membrane where a lipid carrier called bactoprenol carries peptidoglycan precursors through the cell membrane. Bactoprenol will attack the UDP-MurNAc penta, creating a PP-MurNac penta, which is now a lipid. UDP-GlcNAc is then transported to MurNAc, creating lipid-PP-MurNAc penta-GlcNAc, a disaccharide and also a precursor to peptidoglycan. How this molecule is transported through the membrane is still not clear. However, once it is there, it is added to the growing glycan chain. The next reaction is known as transglycosylation. In this reaction, the hydroxyl group of the GlcNAc will attach to the MurNAc in the glycan, which will displace the lipid-PP from the glycan chain. The enzyme responsible for this reaction is transglycosylase (White 2007).

2.2 Inhibitors for Peptidoglycan Synthesis

Glycopeptides and moenomycins are best-studied groups of antibiotics which interfere with the transglycosylation reaction (Reynolds 1989) though their mode of action varies, but they have certain common features such as large molecular masses, no cell penetration, preferential in vivo action on gram-positive

microorganisms, and an accumulation of peptidoglycan precursors in intact cells and in cell-free systems. The non-penetration of such inhibitors further substantiates the localization of the polymerization reactions on the outside of the cytoplasmic membrane. Realizing this, Salton and Kim (1996) stated that there are certain antibacterial drugs such as penicillin which interferes with the production of peptidoglycan by binding itself to the bacterial enzyme such as transpeptidases or penicillin-binding protein or carboxypeptidases and thereby forming the loosely woven structure by inhibiting the cross-linking of tetrapeptide chain with another chain. Vancomycin binds or forms complex with D-Ala-D-Ala dipeptide of exported lipid II or with those of nascent peptidoglycan. It is either both mechanisms are functioning in vivo or one of them is prominent. In the first case, wherein antibiotic complexed with lipid II, the resulting segregation of the lipid substrate will lead to an arrest of the glycan chain elongation, while, in the second case, the binding with peptide subunits of growing chains can have a shielding effect by steric hindrance on both transglycosylation and transpeptidation reactions. While mutation in the gene which codes for transpeptidases or ligase leads to reduced interaction with an antibiotic, a strong and rigid 3D mesh-like layer is formed which could be due to the phenomenon known as antibiotic resistance (Spratt 1994).

In nature, human body synthesized the antibiotic lysozyme found in tears which breaks the β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNaC) in peptidoglycan primarily to destroy the bacterial cells. This phenomenon has been explored in research laboratories mainly to break the cell walls of gram-negative bacteria as lysozyme-treated bacterial cells are osmotically sensitive and easily disrupted by shearing. Homogenization followed by density gradient centrifugation separates the cytosol and inner membrane as well as outer membrane. Certain archaea have shown the presence of a similar layer of pseudopeptidoglycan or pseudomurein wherein the sugar residues are β-1,3-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNaC).

3 Teichoic Acids

Teichoic acid is a major class of cell surface glycopolymers and is found within the cell wall of gram-positive bacteria such as species in the genera *Staphylococcus*, *Streptococcus*, *Bacillus*, *Clostridium*, *Corynebacterium*, and *Listeria* and appears to extend to the surface of the peptidoglycan layer. These are linear polymers of polyglycerol or polyribitol substituted with phosphates and a few amino acids and sugars linked via phosphodiester bonds. There are two types of teichoic acid: the lipoteichoic acid (LTA) polymers which contain a reducing terminal phosphatidic acid, occasionally anchored to the plasma membrane and is extended from the cell surface into the peptidoglycan layer, and the wall TAs (WTAs) which are covalently attached to peptidoglycan and extended through and beyond the cell wall. All the teichoic acids bound to peptidoglycan appear to be linked by a conserved linkage unit consisting of ManNAc-GlcNAc-1-P linked to C6 of a MurNAc residue. The chemical structure of WTAs is composed of ManNAc-(β 1→4)-GlcNAc

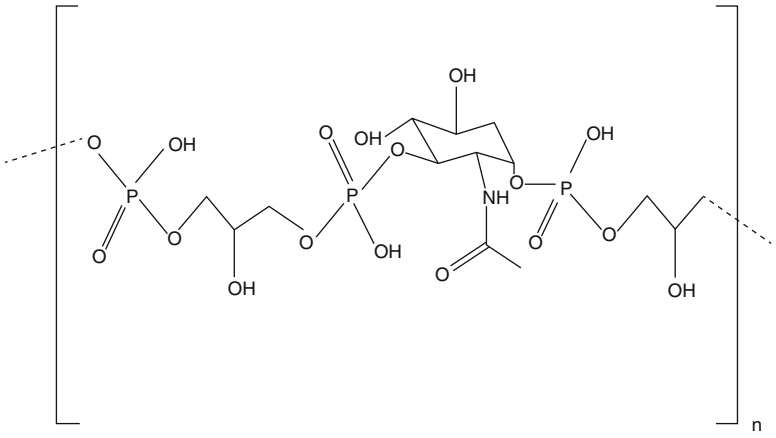

Fig. 2 Structure of teichoic acid

disaccharide with one to three glycerol phosphates attached to the C4 hydroxyl of the ManNAc residue (the "linkage unit") followed by a much longer chain of glycerol or ribitol phosphate repeats (the "main chain") (Fig. 2).

The exact function of teichoic acid in bacterial physiology is still not known though it is believed to be essential for the viability of gram-positive bacteria. There are certain evidences which elucidate the understanding of the important functions involved behind teichoic acids. The WTAs provide a channel of regularly oriented negative charges for threading positively charged substances through the complicated peptidoglycan network. The network of WTA-coordinated cations affects the overall structure of the polymers and this in turn influences the porosity and rigidity of the cell envelope. The WTA plays an important role in terms of cation homeostasis in gram-positive bacteria and provides a reservoir of ions close to the cell surface that might be required for enzyme activity (Marquis et al. 1976; Swoboda et al. 2010). In the past few years, researchers have tried to focus on the three-dimensional structure of WTAs and their bound cation group so as to elucidate the modes of cation binding to WTA polymer phosphate groups and to get an insight for designing the novel antimicrobials (Wickham et al. 2009). The other function is that wall teichoic acids (WTAs) are involved in the interaction of bacterial cells with the environment. WTAs in certain ways are involved in the regulation and assembly of muramic acid subunits on the outside of the plasma membrane. There are instances mainly for *Streptococci*, wherein teichoic acids have been involved in the adherence to the tissue surfaces. If these gram-positive bacteria lack or have defective WTAs, then there are a reduced initial adherence to artificial surfaces and also impaired ability to form biofilms. Contrary to other findings, Aly et al. (1980) mentioned in their study that impaired WTA does not exhibit reduced production of poly-*N*-acetylglucosamine (PNAG), the exopolysaccharide which is mainly involved in biofilm formation. Therefore, this suggests that WTAs played an independent role for biofilm formation. WTA is believed to be a virulent factor

and is required for host infection. Therefore, the enzymes involved in WTA biosynthesis were thought to be the targets for novel antimicrobials that could slow down colonization by gram-positive bacteria in the host cell. LTAs and WTAs have also shown to possess an important role in cell growth, division, and morphogenesis. In this context, Schirner and their coworkers indicated that in the absence of WTA expression, round and severely defective progeny is produced, while, during a defective gene expression of the biosynthesis of LTA, could hinder in septum formation and cell separation (Schirner et al. 2009). It was also suggested that the biosynthetic enzymes of WTA are associated with the machinery involved in elongation, while the biosynthetic enzymes involved for LTA might be associated with the machinery involved in septation and cell division.

3.1 Biosynthesis of Teichoic Acid

The pathway for WTA biosynthesis has been discussed here for *B. subtilis* 168 [synthesizes poly (glycerol phosphate) WTA], *B. subtilis* W23, and *S. aureus* [synthesizes poly (ribitol phosphate) WTA].

3.1.1 Biosynthesis of Poly (Glycerol Phosphate) WTA

Ward (1981) described that the pathway for the biosynthesis of poly (glycerol phosphate) WTAs was first characterized in *B. subtilis* 168. *tag* genes (also known as teichoic acid glycerol genes) were involved during biosynthesis. The pathway begins in the cytoplasm wherein the reversible enzyme TagO, which belongs to the family of phosphosugar transferases, could transfer GlcNAc phosphate to an undecaprenyl phosphate (also known as bactoprenyl phosphate) carrier which is anchored in the bacterial membrane. After catalyzing the reaction by TagO, the GlcNAc-PP-lipid is formed. Another enzyme, *N*-acetylmannosaminyl transferase (TagA), catalyzes the transfer of ManNAc from UDP-ManNAc to the C4 hydroxyl of the GlcNAc residue in order to form a β-linked disaccharide, which will now act as the substrate for the next enzyme in the pathway, TagB (Zhang et al. 2006). In order to complete the synthesis of the linkage unit, TagB, also known as glycerophosphate transferase enzyme, is involved which could transfer a single phosphoglycerol unit from CDP-glycerol to the C4 hydroxyl of ManNAc (Bhavsar et al. 2007). After the linkage unit formation, TagF (polymerizing cytidylyltransferase) could catalyze the attachment of 35 or more glycerol phosphates to the linkage unit in order to form an anionic polymer (Schertzer and Brown 2008). Once assembled, the lipid-linked WTA polymer is putatively modified by a glycosyltransferase (TagE) and then exported to the external surface of the bacterial membrane by a two-component ABC (ATP-binding cassette) transporter, TagGH (Lazarevic and Karamata 1995). The anomeric phosphate of the GlcNAc residue couples the polymer to peptidoglycan and esterified with D-alanine residues. The enzyme which could catalyze this reaction has till date not been identified though it belongs to the class of transferase enzyme.

3.1.2 Biosynthesis of Poly (Ribitol Phosphate) WTA in *B. subtilis* W23

Lazarevic and coworkers studied the pathway for the biosynthesis of poly (ribitol phosphate) WTA in *B. subtilis* W23 and reported that the genes involved were designated as *tar* genes (for teichoic acid ribitol) (Lazarevic et al. 2002). The initial three steps which were mediated by TarO, TarA, and TarB were identical to those in *B. subtilis* 168. Thereafter, pathways diverged wherein the TarF acts as primase and adds one additional glycerol phosphate unit to the 168-type linkage unit. Once the linkage unit is complete, the poly (ribitol phosphate) main chain is assembled. Lazarevic et al. proposed that the assembly of this poly (ribitol phosphate) chain requires two enzymes: TarK, which transfers a single ribitol phosphate residue to the linkage unit, and TarL, which carries out the polymerization of the ribitol phosphate chain. TarK and TarL in *B. subtilis* W23 were thus suggested to function as a primase/polymerase pair, analogous to the primase/polymerase pair (TagB/TagF) that assembles the poly (glycerol phosphate) chain in strain 168. Once the poly (ribitol phosphate) WTA polymer is assembled, the remaining steps are thought to be similar to those in strain 168. That is, the WTA polymer is glycosylated, transported through the bacterial membrane by a two-component transporter, TarGH, attached to peptidoglycan by an unidentified transferase, and esterified with D-alanine residues.

3.2 Inhibitors for Wall Teichoic Acid Biosynthesis

In the past few years, speculations are on that WTA biosynthetic pathway could be the site for the antibiotic, though still only one specific inhibitor has been reported (Swoboda et al. 2010). Swoboda and coworkers also stated that there are two distinct types of antimicrobial targets in the pathway: (1) antivirulent targets (TarO and TarA) and (2) antibiotic targets due to a mixed gene dispensability pattern. The inhibitors of the former slow down colonization and spread of infection, while inhibitors of the latter have shown to prevent bacterial growth. In this context, the inhibitors were evaluated for both target points.

Weidenmaier and coworkers were the first to suggest that the pathway for WTA biosynthesis is the site for antivirulence and had studied it for the case of *S. aureus* (Weidenmaier et al. 2004). Several researchers reported that there is a potent natural product inhibitor, the uridine-containing antibiotic tunicamycin for WTA biosynthesis (Hancock et al. 1976; Wyke and Ward 1977). Tunicamycin inhibits MraY, an enzyme, phosphosugar transferase activity (involved in the peptidoglycan biosynthetic pathway) which couples sugar phosphates to membrane-embedded lipid phosphates. Besides this, tunicamycin also inhibits TarO (Price and Tsvetanova 2007). It has been observed that tunicamycin is more selective for TarO over MraY, and due to this selectivity, it could shut off the in vitro WTA expression without affecting bacterial growth rates. Till date, researchers could not develop any other specific inhibitor or its homologue which could be used in place of tunicamycin for in vivo studies as this inhibitor is toxic to eukaryotes, so this strategy of inhibiting TarO gene involved during WTA biosynthesis could not be

worked out for treating *S. aureus* infections in humans. Therefore, there is a need to identify the nontoxic, selective inhibitors of TarO gene which are involved during the WTA biosynthetic pathway in order to cure different diseases.

The next target in the WTA biosynthetic pathway is the antibiotic target which was reported by Swoboda and coworkers (2010). Peschel and coworkers reported that *S. aureus* strains lacking WTAs are incapable of colonizing a host; however, these resistant mutants are not expected to survive in vivo (Peschel et al. 1999). But, Swoboda et al. pointed out that there are numerous other pathways that contain conditionally the essential enzymes linked to virulence factor expression. Many of these enzymes could be good antibiotic targets provided that the major mechanism for resistance involves deletion of the pathway and results in the production of avirulent organisms (Swoboda et al. 2010).

4 Bacterial Glycoproteins

Glycans are an important constituent of bacterial cell wall and of surface antigens such as lipopolysaccharides (LPSs) and capsular antigens which are attached by lipid anchors (lipid A or diacylglycerophosphates) in gram-negative bacteria (Gotschlich et al. 1981; Schmidt et al. 2003). These carbohydrate conjugates function as virulent factors which have been well documented in literature, while the structure-function relationships of bacterial cell surface lipoglycans have been elucidated. It aids in maintaining structural integrity and correct protein localization and in the induction of host responses (Beutler and Rietschel 2003).

In the past few decades, researchers thought that prokaryotes are incapable of glycosylating the proteins. But now, researchers have firmly established that there is a glycosylation of proteins in eubacteria which is due to the glycoprotein synthetic pathway being operated inside the prokaryotic cell (Power and Jennings 2003; Upreti et al. 2003). The protein glycosylation has initially been reported in Archaea and later in *Clostridia* which exhibited the glycosylated surface (S) layer proteins (Schaeffer and Messner 2001). Earlier, the presence of glycoproteins in bacteria was detected either through the formation of Schiff bases or by the specific binding of lectins. The type of glycosylation either N or O type could be distinguished by using endoglycosidases F (Endo F1, F2, F3, and D), PNGase F, or O-glycosidase in combination with the inhibitors such as bacitracin or tunicamycin which inhibits glycosylation.

4.1 Importance of Protein Glycosylation in Gram-Negative Bacteria

4.1.1 Flagellar Glycosylation and Motility

The *N*- or *O*-glycosylation of the peptide in bacteria could induce the host response by causing infection. In this context, in order to study the effect of protein glycosylation in bacteria on host's mechanism, *Campylobacter* spp. is considered

to be a prominent example as it is believed to be an agent which generally causes diarrhea. The flagellin, an immunodominant protein, is recognized as an essential constituent in flagella which could act as a virulence factor. The flagellins of *C. jejuni* and *C. coli* were found to be extensively glycosylated as reported by Guerry and coworkers (1996). In the case of *C. jejuni*, the glycoprotein structure has been revealed by Szymanski et al. wherein it was indicated that the carbohydrate structure has pseudaminic acid or its derivatives Pse5Am7Ac and Pse5Pr7Pr, which are linked to approximately 19 serine or threonine residues. These immunogenic substituents are clustered in the hydrophobic central core region, and the surface is exposed with flagella filaments (Szymanski et al. 2003). Reports suggest that adjacent to the flagellin structural genes in the genome of *C. jejuni* (NCTC 11168) lies the locus containing approximately 50 genes which represents the flagellin glycosylation machinery (Parkhill et al. 2000).

The other protein glycosylation locus (pgl) of 17 kb mediates the *N*-glycosylation of multiple proteins in *C. jejuni* and represents the first such pathway in gram-negative pathogens (Young et al. 2002). However, mutation in pgl genes affects glycosylation and also abolishes the recognition by polyclonal rabbit antiserum and by antibodies from humans which were infected with *C. jejuni*. Furthermore, insertional inactivation of pgl genes resulted in decreased adherence, invasion in vitro, and loss of intestinal colonization in mice (Szymanski et al. 2002). In addition to the nonflagellar glycoproteins PEB3, an immunoreactive surface protein (Cj0289c; Cj1670c) along with 22 periplasmic glycoproteins was identified. All the glycoproteins have a common oligosaccharide structure a-D-Galp-NAc-(1-4)-a-D-GalpNAc-(1-4)-a-D-GalpNAc [b-D-Glcp-(1-3)]-(1-4)-a-D-GalpNAc-(1-3)-b-D-Bac-(1,N)-Asn Xaa, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucopyranose). Bacillosamine has also been found to be a constituent of LPS and capsular polysaccharide in other prokaryotes (Schaeffer et al. 2001; Schmidt et al. 2003). Szymanski and coworkers reported that PglB is a key enzyme in the pgl locus and probably acts as an oligosaccharide transferase mediating the N-linkage to the peptide backbone; however, any mutation in the pglB gene could totally abolish the glycosylation (Szymanski et al. 2003).

Motility is an important factor in *Helicobacter*-associated diseases. Flagella of gastric *Helicobacter* spp. (*H. pylori*, *H. mustelae*, and *H. felis*) exhibit two distinct flagellins, FlaA and FlaB. Mutants lacking FlaA have impaired motility and shortened flagella, while deletion of FlaB gene has shown only moderate effect (Schmidt et al. 2003).

4.1.2 Pili and Adhesions in Gram-Negative Bacteria

There are several gram-negative microorganisms wherein protein glycosylation in pili influences pathogenesis. Certain examples have been discussed here in this chapter.

In the case of *Haemophilus influenzae*, the infection is being caused through its attachment to the human epithelial cells which occurs by two related adhesions HMW1 and HMW2 being encoded by hmw1 and hmw2, and both genes are associated with hmwB and hmwC (Schmidt et al. 2003). Grass et al. described

that HMW1 is glycosylated and that HMWIC is needed for glycosylation. It was observed that the glycan moiety of HMW1 contains galactose (Gal), glucose (Glc), and mannose (Man) residues and requires the activity of phosphoglucomutase, which is involved in the lipooligosaccharide (LOS) biosynthesis pathway. Shedding of the non-glycosylated adhesins decreases the adherence to epithelial cells. Therefore, glycosylation directly modulates cellular interactions and might also influence pathogenesis (Grass et al. 2003).

In *Neisseria*, type IV pili aid in bacterial attachment to the host cell through the glycosylation of adhesins. Stimson et al. (1995) revealed that in *N. meningitidis* strain C311, there is an O-linked glycan substitution at serine residue 63 (Ser63) wherein pglA or pgtA (pilin glycosylation) gene could transfer the first a-(1, 3)-linked galactose to the diacetamido trideoxyhexose Ser63-substituent. On the other hand, certain researchers observed that strains which are deficient in pilin glycosylation could express more type IV pili along with an increased adherence to the epithelial cells but less resistance against solubilization were present (Marceau et al. 1998). Parge and coworkers deduced the structure of glycoprotein through electron density maps as a-Gal-(1, 3)-GlcNAc disaccharide which is being linked to Ser63 in *N. gonorrhoeae* strain MS11 pili (Parge et al. 1995). The homopolymeric guanosine tract identified in certain strains is basically involved in the phase variation of pglA expression which is associated with the conversion from uncomplicated gonorrhea to disseminated gonococcal infections (Banerjee et al. 2002).

4.1.3 Glycosylation of Surface and Membrane Proteins

Borrelia burgdorferi is the causative agent of Lyme disease. In this microbe, the major surface (lipo) proteins, i.e., OspA and OspB (31 and 34 kDa, respectively), are differentially expressed during the infectious cycle. However, at the time of infection, the presence of anti-Osp antibody prevents the disease by largely reducing or killing the spirochete population in the tick before transmission to the host (Schwan and Piesman 2002). In the present scenario, the human vaccine is based on OspA.

4.1.4 Pili and Adhesion in Gram-Positive Bacteria

Streptococcus parasanguis, being a gram-positive bacterium, colonizes on the tooth surface and plays a pivotal role in the development of dental plaque. Adhesion is mediated by the major fimbrial subunit Fap1, wherein 80 % of the Fap1 protein consists of dipeptide serine repeats. The glycopeptide showed the presence of Rha (rhamnose), Glc, Gal, GlcNAc, and GalNAc. The adherence properties of Fap1 are dependent on its glycan modification because monoclonal antibodies that blocked adhesion did not recognize recombinant Fap1, but were inhibited by a Fap1-derived glycopeptide and showed reduced binding to periodate-oxidized Fap1 (Stephenson et al. 2002).

4.1.5 Glycosylation of Cell Wall and Secreted Proteins

Streptococcus mutans is largely responsible for human dental caries and occasionally causes infective endocarditis in predisposed patients. An immunodominant cell

wall-associated glycoprotein of 60 kDa (IDG-60) carrying NeuNAc (*N*-acetylneuraminic acid), Man, and Gal residues has been identified by screening a genomic library of *S. mutans* strain GS-5. This protein is essential for maintaining cell wall integrity and cell shape; however, the role of the glycan modification has not been elucidated (Chia et al. 2001).

4.1.6 Molecular Mimicry of Eukaryotic Glycoproteins by Bacteria

With time, bacteria have developed glycoconjugates which are closely related to those found in mammals. *Chlamydia trachomatis* synthesizes high-mannose-type *N*-glycan on glycoprotein which is similar to the type assembled on eukaryotic glycoproteins which also produce heparin-like molecule causing infection in the eukaryotic host. *Nisseria meningitidis* produces adhesive fimbriae that contain O-glycan in a pili subunit. The assemblies of eukaryotic-like glycans by pathogenic bacteria may be a form of molecular mimicry for bacteria in order to avoid or to compromise the immune system of the host (Varki et al. 1999).

5 Membrane-Derived Oligosaccharides

Membrane-derived oligosaccharides (MDOs) were named due to the membrane origin of the phosphoglycerol substituents. These are present in the periplasmic space and are widely distributed in gram-negative bacteria. These are also known as the osmoregulated periplasmic glucans (OPGs) since the synthesis and accumulation of MDOs are inversely proportional to the osmotic strength of the environment (Bohin 2000). MDOs are a heterogeneous family of closely related, highly branched oligosaccharides containing glucose as the sole sugar. They are substituted with sn-1-phosphoglycerol, phosphoethanolamine, and 0-succinyl ester residues, giving these molecules a net negative charge. MDOs contain 6–12 glucose residues joined by K-1,2 and K-1,6 linkages, with the principal species containing eight to nine glucose units (Kennedy 1996). Several reports suggest the important functions of MDOs in *E. coli*, such as its role in cell to cell signaling, chemotaxis, lysis induction by bacteriophages, the regulation of capsular polysaccharide synthesis, and the osmoregulation of the outer membrane protein expression (Geiger et al. 1992). Bohin and coworkers stated that when *E. coli* cells were grown in low-osmolarity medium, the MDOs in the periplasmic space represent 3.5–5 % of the cell dry weight, while the cells when grown in the medium with higher osmolarity exhibit a drastic decrease in MDO content (Bohin 2000; Bohin and Lacroix 2006).

5.1 Biosynthesis of MDOs

Limited reports are available on the biosynthesis of MDOs; however, the pioneering study was reported by Dedonder and Hassid (1964). In the past decade,

researchers investigated the synthesis of cyclic glucans in *Rhizobiaceae* and established that membrane-bound glucosyltransferase system catalyzes an essential step in the biosynthesis of MDOs wherein glucose residues are transferred from UDP-glucose to octyl β-D-glucoside (acceptor) with the formation of β 1→2-linked polyglucose chains linked to the acceptor molecule (Weissborn and Kennedy 1984). It was also reported that any mutation in the mdoA locus leads to the loss of membrane-bound glucosyltransferase activity and thereby prevents the formation of MDO chains in vivo. Besides the membrane fraction, a soluble protein identified as acyl carrier protein (ACP) from the cytosolic fraction is also required during the process of biosynthesis. Jackson and Kennedy (1983) proposed a two-step model wherein phosphoglycerol transferase I, an enzyme of the inner membrane, catalyzes the transfer in vitro phosphoglycerol residues from phosphatidylglycerol to nascent MDOs (membrane-linked newly synthesized glucans) or to the synthetic β-glucoside acceptors such as arbutin. Then, the periplasmic phosphoglycerol transferase II would transfer those residues from one molecule of OPG (potentially still an acceptor in the first step) to another already liberated in the periplasmic space. The sn-1,2-diglyceride that is the product of this reaction is normally phosphorylated by the enzyme diglyceride kinase in a salvage reaction, leading to the formation of phosphatidic acid, which then can be used for the synthesis of cellular phospholipids (Jackson et al. 1984).

5.2 Inhibitors of MDO Biosynthetic Pathway

The membrane-bound transglucosylase system is inhibited in a dose-dependent manner by antibiotics such as bacitracin and amphomycin. These antibiotics generally function in a cell-free transglucosylase enzyme system by forming specific complex with polyisoprenyl phosphate derivatives (Banerjee 1989).

6 Lipopolysaccharides

A lipopolysaccharide (LPS) or lipoglycan molecule is made up of a lipid and a polysaccharide which are joined together by a covalent bond. LPSs are present in the outer membrane of the gram-negative bacteria. The low molecular weight form of bacterial polysaccharides is known as lipooligosaccharides (LOSs). The toxicity of LPS was discovered by Richard Friedrich Johannes Pfeiffer and termed as endotoxin. It was reported that endotoxins are toxins released by the bacteria into the environment and are kept within the bacterial cells and are released only after bacterial cell wall destruction (Parija 2009). Researchers used the term endotoxin as a synonym with LPS. The important function of LPS is to provide structural integrity to bacteria and its membrane protection from certain kinds of chemical attack. LPS also aids in membrane stabilization by increasing the negative charge on the cell membrane. This molecule is also believed to be a molecule of life for the

gram-negative bacteria since any sort of mutation or removal could lead to its death. LPS could also illicit a strong response from normal animal immune system. Besides its pathogenic nature, the nonpathogenic aspects have also been studied such as surface adhesion, bacteriophage sensitivity, and interactions with predators such as amebae.

Lipopolysaccharide (LPS) comprises of three parts: (i) "O" antigen, (ii) core polysaccharide, and (iii) lipid A.

6.1 "O" Antigen

It is also named as "O" antigen, "O" polysaccharide, or "O" side chain of the bacteria; it is basically a repetitive unit of glycans and forms a polymer. It is exposed on the very outer surface of the LPS molecule and is attached to the core oligosaccharide in a bacterial cell. The composition of "O" chain varies from strain to strain. Rittig et al. (2004) mention two types of LPS, smooth and rough on the basis of the presence or the absence (or reduced form) of "O" chain respectively. It was also observed that bacteria with rough LPS are more hydrophobic and have a more penetrable cell membrane for hydrophobic antibiotics (Tsujimoto et al. 2003).

6.2 Core Polysaccharide

The core generally contains the oligosaccharide component that attaches itself to lipid A. The sugars commonly present are heptose and 3-deoxy-D-mannooctulosonic acid (also known as KDO, ketodeoxyoctulosonate) (Hershberger and Binkley 1968). In most of the bacteria, a noncarbohydrate component is also present in the LPS core such as phosphate, amino acids, and ethanolamine substituents. In the case of *Neisseria meningitidis*, the inner core is composed of 3-deoxy-D-manno-2-octulosonic acid (KDO) and heptose (Hep) moieties.

6.3 Lipid A

Generally, the lipid A moiety is a much conserved component of the LPS. It is a phosphorylated glucosamine disaccharide decorated with multiple fatty acids. These hydrophobic fatty acid chains anchor the LPS into the bacterial membrane, and the rest of the LPS projects from the cell surface. The lipid A domain is mainly responsible for much of the toxicity of gram-negative bacteria. When bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhea, and possible fatal endotoxic shock (also called septic shock) (Tzeng et al. 2002).

6.4 Lipooligosaccharides (LOSs)

These are the glycolipids which are present in the outer membrane of certain types of gram-negative bacteria such as *Neisseria* spp. and *Haemophilus* spp. It is basically a low molecular weight lipopolysaccharide. LOS helps in maintaining the structural integrity and functionality of the outer membrane in gram-negative microorganisms. Lipooligosaccharides act as immunostimulators and immunomodulators; therefore, its important role in the pathogenesis of certain bacterial infections has been reported (Moran et al. 1996). In certain bacterial strains, LOS molecules display molecular mimicry and antigenic diversity, aiding in the evasion of host immune defenses and thus contributing to the virulence of these bacterial strains. In contrast to lipopolysaccharides, lipooligosaccharides lack "O" antigens and possess only a lipid A-based outer membrane-anchoring moiety and an oligosaccharide core (Kilár et al. 2013).

6.5 LPS Modifications

With time, the host system could manage the toxic effects of gram-negative bacteria which are generally present in the small intestine. In the past few years, it has been reported that a specific enzyme in the host's intestine, i.e., alkaline phosphatase, could detoxify the toxic effect caused by lipopolysaccharide through the removal of two phosphate groups being present on the LPS carbohydrates (Bates et al. 2007). The other route for detoxification could be the addition of more specific sugars using glycosyltransferases in order to modify the structure of LPS.

6.6 Biosynthesis of Lipopolysaccharides

It has been observed that in *Salmonella* all *wbaP* (*rfbP*) genes encode for an enzyme (glycosyltransferase) that transfers Gal-1-P to undecaprenol-P as an initiating step of "O" units, and all have sequences that are readily aligned. It has also been reported that in certain *Salmonella* strains like from group D and group B strains, gene *wbaV* (*rfbV*) encodes for tyvelose transferase and an abequose transferase respectively, and each could carry out functions in the presence of appropriate precursors.

The genes for saccharide processing (including export, polymerization, and assembly of complex polysaccharides such as LPS) commonly occur in families of homologous genes that perform same general functions. Two genes with the same name may have specificity for different oligosaccharides and are distinguished by including the species or some other relevant information as subscript.

Most "O"-antigen gene clusters have genes currently known as rfc, rfbX and cld, or rol. Each could be recognized by the topological features of the encoded protein

although there is a certain sequence similarity for the proteins in the first two families. The wzy genes are generally found within the "O"-antigen gene cluster (Reeves et al. 1996).

6.7 Functions of Lipopolysaccharides

6.7.1 Biological Effects of Lipopolysaccharides on Hosts

Lipopolysaccharides in gram-negative bacteria usually act as a candidate target for new antimicrobial agents. LPS generally acts as endotoxin as it binds to the cell receptor (CD14/TLR4/MD2) complex in monocytes, dendritic cells, macrophages, and B cells, which thereby promotes the secretion of proinflammatory cytokines, nitric oxide, and eicosanoids (Abbas 2006). The TLR4 as the LPS receptor was demonstrated by Bruce Beutler, a noble laureate for this work in the year 2011 in Physiology or Medicine (www.nobleprize.org/nobel_prizes/medicine/laureates/2011/press.html). LPS also acts as an exogenous pyrogen or as the external fever-inducing substance. The functions of LPS are under experimental research for several years as it activates many transcription factors. LPS produces different types of mediators which are involved in septic shock. In this context, Warren et al. (2010) observed that humans are more sensitive to LPS as compared to other animals and reported that a minimal dose of 1 μg/kg induces shock in humans, wherein mice could tolerate a dose which is a thousand times higher compared to the humans' minimal dosage. This difference could be due to the level of circulating natural antibodies (Reid et al. 1997).

Endotoxins could aggravate the pathogenic effect by *Neisseria meningitidis*, a gram-negative microorganism, and could cause meningococcal disease, including meningococcemia, Waterhouse-Friderichsen syndrome, and meningitis.

Certain portions of LOS in some bacteria have shown similarity in terms of their chemical structure with the human host cell surface molecules. It is termed as **molecular mimicry**. Moran and coworkers observed and reported that in the case of certain strains of *Neisseria meningitidis*, the terminal tetrasaccharide portion of the oligosaccharide (lacto-*N*-neotetraose) is the same tetrasaccharide as that found in paragloboside, a precursor for ABH glycolipid antigens found on human erythrocytes. Furthermore, the terminal trisaccharide portion (lactotriaose) of the oligosaccharide from the pathogenic *Neisseria* spp. is also present in the lactoneoseries of glycosphingolipids from human cells. Besides this, the presence of these human cell surface mimics may also act as a camouflage from the immune system and could abolish the immune tolerance when infecting hosts with certain human leukocyte antigen (HLA) genotypes, such as HLA-B35 (Moran et al. 1996).

6.7.2 Effect of LPS Variation on Immune Response

The outer carbohydrate portion or "O" antigen is believed to be the most variable portion of the LPS molecule, and this thereby imparts the antigenic specificity. In contrast, lipid A portion is the most conserved part in LPS, but this portion could also vary in number and nature of acyl chains present within or different genera. These variations in lipid A could impart antagonistic properties to LPS like in the case of

Rhodobacter sphaeroides, wherein diphosphoryl lipid A (RsDPLA) is a potent antagonist of LPS in human cells but is an agonist in hamster and equine cells. In the normal human blood serum, anti-LOS antibodies are present which are bactericidal, while the patients who are suffering from infections arising out of serotypically distinct strains possess anti-LOS antibodies that differ in their specificity primarily within the structure of the oligosaccharide portion of the LOS molecule (Yamasaki et al. 1994). During infection, the variation in the antigenicity of LOS was observed which could be due to the ability of bacteria to synthesize more than one type of LOS molecule through sialylation, which might lead to an increase in resistance to complement-mediated killing (Yamasaki et al. 1994) or can down-regulate the complement activation (Moran et al. 1996) or even it can evade the effects of bactericidal antibodies.

6.7.3 Health Effects of Lipopolysaccharide on Host

The presence of endotoxins in the blood is called endotoxemia. It could lead to septic shock, if the immune response is severely pronounced (Opal 2010). Endotoxemia in the intestine could lead to the development of alcoholic hepatitis (Ceccanti et al. 2006). It has also been reported that gonococcal LOS can cause damage to the human fallopian tubes.

The molecular mimicry of some LOS molecules is thought to cause autoimmune-based host response. The bacteria's mimicry to the host structures via LOS is found with *Helicobacter pylori* and *Campylobacter jejuni*, the organisms which cause gastrointestinal disease in humans, and *Haemophilus ducreyi* which causes chancroid. Certain *C. jejuni* LPS serotypes (attributed to certain tetra- and pentasaccharide moieties of the core oligosaccharide) have also been implicated with Guillain-Barré syndrome and a variant of Guillain-Barré called Miller Fisher syndrome (Moran et al. 1996).

An increase in the endotoxin load due to an increased population of endotoxin-producing bacteria in the intestinal tract is associated with obesity-related patients. This clearly shows that the presence of a high level of endotoxin from *Escherichia coli* could induce obesity as has been observed by Cani and coworkers through experimental studies wherein they injected purified endotoxin from *Escherichia coli* into germ-free mouse models (Cani et al. 2007). It has also been reported that endotoxin is associated with obesity because it induces an inflammation-mediated pathway. Recently, it was observed that *Enterobacter cloacae* B29 could also contribute toward the obesity and insulin resistance in human patients (http://www.nature.com/ismej/journal/vaop/ncurrent/full/ismej2012153a.html).

Endotoxins could be detected through the standard assay known as limulus amebocyte lysate (LAL) assay utilizing the blood from the horseshoe crab (Iwanaga 2007).

7 Capsular Polysaccharide

The capsular polysaccharide constitutes the outermost layer of the bacterial cell and mediates its direct interaction with the environment. Due to these interactions, polysaccharide capsules have been implicated as an important virulence factor for

many bacterial pathogens. The capsular polysaccharide is mainly found in gram-negative bacteria such as in *E. coli*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Salmonella*. In certain gram-positive bacteria, these capsular polysaccharides were also observed such as in the case of *Bacillus megaterium* (could synthesize a capsule composed of polypeptide and polysaccharides), *Streptococcus pyogenes* (synthesizes hyaluronic acid capsule), *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Staphylococcus epidermidis*. Besides this, the capsular polysaccharide was also observed in yeast particularly in *Cryptococcus neoformans* which has a capsular polysaccharide similar to that of bacteria. Generally, it is very difficult to distinguish between the capsular polysaccharide (CPS) and other surface polysaccharides, such as the "O"-antigenic moieties of lipopolysaccharide (LPS), as CPS is found to be associated with LPS (Whitfield 1988). The capsule could be identified either through India ink staining or through serological methods wherein the capsular material acts as antigen and is mixed with specific anticapsular sera. When it was observed under a microscope, the capsule appears swollen due to an increased refractivity. This phenomenon is known as Quellung reaction.

Capsular polysaccharides are the hydrated molecules that have more than 95 % water. These molecules are linked to the bacterial cell surface via covalent attachment to either phospholipids or lipid-A molecules, while, in certain cases, it was observed that CPS may be associated directly with the cell without any membrane anchor (Whitfield 1988; Whitfield and Valvano 1993). The capsular polysaccharide could either be homo- or heteropolymers composed of repeating monosaccharides joined by glycosidic linkages (Roberts 1996). The two monosaccharide units may be joined in a number of configurations which may lead to a large structural diversity in CPS among species, while chemically identical capsular polysaccharides may also be synthesized by different bacterial species. It was identified that certain CPS or K antigen is associated with certain infections. The *Escherichia coli* K1 antigen, a homopolymer of α2,8-linked *N*-acetylneuraminic acid, is the major cause of neonatal meningitis (Robbins et al. 1974). The capsular polysaccharide of *Neisseria meningitidis* group B is identical to the K1 polymer of *E. coli* (Grados and Ewing 1970).

The bacterial capsular polysaccharide could perform a number of functions such as prevention of desiccation, adherence, resistance to nonspecific host immunity, and resistance to specific host immunity and thereby mediating the diffusion of molecules through the cell surface (Whitfield 1988; Roberts 1996). The encapsulated bacteria could sustain or survive outside the host and thereby promotes the transmission of pathogenic bacteria from one host to another; it could be due to the formation of hydrated gel around their cell surface which protects the cell from the effects of desiccation. Capsular polysaccharide promotes biofilm formation as this molecule has the tendency to adhere the bacterial cell either to the surface or with other bacterial cells which facilitates the colonization (Costerton et al. 1987).

During the invasive bacterial infections, interactions between the capsular polysaccharide and the host's immune system can decide the outcome of the infection

(Roberts et al. 1989; Roberts 1996). In the absence of specific antibody, the presence of capsule confers resistance to the nonspecific host defense mechanisms. These responses include the activation of the complement cascade via the alternative pathway and of the C3b-mediated opsonophagocytosis by polymorphonuclear leukocytes. Both these responses provide protection in the preimmune host when specific antibodies are absent. The alternative pathway is initiated by the binding of C3b to the bacterial cell surface. The bound C3b is activated by factor B and thereby forms C3convertase C3bBb. This now allows the binding of more C3 and the formation of membrane attack complex on the outer membrane of the bacteria which leads to lysis and death (Finne 1982; Roberts 1996). This phenomenon could be inactivated by capsule which could provide a cell permeability barrier to complement components, thereby masking the C3b deposited on cell surface structures from C3b receptors on the phagocyte cell surface that could otherwise be potent activators of the alternative pathway (Howard and Glynn 1971). The net negative charge is being conferred on to the cell surface by the capsular polysaccharide in order to confer resistance toward phagocytosis. The net charge is directly proportional to the conferred resistance toward phagocytosis.

In most of the cases, capsular polysaccharides could elicit an immune response while certain are poorly immunogenic. The poor immunogenicity has been observed in *E. coli* KI capsule, *E. coli* K5 capsule, and *Neisseria meningitidis* serogroup B primarily due to the presence of NeuNAc in the capsules which is similar to desulfoheparin (Bhattacharjee et al. 1975). These capsules are structurally similar to the polysaccharides encountered on host tissue; therefore, these capsules are poorly immunogenic and thereby elicit a poor antibody response in the host (Wyle et al. 1972). *Burkholderia pseudomallei* is believed to be the causative agent of melioidosis which is due to direct inoculation into wounds and skin abrasions or due to the inhalation of a contaminated material. This disease is present as an acute pneumonia or as an acute septicemia, a more severe form of disease. The chronic and the subclinical forms generally remain undetected until activated by a traumatic event or decrease in immunocompetence (Ip et al. 1995). *B. pseudomallei* is resistant to a large number of antibiotics; even with the aggressive antibiotic therapy, the mortality rate remains high and the incidence of relapse is common (Currie et al. 2000). Reckseidler-Zenteno reported that certain cell-associated antigens were identified in *B. pseudomallei*. The cell-associated antigens include capsular polysaccharides and lipopolysaccharides (Reckseidler-Zenteno 2012). The capsular polysaccharide produced by *B. pseudomallei* is determined to be an unbranched polymer of repeating tetrasaccharide units with a structure -3)-2-O-acetyl-β-D-Galp-(1-4)-α-D-Galp-1-3)- β-D-Galp-(1-5)- β-D-KDOp-(2- (Masoud et al. 1997). The exact role of CPS in terms of virulence was not known but the sera of patients suffering with melioidosis have shown the presence of antibodies against CPS. The two other CPS structures were also identified: a branched 1,4-linked glucan polymer (CP-1a) and a triple-branched heptasaccharide repeating unit composed of rhamnose, mannose, galactose, glucose, and glucuronic acid (CP-2) (Perry et al. 1995). The genes involved in the synthesis of these capsules and the role of these capsules in virulence had not been identified.

7.1 Biosynthesis of Capsular Polysaccharide

The capsular polysaccharide is produced by many gram-negative bacteria by one of the two assembly systems, i.e., either by Wzy-dependent pathway or ATP-binding cassette (ABC)-transporter-dependent pathway (Cuthbertson et al. 2009; Willis and Whitfield 2013).

Among the gram-negative bacteria, the capsular K antigens of *E. coli* are widely studied, and the capsule system is differentiated into four groups based on assembly system, key genes organization, and regulatory features (Whitfield 2006). The Wzy-dependent pathway is used for groups 1 and 4 capsules wherein the isolates are associated with gastrointestinal infections. In this pathway, the initial synthesis took place in the cytoplasm wherein the repeating units of polyprenol-linked CPS are synthesized which are then flipped by a Wzx protein across the inner membrane and further polymerized into the full length CPS by a Wzy protein. The CPS chains are assembled into a capsular structure onto the cell surface, but how it is retained in the cell surface is not entirely clear though it is a multifactorial process. The similar process exists in genera such as *Klebsiella* and *Erwinia*.

The capsular system of groups 2 and 3 in *E. coli* requires ABC-transporter-dependent assembly. This system is found in mucosal pathogens which include *Campylobacter jejuni*, *N. meningitidis*, *H. influenzae*, *Mannheimia haemolytica*, etc. These bacteria caused variety of diseases in humans like septicemia, meningitis, urinary tract infections, gastrointestinal infections, and otitis media (Agrawal and Murphy 2011). The CPS assembled by the ABC-transporter-dependent pathway is in the presence of phospholipids at the reducing end of the polysaccharide chain. Willis and Whitfield (2013) reported that the terminal lipid is attached to the repeating units of polysaccharide chain in *E. coli* K1 and K5 to the cell surface. The phospholipid is lyso-phosphatidylglycerol (PG) which is attached to the CPS chain via a novel β-linked poly-3-deoxy-D-manno-oct-2-ulosonic acid (kdo) linker. The amount of CPS associated with the cell is very difficult to measure since the linkage between the lipid and CPS is unstable; however, other mechanisms are also there which allow the CPS to link to the cell membrane such as the ionic interactions between CPS and the core region of LPS as observed in *E. coli* K1 (Jimenez et al. 2012).

In the ABC-transporter-dependent pathway, CPS is synthesized in the cytoplasm wherein the repeating units of polysaccharide structure are assembled through the action of glycosyltransferase (GT) wherein GTs catalyze the transfer of specific sugars from an activated donor to the non reducing end of the growing CPS glycan (DeAngelis 1999) and are exported across the inner membrane via an ABC transporter comprising of two identical nucleotide-binding domain (NBD) polypeptides (KpsT in *E. coli*) and two integral membrane polypeptides (KpsM). In order to complete the transportation from the periplasm toward the cell membrane, it requires two other characteristic components: a member of the polysaccharide co-polymerase (PCP-3) family (KpsE) and an outer membrane polysaccharide (OPX) protein (KpsD). These proteins are thought to form protein complex, enabling synthesis and translocation from the cytoplasm to the cell surface in a coordinated process.

KpsE, being a PCP family protein, is anchored to the inner membrane with N- and C-terminal transmembrane helices. KpsE interacts with KpsD, the OPX family protein, and this interaction is specific for the cognate pairs of KpsED homologues. In *E. coli* group 1 CPS form, the OPX protein forms an octameric outer membrane channel which could facilitate the CPS efflux. The OPX protein of CPS group 1 is mainly located in the periplasm wherein it interacts with the periplasmic domain of its cognate PCP-2a protein. The KpsD and its homologues possess a motif shared by all the OPX proteins, but they do not form stable multimers in the outer membrane so their 3D structures may differ from group 1 OPX protein. The efflux pump is organized in a tripartite and aids in the CPS export system. Here, the efflux pump is connected to an outer membrane channel such as ToIC via an adaptor protein in order to create a contiguous channel from the cytosol to the exterior of the cell (Willis and Whitfield 2013).

A lot more studies need to be carried out to understand the complete mechanism for CPS biosynthesis, since there is a need to understand the mechanism as to how the substrate for the polymerizing CPS synthases is synthesized, how the ABC transporter recognizes the CPS for export, and how is the polymer transported across the two membranes and the periplasmic space.

8 Future Outlook

The cell-bound polysaccharides as discussed in this communication are evolved in the prokaryotes to perform both structural and functional roles in cell growth and division. In this context, in the past few years, the entire genome of the bacteria is available, and a survey of open reading frames indicates that proteins involved in carbohydrate metabolism, carbohydrate-binding proteins, and cell wall assembly proteins constitute the major families of the expressed genes. Therefore, the genes encoding different enzymes or precursors which are responsible during the biosynthetic pathway of these cell-bound polysaccharides in bacteria which act as virulence factors to cause pathogenesis in host cell could be a good target for the development of antibiotic drugs. The structure and function of polysaccharides and the biosynthetic pathways involved in their synthesis would provide an important clue for designing the therapy to combat the infectious diseases caused by the bacteria. Till date, the mechanisms involved in biosynthetic pathways are not completely explored, and certain precursors are still not known thereby it remains a highly challenging problem which could prove to be a promising area for future work.

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Algal Polysaccharides and Health

4

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Contents

Abstract

Freshwater and marine algae cover a wide group of various organisms living in diversified, terrestrial, or water habitats. Various environment conditions are important factors for the formation of many defense mechanisms to survive unfavorable climate leading to a wide scale of chemical compounds created by algae. Many of them show interesting and important biological activities with health benefits, which are the reason of algae being in the focus of scientists worldwide. Generally, algae can be considered as an abundant source of many nutrients, besides the polysaccharides, responsible for their different physico-chemical properties with health beneficial effects. Fundamental seaweed polysaccharides with economic impact are presented by alginates, agars, carrageenans, ulvanes, and fucoidans used as a raw material mostly in the food and pharmaceutical industry. From medicinal point of view, especially sulfate polysaccharides are an important source of bioactive natural compounds

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exhibiting anticoagulant, antithrombotic, antitumor, antimicrobial, antimutagenic, anti-inflammatory, immunomodulatory, and antiviral activities. Thus, significant attention of this chapter has been focused on sulfate polysaccharides derived from algae with anticoagulant activities.

Keywords
Freshwater algae • Seaweed • Sulfate polysaccharides • Anticoagulants • Heparin

1 Introduction

Increasing interest in healthy human food across the world introduces also a rising consumption of algae due to the presence of many bioactive compounds. Algae represent a wide group of very diverse organisms formed by more than thirty thousand of species of microscopic or huge dimensions both freshwater algae and seaweed. According to the scientific classification, algae belong to the domain Bacteria with prokaryotic cells and Eucarya with eukaryotic cells. They are able to colonize different types of habitats from terrestrial types to rivers, lakes, seas, oceans, and hot springs, and they live in all biogeographic areas from tropic to polar areas. Various environment and living conditions of algae are responsible for enormous algal diversity resulting in different dimension, shapes, colors, and heterogeneous chemical composition (Dawczynski et al. 2007; Marsham et al. 2007; Ogbonda et al. 2007). Frequently, general classification according to their pigments as red (Rhodophyta), brown (Phaeophyta), green (Chlorophyta), and blue-green (Cyanophyceae) algae is used. Microalgae cover unicellular green and red algae as well as cyanobacteria (known as blue-green algae) and also diatoms and dinoflagellates. Macroalgae are represented by three groups of brown (Phaeophyceae), red (Rhodophyceae), and green (Chlorophyceae) seaweed.

Different biomes of algae together with many abiotic and biotic factors, such as geographic location, water temperature, light intensity, a level of nutrition in water, algal species, the time of harvest, and stage of life cycle, predominantly determine their different structure and significantly affect the occurrence of miscellaneous compounds in various concentration in algae. The high nutritional value of algae results from the content of proteins with essential amino acids, minerals, essential fatty acids, and vitamins. Many other compounds present in algal biomass possessing health beneficial impacts on human body, such as phenolic compounds, are secondary metabolites evolved as protective agents toward unfavorable environmental conditions (Dawczynski et al. 2007; El Gamal 2010; Marsham et al. 2007; MacArtain et al. 2007; Mišurcová 2011, Mišurcová et al. 2011a, b, 2014).

Among the fundamental compounds, i.e., polyphenols, vitamins, minerals, and proteins, algal polysaccharides have been in the focus of researches due to many important beneficial impacts on human health. Algal polysaccharides are located mostly in algal cell walls as structural compounds. Their structure and composition were found crucial for their activities on signaling pathway regulating defense of

algal cells of unicellular organisms or plant tissue of multicellular algae against the environmental surroundings (Jaulneau et al. 2010; Patron and Keeling 2005; Aquino et al. 2011; Rodrigues et al. 2012). Polysaccharides do not participate on the nutritional value of algae. Therefore, algal polysaccharides are considered as a source of dietary fiber resistant to enzymatic hydrolysis of intestinal microflora of human digestive tract (Jiménez-Escrig and Sánchez-Muniz 2000; Warrand 2006; Kim 2011). Polysaccharides of seaweed have been widely investigated for their chemical properties and important biological effects in recent years. Chemical composition of algal polysaccharides is responsible for their different functions. Exceptionality and variety of algal polysaccharides functions are based on the ability to bind sulfate groups to hydroxyl groups of sugar molecules so their biological activities are caused mainly by their diverse composition and the extent of sulfation (Percival 1979 ; Athucorala et al. 2007; Costa et al. 2010; Damonte et al. 1994; De Zoysa et al. 2008). Generally, acidic sulfated polysaccharides, such as alginic acids and carrageenans, are able to reduce cholesterol absorption in the gut due to their production of indigestible ionic colloid and in the case of neutral polysaccharides agars, thanks to their water dispersibility (Jiménez-Escrig and Sánchez-Muniz 2000). Further, algal polysaccharides are in the focus of many researches as they are an important source of bioactive natural compounds with specific biological functions; they show many health benefits provided by their anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, and antiviral properties (Mišurcová et al. 2012; Costa et al. 2010). This chapter gives details on the characterization of algal polysaccharides and their physiological function as anticoagulants.

2 Chemical Composition of Algal Polysaccharides

Different phylogenesis of many cyanobacterial and algal species determines various chemical composition and structure of polysaccharides that are extensively studied for their different physiological functions. Polysaccharide composition of algae has been described in many research papers. Common feature evaluated from these investigations is their enormous variability stemming from different algal species; further, cultivation methods of freshwater algae as well as different algal habitats and other environmental conditions affect their growth (Aquino et al. 2011; Rodrigues et al. 2012; Rodrigues and da Silva Bon 2011; Becker 2007; Cheng et al. 2011). Moreover, composition and proportions of algal polysaccharides vary with the morphological phase of algal life stages, and their location in algal cells determines also their functions (Usov 1998). Storage polysaccharides are the main source of energy, while structural polysaccharides have solidifying and protective functions.

2.1 Storage Polysaccharides of Algae

Fundamental energy storage polysaccharides could be divided into three groups according to their synthesis, different structures, and localization in cell bodies.

Thus, starch is a storage polysaccharide of green plant including algae, floridean starch is deposited in red algae, and glycogen is a storage polysaccharide of blue-green algae belonging to Cyanobacteria. Storage polysaccharides of freshwater blue-green and green algae are glycogen and starch, respectively. The molecules of both polysaccharides, starch and glycogen, are formed by α-(1,4)-linked glucose units with α-(1,6)-branch points, whereas length and number of branches alter in dependence on the species of organisms (Chao and Bowen 1971). The fundamental difference between starch, floridean starch, and glycogen is their distinct positions in algal cells. While starch synthesis is localized within the plastids because of relocalization of green algal branching enzymes and probably phosphoglucomutase to the plastid, floridean starch and glycogen syntheses are accumulated in the cytosol (Patron and Keeling 2005).

2.1.1 Starch

Further, studies concerning starch structure diversity among the various species of green algae have shown a highly various percentage of amylose. Green algal starch consists of about 70 % of branched polymer amylopectin, the remaining 30 % is a non-branched or slightly branched polymer of amylose. However, differences of starch structure also depend on the established cultivation conditions. Environmental factors, mainly temperature, light, and nutrient content in growth medium, are responsible for the quantitative distribution of amylose. Saturated cultures of three green algae *Chlamydomonas reinhardtii*, *Dunaliella bioculata*, and *Haematococcus pluvialis* grown on acetate and light accumulated starch with a low concentration of up to 5 % of amylose. On the other hand, nutrient-starved cells accumulated significantly a higher amount of amylose, from 15 % to 30 % (Ball and Deschamps 2009). Polymodal distribution of chain lengths within amylopectin molecules allows them to form granules in the matrix consisting of alternating, concentric, amorphous, and semicrystalline lamellae (Myers et al. 2000). Amylopectin synthesized by green plant has highly organized tandem-cluster structure while the bacteria and animals continue to produce random branched glycogen (Nakamura et al. 2005). Further, similar features between starch synthesis in Chlorophyta and glycogen synthesis in bacteria are attributable to the origin of chloroplasts from endosymbiotic cyanobacteria (Viola et al. 2001).

2.1.2 Glycogen

The main storage polysaccharide of blue-green algae is glycogen, and its production is strongly dependent on the conditions of algal cultivation, nitrate concentration, and light intensity. Higher production of glycogen may be caused by nitrate deficiency. Low nitrate concentration favors the accumulation of glycogen; however, it leads to lower biomass production (Aikawa et al. 2012).

2.1.3 Floridean Starch

The main storage polysaccharide of red seaweed is floridean starch. It has a similar structure as starch of green seaweed and plant, however, without amylose. Nevertheless, it was confirmed that some species of red algae form also amylose units

(McCracken and Cain 1981). Another difference is the imposition of granules of floridean starch outside the plastids (Shimonaga et al. 2007; Viola et al. 2001). In red algae, isoamylases and starch synthases are plastid-derived enzymes operating in the cytosol where they use uridine diphosphate (UDP) glucose as a glucan donor, resulting in cytosolic starch synthesis. Thus, starch granules in red algae are exclusively synthesized in the cytoplasm (Patron and Keeling 2005; Viola et al. 2001).

2.1.4 Laminaran

The main storage polysaccharide of brown seaweed is laminaran, also called laminarin, and its chemical structure is formed by (1,3)-β-D-glucan with β-(1,6) branching with different reducing endings that can have either mannitol or glucose residues. Laminaran contains also a large amount of neutral sugars with a low concentration of uronic acid, whose proportions vary according to different species (Rioux et al. 2007). The extent of branching predestinates different solubility of laminarans. Highly branched laminaran is soluble in the cold water, whereas a lower degree of branching enables solubility only in the warm water (Jaulneau et al. 2010; Rupérez et al. 2002). As well as previous mentioned storage polysaccharides, also laminaran content varies in dependence on the season, age of population, seaweed species, and geographic location. Seasonal changes of laminaran and mannitol in different species of brown seaweed have also been reported, concerning a different stage of the life cycle (Iwao et al. 2008; Zvyagintseva et al. 2005).

2.2 Structural Polysaccharides of Algae

Structural polysaccharides have the main function to protect algal cells and tissues. Obviously, algal structural polysaccharides are formed as miscellaneous mixtures of heterogenic compounds with sulfated and branched polysaccharides, proteins, and also inorganic ions, such as calcium and potassium.

2.3 Freshwater Algae

The cell wall of blue-green alga *Spirulina platensis* is formed by four layers marked as LI, LII, LIII, and LIV without cellulose. All layers are very weak except for the layer LII consisting of peptidoglycan that gives the cell wall its rigidity. The LI layer consists of β-(1,2)-glucan, a polysaccharide that is nondigestible by human gastrointestinal tract. LIII is possibly composed of protein fibrils, and the most external membrane layer LIV is composed of material arranged straight, parallel with the trichome axis, and which is considered to be analogous to that one present in the cell wall of gram-negative bacteria (Ciferri 1983; Mišurcová et al. 2012). It was reported that the variable sugar content is in the range of 8–14 % in biomass of *Spirulina platensis* in dependence on different parts of cell structures (Becker 2007)

and also on various species, when in algal biomass of *Spirulina maxima* a lower amount of 2.0 % of polysaccharides presented by xylose, rhamnose, and glucose was observed. The cell wall contains of 10.0 % of polysaccharides with glucose being the major representative. In external cellular wall layers, there have been established polysaccharides formed by a mixture of six neutral monosaccharides including fucose, rhamnose, xylose, mannose, galactose, and glucose, and finally, two uronic acids have been presented by glucuronic and galacturonic acids (Nie et al. 2002).

The cell walls of the freshwater green algae of *Chlorella* strains are composed of up to 80 % of polysaccharides including cellulose, and they may be constructed by three different types of structures varying among the miscellaneous strains (Rodrigues and da Silva Bon 2011). The first type of cell structure is formed by a trilaminar outer cell wall layer, the second by a thin outer monolayer, and the third is without an outer layer (Yamada and Sakaguchi 1982).

Trilaminar cell wall consists of algaenan, generally known as non-hydrolyzable macromolecular components, such as glycoproteins and glucosamine-containing biopolymers (Burczyk et al. 1999). The second cell wall type consists of an outer non-trilaminar layer and inner microfibrillar layer, which is probably composed of β-linked polysaccharides, such as cellulose, and of a little amount of pectin. The third type of cell wall consisted of one microfibrillar layer in which a bigger amount of pectin and a small amount of β-linked polysaccharides were established (Yamada and Sakaguchi 1982; Mišurcová et al. 2012). The chemical composition of the cell walls of green freshwater algae would vary depending on algal species and other types of their cell wall structure, but it has been also reported that cultivation conditions have the impact on chemical composition of the cell wall in *Chlorella variabilis* NC64A; different levels of neutral sugar, uronic acid, and amino sugar in the cell wall have been found when cultured in different nitrogen sources and concentrations (Cheng et al. 2011). Rigid cell walls of *Chlorella* species contain mannose as a major sugar component, further glucose, and glucosamine. Rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose have been determined in the wall matrix (Takeda 1996).

2.4 Seaweed

Structural polysaccharides of seaweed cell walls usually consist of an outer amorphous mucilage matrix commonly formed by linear sulfated galactan polymers (carrageenans, agars, and alginates) and of an inner rigid component from cellulose microfibrils (Arad and Levy-Ontman 2010). However, cellulose as a neutral structural polysaccharide is rarely present in the cell walls of red and brown seaweed as a pure β-(1,4) glucan; more frequently the wall contains other sugars than glucose and is also presented in lower levels than in higher plants, in a relatively small amount of 2–10 % in the majority of red algae. Moreover, its amount and configuration of microfibrils differs according to different life stage of algae. Whereas the concholices phase of red algae *Porphyra tenera*, *Bangia atropurpurea*, and *Bangia*

fuscopurpurea contains cellulose and minor amount of mannan in their cell walls, the generic phase of the same species contains (1,4)-linked β-D-mannan as the main structural polysaccharide of their cell walls (Gretz et al. 1980; Usov 2001). Further, the cell wall of red seaweed *Palmaria palmata* contains cellulose in a small amount, and in the genus *P. tenera*, it has been even replaced by insoluble mannose, galactose, and xylose (Deniaud et al. 2003; Rupérez and Toledano 2003). Galactose and glucose are determined as the main neutral sugars in cell walls of red seaweed *Chondrus crispus* (Rupérez and Toledano 2003).

The cell walls of red microalgae are without cellulose microfibrils. They are rather encapsulated within the gel matrix from sulfated polysaccharides (Arad and Levy-Ontman 2010). The main part of red and brown seaweed cell walls is represented by sulfated galactans, which are known as agar, alginate, and carrageenan; fucans described as fucoidin, fucoidan, ascophylan, sargassan, and glucuronoxylofucan; and also cellulose. However, their amounts and distribution are variable due to enormous algal diversity and also during different stages of their live cycles (Popper and Tuohy 2010; Percival 1979).

2.4.1 Cellulose

The main structural polysaccharide of some algal species as well as terrestrial plants is cellulose. Its linear molecules are formed by the condensation of D-glucose units through β-(1,4) glycosidic bonds. The hydrogen bonding patterns in cellulose are considered as one of the most important factors on its physical and chemical properties including the solubility, crystallinity, and hydroxyl reactivity. Several different crystalline structures are known in dependence on the location of hydrogen bonds between and within cellulose fibrils. Cellulose I with structures I_α and I_β is natural and I_α occurs in bacteria and some algae and the latter in higher plants (Nishiyama et al. 2003; Kroon-Batenburg and Kroon 1997). Cellulose obtained from algal species is substantially different from cellulose in higher plant; it has a porous or spongy structure.

2.4.2 Alginate

Alginates without sulfate groups are constituents located in the cell wall and in the matrix of brown seaweeds together with fucans and heteroglycans rich in sulfated L-fucoses. Alginates consist of two chain-forming heteropolysaccharides made up of blocks of β-(1,4)-linked D-mannuronic (M) and α-(1,4)-linked L-guluronic (G) acids, and their structure varies in dependence on the monomer position in the chain, forming either homopolymeric (MM or GG) or heteropolymeric (MG or GM) blocks (Rioux et al. 2007; Miller 1996). The alginate composition is significantly dependent on the algal species, diverse location, various season, as well as different parts of thallus from which the extraction of alginate is made, i.e., the stipe has other mechanical requirements in comparison with the fronds. Thus, a higher content of guluronate has been required in plant parts with higher rigidity. Physical properties of alginates as well as formation of gels depend on the relative proportion of these blocks. The ability of alginates to form gels in the presence of divalent

calcium ions is one of their main biofunctional properties and has a great industrial significance (Larsen et al. 2003). The evaluation of the M/G ratio is fundamental for the detection of gel properties. High M/G ratio is the signature of alginate giving elastic gels, while low M/G ratio provides brittle gels (Fenoradosoa et al. 2010).

3 Sulfated Polysaccharides

From a wide scale of different algal polysaccharides, sulfated polysaccharides are the most important from biological activity point of view. They are observed in all groups of algae in contrast to terrestrial plants. Their occurrence in marine algae is connected with a possible correlation with salt stress, and these compounds are also related to mechanical, ionic, and osmotic regulation, helping the survival of algae in the marine environment (Aquino et al. 2011; Rodrigues et al. 2012). The nomenclature of algal sulfated polysaccharides has been often based on the name of algal species, such as spirulan, furcellaran, ulvan, fucan, etc. Generally, sulfated polysaccharides, naturally occurring glycosaminoglycans, are a class of compounds containing hemiester sulfate groups in their sugar residues (Shanmugam and Mody 2000). Red algae produce sulfated galactans consisting entirely of the β-galactose or α-galactose units. The first always belongs to D-series, while the latter is either D-series in carrageenans or L-series in agars (Usov 1998). Sulfated glucans, sulfated galactans, and sulfate arabinogalactans are produced by green algae, and spirulan is known as sulfated polysaccharides of blue-green algae (Aquino et al. 2011; Costa et al. 2010; Shanmugam and Mody 2000).

3.1 Red, Green, and Blue-Green Algae Sulfated Polysaccharides

Sugar presentations of sulfated polysaccharides of some red and green seaweed and of some blue-green algae are shown in Table 1. It is evident that sugar composition as well as the extent of sulfation is very miscellaneous not only across the algal species but also within the same algal strains. While the abundant galactose residues have been established in red seaweeds, rhamnose and glucose residues and uronic acids have been determined in higher amounts in most green seaweeds. Different localities of *Ulva conglobata* affect the composition of their sulfated polysaccharides, especially in the amount of sulfate (Mao et al. 2006). Apparently, the separation processes are also responsible for the different amounts and distribution of neutral sugar and amounts of sulfate in polysaccharide molecules. Mostly hot or cold water is used for the extraction of sulfate polysaccharides from seaweeds. While in species, i.e., *Codium dwarkense*, *C. tenue*, *Avrainvillea erecta*, cold water extraction has been more effective than hot water extraction on the sulfate amounts, in the second seaweed group presented by *Udotea indica* and *Halimeda gracilis*, significant differences in chemical composition of sulfated polysaccharides in hot or cold water extracts have not been observed (Shanmugam et al. 2002).

Table 1 Chemical composition of sulfate polysaccharides of some blue-green algae and some red and green seaweeds

Algal species	Neutral sugar residues (NSR)						Unit of NSR	UA	S	Reference
	Rha	Fuc	Xyl	Man	Glu	Gal		(%)		
Red seaweeds										
Schizymenia binderi			1.8		0.5	49.8	%	4	24.7	Zúniga et al. 2006
Chondrus crispus[E]	3.6	0	2.8	1.4	5.4	120.7	g/kg			Rupérez and Toledano 2003
Porphyra tenera[E]	4.3	0.5	2.7	1.9	4.8	107	g/kg			Rupérez and Toledano 2003
Nothogenia fastigiata[H]			21.5	62.3		16.2	mol %		17.6	Kolender et al. 1997
Green seaweeds										
Ulva conglobata[H]	71.9	0.82	1.06	3.32	20.77	2.13	mol %	12.6	35.2	Mao et al. 2006
Ulva conglobata[H]	72.26	1.02	1.98	1.51	22.03	1.2	mol %	14.9	23	Mao et al. 2006
Monostroma latissimum	86.77		6.29		6.94		mol %	3.24	23.6	Mao et al. 2009
Monostroma latissimum	78.65		7.83	2.03	11.49		mol %	10.8	21.2	Zhang et al. 2008
25.4 kDa[W]										
Monostroma latissimum	85.77		5.72		7.29	1.22	mol %	12.1	25.5	Zhang et al. 2008
61.9 kDa[W]										
Monostroma latissimum	80.35		5.28	1.43	10.07	2.87	mol %	14.6	27.3	Zhang et al. 2008
26 kDa[W]										
Monostroma latissimum	78.28		8.6	1.66	10.02	1.44	mol %	13.5	24.3	Zhang et al. 2008
10.6 kDa[W]										
Monostroma nitidum	79.4		5.2		10.1	5.3	mol %	7.92	28.2	Mao et al. 2008
870 kDa[W]										
Monostroma nitidum	78.2		14.7	3.4	3.7		mol %	6.76	34.4	Mao et al. 2008
70 kDa[W]										
Codium dwarknese[C]						43.62	mol %	1.79	28.5	Shanmugam et al. 2002
Codium dwarknese[H]	1.9			32.4	7.68	12.23	mol %	3.23	19.5	Shanmugam et al. 2002
Codium tenue[C]							mol %	1.66	31.1	Shanmugam et al. 2002
Codium tenue[H]							mol %	2.91	24.4	Shanmugam et al. 2002

(*continued*)

Table 1 (continued)

Algal species	Neutral sugar residues (NSR)						Unit of NSR	UA	S	Reference
	Rha	Fuc	Xyl	Man	Glu	Gal		(%)		
Avrainvillea erecta^{C}							mol %	5.6	32.1	Shanmugam et al. 2002
Avrainvillea erecta^{H}	9.68		7.56	8.1	66.32	7.94	mol %	7.68	27.9	Shanmugam et al. 2002
Udotea indica^{C}							mol %	2.37	12.5	Shanmugam et al. 2002
Udotea indica^{H}							mol %	1.43	12.6	Shanmugam et al. 2002
Halimeda gracilis^{C}							mol %	7.15	13.5	Shanmugam et al. 2002
Halimeda gracilis^{H}							mol %	6.79	14.4	Shanmugam et al. 2002
Blue-green algae							**GUA**			
Arthrospira platensis^{UF}	49.7		5.9	0.9	4.3	5.8	%	32.0		Majdoub et al. 2009
Spirulina platensis^{H}	1				0.16		mg/g	18.30	2.35	Abd El Baky et al. 2013
Spirulina platensis^{Et}					0.06	0.57	mg/g	2.46	5.02	Abd El Baky et al. 2013

Rha rhamnose, *Fuc* fucose, *Xyl* xylose, *Man* mannose, *Glu* glucose, *Gal* galactose, *UA* uronic acid, *GUA* guluronic acid, *S* amount of sulphate
Superscripts mean typ of extraction: *W* water, *H or C* hot or cold water, *E* enzymatic, *UF* ultrafiltration, *Et* ethanol

Two main sulfated polysaccharides of red seaweed are agar and carrageenan known as hydrocolloids. They are widely used as texturing agents for many applications in food industry, such as gelling, thickening, and stabilizing agents in different food production and also in many nonfood applications (Lahaye 2001; Sartal et al. 2011). Structural variability of these sulfated galactans occurs among diverse algal species. It is based on different environmental conditions, the season of the collection, and on the extraction methods. Furthermore, various hydroxyl groups may be substituted by sulfate ester, methyl groups, pyruvic acid acetal, or additional monosaccharides. However, the major structural variation is the sulfation pattern (Pomin and Mourão 2008; Usov 1998).

3.1.1 Agar

Generally, agar consists of two major polysaccharides, neutral agarose as a gelling fraction and charged acid agaropectin as a non-gelling fraction. Its molecule is composed of a linear chain of alternating 3-linked β-D-galactopyranosyl and 4-linked of 3,6-anhydro-α-L-galactopyranosyl residues. This backbone may be substituted in varying percentages of half-ester sulfate, methoxyl, or pyruvate groups, and the character of backbone modification may strongly influence gelling properties, i.e., a high content of 3,6-anhydrogalactose and low sulfate content are necessary for a gelling ability (Miller et al. 1993; Usov 1998). Agar is localized in extracellular matrix and is secreted by the Golgi apparatus, and its composition has been documented as very changeable in dependence on the season, seaweed species, different life phases of algae, and also the extraction methods (Praiboon et al. 2006).

3.1.2 Carrageenan

Other sulfated galactan is carrageenan extracted from red seaweed, especially species belonging to the family Gigartinaceae. This galactan consists of linear chains of repeating disaccharide units with alternating 3-linked β-D-galactopyranose (G-units) and 4-linked α-D-galactopyranose (D-units) or 3,6-anhydro-α-D-galactopyranose (DA-units). Furthermore, they usually contain more sulfate than agars in the range of 22–35 % (Jiao et al. 2011; Shanmugam and Mody 2000). Their classification into fifteen different groups, i.e., kappa, iota, lambda, gamma, theta, epsilon, and mu (κ, ι, λ, γ, τ, ε, and μ), is based on the presence and localization of sulfate esters and on the presence of the 3,6-anhydro-D-galactose linked in (1,4) (Shanmugam and Mody 2000; Usov 1998; Lahaye 2001). The main copolymers from industrial point of view are kappa, iota, and lambda carrageenans showing a different ability to form gels with dissimilar characteristics. Thus, κ-carrageenan forms strong rigid gels, ι-carrageenan forms soft elastic gels, and λ-carrageenan does not form any gels but produces the highest viscosities in the water (Sartal et al. 2011). While the main repeating dimer structure of κ-carrageenan is G4S-DA, in ι-carrageenan, it is repeating disaccharide structure of G4S-DA2S, and finally, λ-carrageenan consists of G2S-D2S,6S of dimer structure (De Ruiter and Rudolph 1997; Lahaye 2001). Variations in carrageenan structures occur not only between different species of the Gigartinaceaes but also within the same species in dependence on different life stages.

3.1.3 Furcellaran

Furcellaran, known as Danish agar, is extracted from red seaweed of genus *Furcellaria*. Besides agar and carrageenan, furcellaran is further anionic sulfated polysaccharide that is considered to be a copolymer of β- and κ-carrageenan. The composition of furcellaran extracted from red seaweed *F. lumbricalis* consists mainly of 3-linked β-D-galactopyranose, 4-linked 3,6-anhydro-α-D-galactopyranose, and 3-linked β-D-galactopyranose 4-sulphate (Laos and Ring 2005; Laos et al. 2005). Hydroxyl groups in polysaccharide chain may be substituted by sulfate, methyl groups, and other sugar monomers, such as xylose and glucose. Furcellaran can be commercially used as a gelling agent for its ability to form gels in the presence of specific gel-promoting cations, especially K^+ and Ca^{2+} (Laos et al. 2005).

3.1.4 Porphyran

Porphyran as the main polysaccharide of red alga *Porphyra umbilicalis* has been established by structural analysis. It has been observed that it consists of D- and L-galactose residues in the amount of 24–45 %; 3,6-anhydro-L-galactose has been present in the amount of 5–19 %, 6-*o*-methyl-D-galactose in the amount of 3–28 %, and ester sulfate in the amount of 6–11 %. Further, the ester sulfate seems to occur always as 1,4-linked L-galactose 6-sulfate, even if its content is variable. 3,6-anhydro-L-galactose and L-galactose 6-sulfate have been interchangeable between the polysaccharides, and D-galactose and 6-*o*-methyl-D-galactose have been related in a similar way. Moreover, 3,6-anhydro-L-galactose and L-galactose 6-sulfate introduce approximately a half of sugar units, and D-galactose together with 6-*o*-methyl-D-galactose form the other half of sugar units (Rees and Convay 1962).

3.1.5 Ulvan

The cell wall matrix of green seaweed contains highly sulfated complex of heteropolysaccharides named ulvans whose molecules consist of different sugar residues in dependence on the seaweed strain mainly of the order Ulvales (*Ulva* and *Enteromorpha* sp.). Ulvans are water-soluble polysaccharides consisting mainly of rhamnose, xylose residues, iduronic and glucuronic acids, and sulfate groups. These sulfated galactans tend to be more complex and heterogeneous in the structure than sulfated galactans from red seaweed (Jiao et al. 2011). The main ulvan constituents are sulfated rhamnose residues linked to uronic acids resulting in repeated disaccharide unit β-D-glucuronosyl-(1,4)-α-L-rhamnose 3-sulfate, called aldobiouronic acid (Jaulneau et al. 2010; Lahaye et al. 1997). Also other neutral sugars, such as xylose, mannose, galactose, and glucose, participate in different amounts on the composition of sulfated polysaccharides of green seaweed (Mao et al. 2006, 2009; Zhang et al. 2008; Shanmugam et al. 2002).

3.1.6 Spirulan

Calcium spirulan (Ca-SP) and sodium spirulan (Na-SP) are sulfated polysaccharides obtained from hot water extracts of blue-green alga *Spirulina platensis*.

They are composed of rhamnose, 3-*o*-methylrhamnose (acofriose), 2,3-di-*o*-methylrhamnose, 3-*o*-methylxylose, uronic acids, sulfate, and calcium or sodium ions. The backbone consists of 1,3-linked rhamnose and 1,2-linked 3-*o*-methylrhamnose units with some sulfate substitution at the 4-position; the polymer is terminated at nonreducing end by 2,3-di-*o*-methylrhamnose and 3-*o*-methylxylose residues (Yamamoto et al. 2003; Lee et al. 2000). In Ca-SP molecules, there are two types of disaccharide repeating units, *o*-rhamnosyl-acofriose and *o*-hexuronosyl-rhamnose (aldobiuronic acid). Component sugar analysis of Ca-SP determines 52.3 of % rhamnose and 32.5 % of 3-*o*-methyl-6-deoxyhexose, together with 4.4 % of 2,3-di-*o*-6-deoxyhexose, 4.8 % of 3-*o*-methylpentose, and trace amounts of other sugars (Lee et al. 1998). Ca-SP has been found as an antiviral agent as well as an anticoagulant with heparin cofactor II-dependent antithrombin activities but also as a potent inducer of tissue-type plasminogen activator (t-PA) production (Lee et al. 1998; Hayakawa et al. 1997). However, Ca-SP has a very low anticoagulant activity in comparison with Na-S, which has been studied for its strong antithrombin activity (Yamamoto et al. 2003).

3.2 Brown Algae Sulfated Polysaccharides

Fucans that include polydisperse molecules based on sulfated L-fucose and also heterofucans called fucoidans are observed in brown seaweeds. The chemical compositions of sulfated polysaccharides of some brown seaweed species are expressed in Table 2. Evidently, fucose is the neutral sugar abundantly presented in sulfate polysaccharides of brown seaweeds, in which different extent of sulfation has been observed. Other sugar residues, such as xylose, mannose, glucose, and galactose, participate on the composition of sulfated polysaccharides of brown seaweeds in different amounts. Galactose and xylose sugar residues occur in higher amounts following the fucose residues, the former especially in species of *Laminaria*, and in *Fucus* alternating galactose and xylose residues (Ushakova et al. 2009; Dürig et al. 1997; Rupérez and Toledano 2003). However, in *Panina* species, xylose has been the second most abundant sugar residue, and even in *Sargassum polycystum*, *Turbinara ornate*, and *Undaria pinnatifida*, galactose has been contained in higher amounts of 13.7 %, 25.6 %, and 6.4 % in relation to fucose which is 20.3 %, 30.3 %, and 7.1 %, respectively (Thuy et al. 2015; Rupérez and Toledano 2003).

3.2.1 Fucoidans

Cell walls of several orders of brown seaweed, particularly Fucales and Laminariales, consist mainly of fucoidans, which are composed from variable amounts of saccharide units, such as fucose, xylose, glucuronic acid, galactose, and mannose with a different degree of sulfation (Berteau and Mulloy 2003). Their various structures derived from different sugar distribution and diverse sulfate group contents have been described as fucoidin, fucoidan, ascophyllan, sargassan,

Table 2 Chemical composition of sulfated polysaccharides of some brown seaweeds

Brown algal species	Neutral sugar residues (NSR)						Unit of NSR	UA	S	Reference
	Rha	Fuc	Xyl	Man	Glu	Gal		(%)		
Laminaria saccharina^{IE}		36.7	1.2	1	2.2	4.6	%	4.8	29.6	Ushakova et al. 2009
Laminaria digitata^{IE}		30.1	1.9	1.7	1.4	6.3	%	7	27.5	Ushakova et al. 2009
Laminaria digitata^{E}	2.9	12.1	1.4	2.6	4.8	6.7	g/kg			Rupérez and Toledano 2003
Fucus distichus^{IE}		40.8	0.8			0.8	%	<1	34.8	Ushakova et al. 2009
Fucus serratus^{IE}		24.8	2.4	2.1	2	4.8	%	8.2	29.2	Ushakova et al. 2009
Fucus evanescens^{IE}		58.7	1.6			1.6	%	<1	36.3	Ushakova et al. 2009
Fucus spiralis^{IE}		33	2.8	1.4	1.2	3	%	8.2	25.9	Ushakova et al. 2009
Fucus vesiculosus^{IE}		26.1	2.4	3.1	2.2	5	%	10.3	23.6	Ushakova et al. 2009
Fucus vesiculosus		86.3				14	%		10.2	Dürig et al. 1997
100 kDa^{IE}										
Fucus vesiculosus		92.5				7.5	%		9.13	Dürig et al. 1997
150 kDa^{IE}										
Fucus vesiculosus		92.3				7.7	%		10.8	Dürig et al. 1997
100 kDa^{IE}										
Fucus vesiculosus		92.6				7.3	%		9.2	Dürig et al. 1997
50 kDa^{IE}										
Fucus vesiculosus		76.1	9.4	7.6		6.9	%		7.6	Dürig et al. 1997
100 kDa^{IE}										
Fucus vesiculosus^{Et}		43.9	4.7	2.7	30	5.6	g/kg			Rupérez and Toledano 2003
Ascophyllum nodosum^{IE}		26.6	4.4	2.6	1.1	4.7	%	9.4	24.4	Ushakova et al. 2009
Ascophyllum nodosum^{Et}								9.3	22.3	Rioux et al. 2007
Chorda filum^{IE}		64	0.6	0.5	0.5	1.3	%		26.5	Ushakova et al. 2009
Analipus japonicus^{IE}		44.1	2.2			5.8	%	5.9	22.9	Ushakova et al. 2009
Punctaria plantaginea^{AqCl}		44.3	17	tr	tr	2.6	%	2.3	19.2	Bilan et al. 2014

Saccharina longicruris^{Et}								8.2	14.2	Rioux et al. 2007
Cladosiphon okamuranus^{IE}		30.9	0.7		2.2		%	23.4	15.1	Ushakova et al. 2009
Padina tetrastromatica^{W}	2	54	18	9	9	9	mol %	14		Karmakar et al. 2009
Panina tetrastromatica^{W}	5	59	23	3	tr	10	mol %	9		Karmakar et al. 2009
Panina tetrastromatica^{W}	tr	72	25			3	mol %	4.5		Karmakar et al. 2009
Panina tetrastromatica^{W}	tr	70	24			6	mol %			Karmakar et al. 2009
Sargassum mcclurei^{MCW}		40	6.2	11	10	20	mol %		30.5	Thuy et al. 2015
Sargassum polycystum^{MCW}		20.3	2.6	1.9	1.1	14	mol %		23.4	Thuy et al. 2015
Turbinaria ornata^{MCW}		30.3	tr	tr	tr	26	mol %		25.6	Thuy et al. 2015
Undaria pinnatifida^{E}		7.1			tr	6.4	g/kg			Rupérez and Toledano 2003

Rha rhamnose, *Fuc* fucose, *Xyl* xylose, *Man* mannose, *Glu* glucose, *Gal* galactose, *UA* uronic acid, *GUA* guluronic acid, *S* amount of sulphate
Superscripts mean typ of extraction: *IE* ion exchange, *W* water, *E* enzymatic, *Et* ethanol, *AqCl* aqueous calcium chloride, *MCW* methanol:chloroform:water (4:2:1)

and glucuronoxylofucan (Percival 1979). Depending on their chemical composition, fucoidans could be further divided into xylofucoglycuronans and glycouronogalactofucans consisting of α-(1,3)-linked sulfated L-fucose as a fundamental subunit and a branch unit of α-D-galactose, D-mannose, D-xylose, and glucuronic acid (Jiménez-Escrig and Sánchez-Muniz 2000; Karmakar et al. 2009; Jiao et al. 2011). Contents, chemical composition, and the structure of fucans are changeable in dependence on different environmental conditions, time of collection, seaweed life stage, and extraction procedures (Silva et al. 2005). Besides, the extent of fucoidan content changes is variable and dependent on the seaweed species. Thus, fucoidans have been classified into two groups derived either from *L. saccharina*, *L. digitata*, *Analipus japonicus* or from *Ascophyllum nodosum* and *Fucus* sp.; and their central chains are composed of (1,3)-linked α-L-fucopyranose residues and of (1,3)- and (1,4)-linked α-L-fucopyranose residues, respectively (Jiao et al. 2011; Ushakova et al. 2009).

Different structures of fucoidans have been reported by Silva et al. (2005) who analyzed the chemical composition of heterofucan obtained from brown algae *Padina gymnospora*. The fraction (18 kDa) of heteroglycans consists of 3- or 4-linked β-D-glucuronic acid with minor amounts of 3- or 4-linked galactose units, where almost 50 % of 3-linked glucuronic acid units are branched at C-2 and the branches of galactoses are at C-6, C-2, or C-3 on disubstituted galactose. The fucose chains are formed by 3- or 4-linked fucose units and minor amounts of 4-linked fucose are branched at C-2 with the chains of xylose and/or fucose. Furthermore, for *F. vesiculosus*, two possible structures have been determined. In the first case, fucoidan has been presented as a polymer consisting of α-(1,2)-linked fucose with sulfate branches in the position of 4, whereas in the second structure, fucoidan possesses α-(1,3)-linked fucose with sulfate branches in the same position of 4 (Percival and McDowel 1967; Pomin and Mourão 2008). Fucoidan from a commercial source extracted from *F. vesiculosus* has α-(1,3)-linkages between fucose units, and the ending fucose units have been observed to hold branching with α-(1,2)-linkages or α-(1,4)-linkages (Patankar et al. 1993).

4 Biological Activities of Algal Polysaccharides

Importantly, algae are known as a great source of enormous compounds necessary to protect themselves from the exposure of external environmental factors, such as pollution, mechanical stress, and UV radiation. Both freshwater algae and seaweed have been found as producers of many bioactive compounds. Among these, structurally diverse polysaccharides stimulate human health because of their antimicrobial, antiviral, antimutagenic, anticancer, blood anticoagulant, immunomodulating, and anti-inflammatory activities, as well as hypolipidemic and hypocholesterolemic effects (Ye et al. 2008; Holdt and Kraan 2011; Costa et al. 2010). Polysaccharides have been considered as a dietary fiber from the nutrition point of view. Although dietary fiber does not belong between nutrients, it forms a very important part of diet, and its low intake in some Western countries is one of the reasons leading to

the growth of the number of incidence and mortality due to cardiovascular diseases and colorectal cancer. Generally, the main physiological functions of different parts of dietary fiber are based on their solubility or insolubility in water and degradability or fermentability by intestinal microflora that was reported earlier (Mišurcová et al. 2012). Besides, the important algal polysaccharides performing biological activities are sulfated polysaccharides that have been developing as a new generation of nutraceuticals and drugs (Holdt and Kraan 2011; Shi et al. 2007; Blunt et al. 2010; Bouhlal et al. 2010; Kim 2011).

4.1 Anticoagulant Activity

The human blood coagulation system is the process leading to the arrest of bleeding (hemostasis) and includes the transformation of liquid blood into a solid state in order to reduce the loss of blood from injured blood vessels. This process covers three mechanisms such as formation of prothrombinase, conversion of prothrombin into the thrombin which is a key protein of coagulating cascade, where thrombin activates a series of coagulant factors, and, finally, conversion of soluble fibrinogen into insoluble fibrin (Fig. 1).

The blood coagulation system consists of cellular elements (blood platelets, white cells, to some extent red cells, and microvascular remnants or microparticles), coagulation enzymes, proteins cofactors, and a number of anticoagulant proteins (Spronk et al. 2003). The mechanism of blood coagulation is based on the enzyme cascade divided in the intrinsic, extrinsic, and common pathway, where a series of coagulation factors promote the formation of the end product fibrin (Spronk et al. 2003; Wijesekara et al. 2011). As it can be concluded from Fig. 1, during the intrinsic pathway activated Stuart-Prower factor (X) can also be activated by the extrinsic pathway. Firstly, the intrinsic cascade begins with the formation of primary complex of collagen by high molecular weight kininogen (HMWK), prekallikrein, and Hageman factor (XII). During the activation, the single-chain protein of the native Hageman factor is divided into two chains of different molecular weights (28 and 58 kDa). However, both chains remain linked by a disulfide bond. The 28 kDa light chain contains the active site, and this molecule is called as activated Hageman factor (XIIa), which can activate plasma thromboplastin antecedent (PTA) or antihemophilic factor – C (XI). Further, HMWK, known as Fitzgerald factor, binds to the factor XI, and in the presence of Ca^{2+} ions, it facilitates the activation process of factor XIa. This factor XIa activates Christmas factor, plasma thromboplastin component (PTC), or antihemophilic B factor (factor IX) in the reaction requiring Ca^{2+} ions, factor VIII, and phospholipids. Antihemophilic factor VIII is obviously an essential factor for this step of coagulation cascade, and its deficiency is associated with hemophilia A, while the deficiency of factor IX is connected with hemophilia B (Adelson et al. 1963). Activated IXa factor further activates Stuart-Prower factor (X) to factor Xa; and factor X is the first molecule of the common pathway of coagulation cascade. The extrinsic pathway could be considered as an alternative

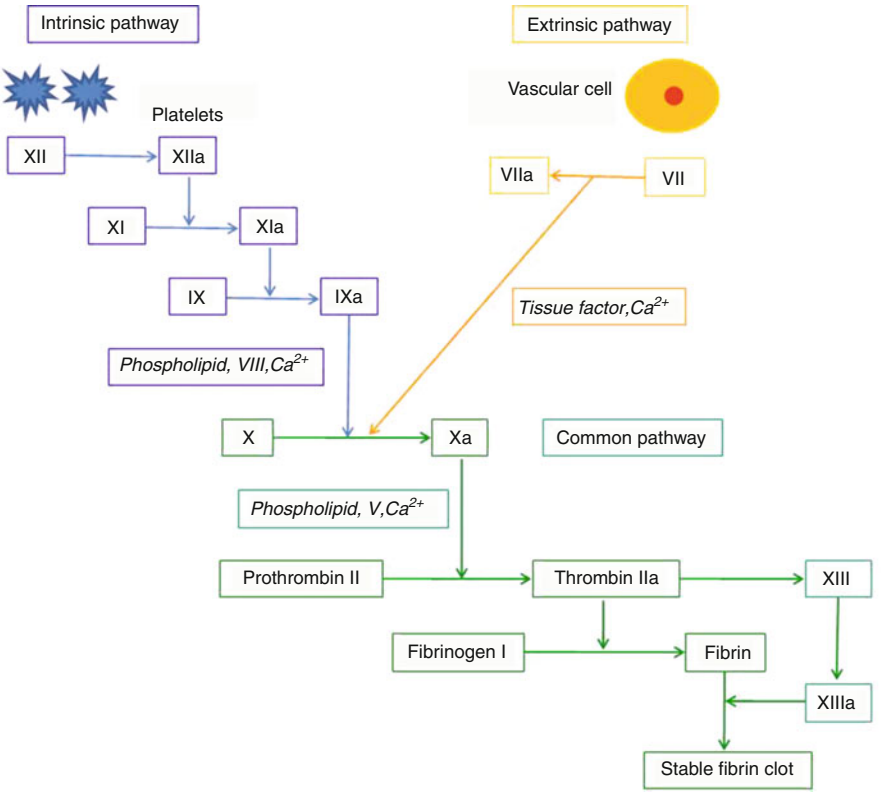

Fig. 1 The scheme of coagulation cascade

way of the activation of factor X in the cooperation with two main components – tissue factor (TF) and factor VII. Blood coagulation factor VII, formerly known as proconvertin, belongs to the serine protease enzyme class, and its main role in extrinsic pathway is to initiate the coagulation process in conjunction with TF.

TF is constitutively present on cell membranes within and around the vessels and serves as the cell surface receptor for serine protease factor VIIa. Carboxylated GLA domain of factor VIIa binds to negatively charged phospholipids in the presence of calcium. Binding of VIIa to negatively charged phospholipids greatly enhances the protein-protein binding of VIIa to TF. Upon a vessel injury, tissue factor, normally found outside of blood vessels, is exposed to the blood where it forms a catalytic complex with factor VIIa activating factor IX and catalyzing the conversion of inactive protease factor X into active protease factor Xa (Spronk et al. 2003; Mirzaahmadi et al. 2011).

Both intrinsic and extrinsic pathways lead to the activation of factor X into factor Xa (the common pathway) which combines with its cofactor – activated proaccelerin (factor Va) – in the presence of calcium and phospholipid to produce thrombin for the conversion of fibrinogen to fibrin. Fibrin monomers spontaneously

polymerize and form an insoluble gel (clots) which is held together by noncovalent and electrostatic forces and is stabilized by fibrin-stabilizing factor XIII catalyzing the formation of peptide bonds between fibrin monomers. Clots together with aggregated platelets (thrombi) block damaged blood vessel and prevent further bleeding (Chatterjee et al. 2010).

Hemostatic abnormalities can lead to serious health problems, such as excessive bleeding or thrombosis. Thus, the human coagulation mechanism has to be strictly regulated by the inactivation of procoagulant enzymes, fibrinolysis, and hepatic clearance of activated clotting factors (Kalafatis et al. 1997) via tissue pathway inhibitor (TFPI), heparin-antithrombin pathway, and protein C pathway (Esmon 2005). The first inhibitor process TFPI inactivates factor VIIa bound to tissue factor (TF). The second antithrombin (ATIII)-heparin mechanism inactivates factor Xa, thrombin, factor IXa, and factor VIIa bound to cell surface tissue factor (Rao et al. 1995). The latter protein C pathway is based on the activation of protein C by the thrombin-thrombomodulin complex on endothelium. This natural anticoagulant system exerts its anticoagulant effect by regulating an activity of factors VIIIa and Va, cofactors in tenase, and prothrombinase complexes, respectively (Dahlbäck and Villoutreix 2005; Esmon 2003).

Heparin, a highly sulfated glycosaminoglycan, is naturally produced by basophils and mast cells; in medicine it is principally used as an anticoagulant to treat and prevent blood clots in the veins and arteries. Heparin molecule possesses a specific local structure, and it is composed of pentasaccharide sequence with a specific pattern of sugar residues along with a sulfation pattern required to induce a conformational activation of antithrombin. Heparin has also an additional anticoagulant mechanism in which polysaccharide brings antithrombin and thrombin in a ternary complex in which both the inhibitor and proteinase are bound to the same polysaccharide chain (Pereira et al. 2002; Streusand et al. 1995). The heparin usage is limited due to several side effects, i.e., a serious side effect resulting in degradation of platelets causing thrombocytopenia (Bick and Frenkel 1999; Castelli et al. 2007).

4.2 Anticoagulant Activity of Algae

Therefore, the requirement of finding alternative sources of anticoagulants has arisen. Heparin-like substances extracted from seaweed have been greatly studied in vitro as potential blood anticoagulants. So far, about 150 species across three major divisions of marine red (Rhodophyta), brown (Phaeophyta), and green (Chlorophyta) algae have been reported to have blood anticoagulant activities. Examples of anticoagulant activities of some algal species across three major divisions of green, red, and brown seaweed as well as blue-green algae are shown in Table 3.

Generally, anticoagulant activity of sulfated polysaccharides has been usually measured by different in vitro method, such as activated partial thromboplastin time (APTT), prothrombin (PT), and thrombin (TT) times. The first assay indicates the

Table 3 Anticoagulant activity of some species of brown, red, and green seaweeds and blue-green freshwater algae

Algae		Polysaccharide	Reference
Brown	*Spatoglossum schroederi*	Sulfated galactofucan	Rocha et al. 2005
	Ecklonia cava	Sulfated polysaccharide	Wijesinghe et al. 2011
	Ascophyllum nodosum	Fucoidan	Chevolot et al. 2001
	Fucus vesiculosus	Fucoidan	Dürig et al. 1997
	Fucus vesiculosus	Fucan	Bernardi and Springer 1962
	Dictyota cervicornis	Sulfated polysaccharide	Costa et al. 2010
	Dictyopteris delicatula	Sulfated polysaccharide	Costa et al. 2010
	Dictyota mertensis	Sulfated polysaccharide	Costa et al. 2010
	Laminaria saccharina	Fucoidan	Ushakova et al. 2009
	Laminaria digitata	Fucoidan	Ushakova et al. 2009
	Fucus distichus	Fucoidan	Ushakova et al. 2009
	Fucus serratus	Fucoidan	Ushakova et al. 2009
	Fucus evanescens	Fucoidan	Ushakova et al. 2009
	Fucus spiralis	Fucoidan	Ushakova et al. 2009
	Lessonia vadosa	Fucoidan	Chandía and Matsuhiro 2008
	Sargassum vulgare	Fucan	Dore et al. 2013
Red	*Porphyra haitanensis*	Porphyran	Zhang et al. 2010
	Schizymenia binderi	Sulfated galactan	Zúniga et al. 2006
	Botryocladia occidentalis	Sulfated galactan	Farias et al. 2000
	Gelidium crinale	Sulfated galactan	Pereira et al. 2005
	Corallina	Sulfated galactan	Sebaaly et al. 2014
	Corallina	Carrageenan	Sebaaly et al. 2014
	Lomentaria catenata	Sulfated galactan	Pushpamali et al. 2008
	Gigartina skottsbergii	Carrageenan	Carlucci et al. 1997
	Grateloupia indica	Sulfated galactan	Sen et al. 1994
	Stenogramme interrupta	Carrageenan	Cáceres et al. 2000
	Nothogenia fastigiata	Xylomannan	Kolender et al. 1997

(*continued*)

Table 3 (continued)

Algae		Polysaccharide	Reference
Green	*Codium dwarkense*	Sulfated polysaccharide	Shanmugam et al. 2002
	Codium indicum	Sulfated polysaccharide	Shanmugam et al. 2002
	Caulerpa prolifera	Sulfated polysaccharide	Costa et al. 2010
	Caulerpa sertularioides	Sulfated polysaccharide	Costa et al. 2010
	Codium isthmocladum	Sulfated polysaccharide	Costa et al. 2010
	Caulerpa cupressoides	Sulfated polysaccharide	Rodrigues et al. 2011
	Ulva conglobata	Sulfated polysaccharide – rhamnose	Mao et al. 2006
	Monostroma nitidum	Sulphated polysaccharide – rhamnose	Mao et al. 2008
	Monostroma latissimum	Sulfated polysaccharide – rhamnose	Mao et al. 2009
	Enteromorpha clathrata	Sulfated polysaccharide	Qi et al. 2012
	Enteromorpha linza	Sulfated polysaccharide – rhamnose	Wang et al. 2013
Blue-green	*Spirulina platensis*	Sulfated polysaccharide	Abd El Baky et al. 2013
	Arthrospira platensis	Sulfated polysaccharide – rhamnose	Majdoub et al. 2009
	Spirulina platensis	Calcium spirulan – sulfated polysaccharide	Hayakawa et al. 2003

precise anticoagulant potency of analyzed polysaccharides, and it is expressed as international units (IU) per mg of polysaccharide using the non-fractionated heparin (HEP, 193 IU/mg). The latter assays (PT, TT) have been performed by using normal human plasma in which algal polysaccharide extract is added and clotting time is recorded after the clotting induction by addition of thrombo reagent (Rodrigues et al. 2011; Padmanaban et al. 2013; Mao et al. 2008). The mechanism of anticoagulant activities of algal sulfated polysaccharides in the clotting cascade could be identified by all assays, APTT, PT, and TT as well. APTT assay could identify the mechanism of anticoagulant activities of algal sulfated polysaccharides in the intrinsic pathway of clotting cascade by inhibiting factors XII, XI, X, IX, VIII, and prothrombin; the PT assay could identify the mechanism of ulvans, fucans, and galactans in extrinsic pathway by inhibiting factors X, V, and prothrombin; and TT assay could be helpful to investigate the effect of sulfated

polysaccharides on thrombin-accelerated clot formation in platelet poor plasma (Padmanaban et al. 2013; Mao et al. 2008). Anticoagulant activity of algal polysaccharides varies depending on their chemical composition and particularly on different contents and positions of sulfate radicals, molecular weight, and sugar position. Moreover, the influence of the extractive methods used for the isolation of algal polysaccharides on anticoagulant activity has been confirmed. Leite et al. (1998) investigated the anticoagulant activity of acidic polysaccharide extracts from brown alga *Spatoglossum schröederi*. Polysaccharide extracts were obtained by ion-exchange chromatography using different salt molarities (0.15–3.0 M NaCl) and lower concentrations from the range of 0.5–0.7 and 1.0–1.5 M of salt resulting in higher content of alginic acid and xylose in obtained extract, respectively, while the extracts enriched with sulfated xylofucan were obtained with using higher concentrations (2.5–3.0 M) of salt. Anticoagulant activities of all polysaccharide fractions were low (0–22.4 IU/mg) in comparison with heparin activity of 150.0 IU/mg. However, all acidic polysaccharide fractions showed the ability to stimulate the synthesis of antithrombotic heparin sulfate produced by the rabbit aorta endothelial cells in culture to the same amount of heparin Leite et al. (1998). Neutral polysaccharides are usually extracted with cold or hot water, due to low or very high molecular weight, respectively. Higher anticoagulant activity of green algae *Codium* spp. and *Udotea* spp. has been reported in the fraction obtained by cold or hot water extraction, respectively (Shanmugam and Mody 2000). Finally, it has been also reported that polysaccharides isolated by proteolytic digestion from green seaweed *Caulerpa cupressoides* have a low anticoagulant potential in relation with polysaccharide extract obtained by ion-exchange chromatography (Rodrigues et al. 2011). Further, the strength of anticoagulant activity is also influenced by algal genus. Generally, sulfated polysaccharide extracts of brown seaweeds exhibit higher anticoagulant activity than red and green algae extracts. However, in comparative analysis, it has been observed that red seaweed *Gelidium* contained higher content of total saccharides and total sulfate content than brown seaweed *Sargassum* and green seaweed *Ulva*, and their anticoagulant activities are in correlation with sugar and sulfate contents. So, the extract from red seaweed *Gelidium* exhibits the highest anticoagulant activity, followed by the extract from brown seaweed *Sargassum*, while the extract from green seaweed *Ulva* has showed the lowest anticoagulant activity (Padmanaban et al. 2013).

4.2.1 Blue-Green Algae

Calcium spirulan (Ca-SP) is sulfated polysaccharide extracted from blue-green alga *Spirulina platensis*. It has been reported that Ca-SP exhibits anticoagulant activity by potential inhibition of thrombin mediated by heparin cofactor II (HCII), however, in a different mechanism from that of heparin (Hayakawa et al. 2003). Heparin cofactor II is a plasma serine protease inhibitor which selectively inhibits thrombin. Its ability to inhibit α-thrombin is accelerated by a variety of sulfated polysaccharides in addition to heparin and dermatan sulfate. The mechanism of activation of HCII is based on the fact that binding of either heparin or dermatan sulfate to HCII

displaces the N-terminal acidic domain of the inhibitor, which normally occupies the glycosaminoglycan-binding site. Then, N-terminal acidic domain binds to anion-binding exosite I of thrombin, facilitating proteolytic attack on the reactive site of peptide bond of HCII resulting in a significant decrease of the rate of α-thrombin inhibition in the presence of either heparin or dermatan sulfate. Hayakawa et al. (2000) has suggested that the stimulatory effect of Ca-SP is not mediated through N-terminal acidic domain of HCII, but the anion-binding exosite I of α-thrombin is essential for the rapid inhibition of the reaction by HCII in the presence of Ca-SP. The allosteric activation of HCII by Ca-SP has been observed through Arg^{103} – Leu mutant bound to Ca-SP-Toyopearl with the normal affinity and inhibited α-thrombin in a manner similar to native rHCII.

Moreover, sodium spirulan (Na-SP) isolated from blue-green alga *S. platensis* has been observed as a strong anticoagulant agent in the blood coagulation-fibrinolytic system. Not only because it activates heparin cofactor II, physiologic inhibitor of thrombin, but it also exhibits fibrinolytic property through varying effects on the endothelial fibrinolytic protein secretion, where it stimulates the release of anticoagulant heparan and dermatan sulfate proteoglycans from the vascular endothelial cell layers (Yamamoto et al. 2003).

4.2.2 Green Algae

The mechanism of anticoagulant activity of green algal sulfated polysaccharides has been assigned to common pathway, primarily heparin cofactor II (HCII)-mediated anticoagulant action (Shanmugam and Mody 2000). According to Wijesekara et al. (2011), sulfated arabinan and sulfated arabinogalactan extracted from two green algae species *Codium dwarkense* and *C. tomentosum* have been the most active coagulants; the former shows higher coagulant activity, while the latter is relatively less active. Further, it has been reported that the coagulant activity is directly proportional to sugar and sulfate contents of proteoglycan and inversely proportional to protein content of proteoglycans. Thus, proteoglycans with 18.4 % content of sulfate show the greatest anticoagulant activity, followed by sulfated polysaccharides with the content of 10.2 % and 7.5 % of sulfate (Wijesekara et al. 2011). Further, highly sulfated galactan (13.1 % of sulfate) containing mainly galactose with a small amount of glucose from green algae *C. cylindricum* has been found as an anticoagulant with the similar activity compared with heparin, but weaker than heparin (Matsubara et al. 2001). Anticoagulant activity of sulfated polysaccharides isolated from green seaweed *Monostroma nitidum* has also been described. Among two sulfated polysaccharides of *M. nitidum* with different structure of their molecules, different extent of anticoagulant activities has been evaluated. Evidently, both polysaccharide fractions exhibit high anticoagulant activities; however, differences between them are directly due to their structural feature discrepancy, and sulfated polysaccharide with a lower molecular size and higher sulfate content show notably higher anticoagulant activity (Mao et al. 2008). Further, sulfated polysaccharide composed mainly of (1,2)-linked L-rhamnose residues with sulfate groups substituted at positions C-3 and/or C-4 isolated from marine green algae *M. latissimum* show high anticoagulant activities proved by APTT and

TT assays. Anticoagulant property of this sulfate polysaccharide has been mainly attributed to powerful potentiation of thrombin by HP II (Mao et al. 2009). The influence of different concentrations of extraction solutions (0.50, 0.75, and 1.00 M NaCl) used for the elution of sulfated polysaccharides of three species – *Caulerpa cupresoides*, *C. racemosa*, and *C. prolifera* – has been confirmed. Dissimilarity in the presence of sulfate groups in chemical structures of their sulfated polysaccharides obtained by ion-exchange chromatography has been observed. Lower content of sulfate groups has been established in *C. cupressoides*, followed by *C. racemosa*, and higher content has been determined in *C. prolifera* which is in accordance with the extent of their anticoagulant activities of 17.37, 22.17, and 25.64 IU/mg, respectively, in comparison with heparin activity of 193 IU/mg (Rodrigues et al. 2012). Green seaweed *Ulva conglobata* collected from three various locations in China (coasts of Quigdao, Yantai, and Rizhao) exhibited different polysaccharide compositions with major representation of rhamnose 71.90, 72.26, and 63.77 mol%, respectively; variable contents of glucose and fucose; and trace amounts of xylose, galactose, and mannose. Their sulfate ester contents were 35.20 %, 23.04 %, and 28.06 %, respectively. The highest anticoagulant activity was established in hot water extract from *U. conglobata* collected from the coast of Quigdao in comparison with extracts from the other locations. Polysaccharide extract from the coast of Rizhao possessed higher content of sulfated ester and lower content of rhamnose than extract from the coast of Yantai, the former showing lower anticoagulant activity than the latter (Mao et al. 2006). The extent of anticoagulant activity of algal polysaccharides varied in dependence on different sugar residues and their proportion, as well as on sulfation content and structural features.

4.2.3 Red Seaweed

Anticoagulant activity has been studied in over 40 species of red algae. Generally, the mechanism of anticoagulant activity of galactan sulfate of red seaweed could be realized through the inhibition of thrombin and could be either directly, even though the mechanism has been still unknown, or indirectly via HCII. Anticoagulant activity of sulfated galactans depends on the nature of sugar residue, sulfate content, and the position of sulfate groups. Furthermore, carrageenans with high molecular weight and high sulfate content have shown higher anticoagulant activity than low molecular weight and low sulfate content (Wijesekara et al. 2011).

The mechanism of anticoagulant activity of carrageenans and other sulfated galactans is exhibited via thrombin inhibition. Carrageenans appear to inhibit amidolysis of thrombin directly and via ATIII; however, only ATIII potentiated Xa amidolysis has been observed (Shanmugam and Mody 2000). Anticoagulant properties of three types, κ-, ι-, and λ-carrageenans with different sulfate amounts of 17.89 %, 27.20 %, and 33.38 %, respectively, were established by APTT and PT assays and compared with unfractionated heparin from bovine lung. The compounds may act only on intrinsic pathways of the blood coagulation system, not on extrinsic pathways which could be confirmed by very low results of PT assay in the case of κ- and ι-carrageenans. Only λ-carrageenan showed similar anticoagulant activity of 120 s at 20 μg, as heparin did. All types of analyzed carrageenans had

very low anticoagulant activity when compared to heparin by APTT assay. While κ- and ι-carrageenans exhibited the anticoagulant activity of 132.2 and 240 s at the concentration of 100 μg, λ-carrageenans displayed elevated anticoagulant activity of 240 s at 20 μg, and heparin showed the anticoagulant activity of 250 s at 2.5 μg (Silva et al. 2010).

Moreover, the strength of anticoagulant activity between different types of carrageenans appeared to be due to various amounts of sulfate content. λ-carrageenan showed greater antithrombotic activity than κ-carrageenan, although it also showed higher toxicity which is dependent on the molecular weight (Shanmugam and Mody 2000).

The extent of anticoagulant activity of carrageenans seemed to be influenced also by a different composition of polysaccharides extracted from red seaweed in various stages of their life cycle. Anticoagulant activity of carrageenans of λ-type and the mixture of κ-/ι-type extracted from cystocarpic (reproductive) and sterile plants of *Chondrus pinnulatus* harvested on the Russian Pacific coast had extremely high values exceeding 600 s established by APTT assay in contrary to that from sterile plants. Since molecular weights of cystocarpic plant carrageenans were almost twice higher (420 and 389 kDa) than molecular weights of carrageenans from sterile plants (290 and 220 kDa), high anticoagulant activity may be caused by high molecular weight of this polysaccharide (Yermak et al. 2006).

Sulfated galactans and carrageenan were extracted from red algae *Corallina* collected at the Lebanese coast in the yields of 2.5 % and 10 %, and their anticoagulant activities were determined by ATPP assay. Carrageenans exhibited more powerful anticoagulant activity from 78.4 to 120 s at doses of 0.05 and 0.5 μg, respectively, than sulfated galactans that reached 104.3 s at their highest dose of 5 μg, but only λ-carrageenan was used for analyses (Sebaaly et al. 2014).

Further, it was reported that 2,3-di-*o*-sulfated D-galactan from red seaweed *Botryocladia occidentalis* exhibited anticoagulant activity comparable to heparin, which appeared to be due to the inhibition of thrombin and factor X. Furthermore, 2,3-di-*o*-sulfated galactose has an amplifying effect on the anticoagulant activity of sulfated galactans. From the three fractions with sulfate contents of 0.69 %, 1.47 %, and 2.08 % eluted by using different concentrations of NaCl 1.0, 2.2, and 3.0 M, respectively, the fraction with the sulfate content of 1.47 % showed the highest anticoagulant activity of 150 IU/mg by APTT assay, while the fraction with the highest sulfate content showed a lower anticoagulation effect of 130 IU/mg and the fraction with the lowest sulfate content showed even no effect. Finally, desulfation had the abolish effect on the anticoagulant activity of the sulfated galactans (Farias et al. 2000). The proportion and/or distribution of 2,3-di-sulfated galactose along the polysaccharide chain modulated the interaction of polysaccharides with specific proteases in the coagulation system. It was reported that 2,3-di-sulfated galactose obtained from *B. occidentalis* inhibited intrinsic tenase and prothrombinase complexes crucial for factor Xa and thrombin generation, respectively (Jiao et al. 2011). Porphyran derivate obtained from red seaweed *Porphyra haitanensis* with fully sulfated modification showed the highest anticoagulant activities by APTT, TT, and PT assays 396.47 s, 311.70 s, and 298.03 s, respectively, in a dose of 100 μg/ml.

Besides the degree of sulfation, distribution of sulfated group was also an important factor influencing the anticoagulant activity of porphyran, which has a linear backbone of alternating 3-linked β-D-galactosyl units and 4-linked α-L-galactosyl 6-sulfate or 3,6-anhydro-α-L-galactosyl units. In the case of alkali treatment of porphyran, 6-*o*-desulphatation in (1,4)-linked residue was carried out, nevertheless almost without any change of anticoagulant activity. Moreover, 6-*o*-sulfated derivate showed a lower anticoagulant effect than 2,2′,4-*o*-sulfated derivate. From these results, it could be concluded that sulfate groups at C-6 were not necessary for the anticoagulant activity, whereas an increase in the overall molecular weight and position of sulfate groups at C-2, C-3, and C-4 was a dominant factor for the anticoagulant effect of porphyran (Zhang et al. 2010).

4.2.4 Brown Seaweed

Anticoagulant activity of fucoidans, like heparin, is based on the inhibition of thrombin activity by influencing directly the enzyme or through the activation of thrombin inhibitors, including antithrombin III (ATIII) and heparin cofactor II (HCII). Some fucoidans activate only antithrombin, whereas others interact with both inhibitors (Cumashi et al. 2007). Almost all fucoidans exhibit anticoagulant activity that may be related to the content and position of the sulfate group, molecular weight, and sugar position (Berteau and Mulloy 2003).

It has been documented that fucans with high sulfate and low uronic acid content show higher anticoagulant activity than those with high uronic acid and low sulfate content (Wijesinghe and Jeon 2012; Cumashi et al. 2007; Shanmugam and Mody 2000). It has been reported that anticoagulant activity of the fucoidans does not depend on the content of neutral sugar residues in their molecules or on the structure of the main chain. However, the degree of sulfation of their molecules has been reported as an important factor to affect their anticoagulant activities (Ushakova et al. 2009). Thus, it has been documented that higher content of sulfate groups is related to higher anticoagulant activity in native fucoidans (e.g., *Hizikia fusiformis*, *Ecklonia kurome*), and furthermore, the position of the sulfate groups on sugar residues is a very important factor of influence on the anticoagulant activity of fucoidans, relating to the concentration especially of C-2 sulfate and C-2,3 disulfate (Li et al. 2008). The structural analysis has established that a central core of fucan obtained from brown algae *Padina gymnospora* is composed of 3- or 4-linked β-D-glucuronic acid with minor amounts of 3- or 4-linked galactose units. High content of nonreducing fucose and xylopyranose terminal residues indicates that these heteroglycans are highly branched polymers. Subsequent desulfation of this heteroglycans fraction obtained by solvolysis in dimethyl sulfoxide caused the reduction of the sulfate groups by about 76 % due to the removal of 3,4-disubstituted fucosyl residues mostly sulfated at C-3, resulting in an abolishment of its anticoagulant activity. Thus, the presence of (1,4)-linked 3-*o*-sulfated fucose could be related to higher anticoagulant activity (Silva et al. 2005). Moreover, sulfated fucan obtained from brown seaweed *Laminaria brasiliensis* shows a strong anticoagulant activity of 30.0 IU/mg; however, desulfation of this fucan totally abolishes its anticoagulant activity (Pereira et al. 1999). According to

Ushakova et al. (2009), fucoidans originated from brown seaweeds *L. saccharina*, *L. digitata*, and *Fucus distichus* contained the highest amount of sulfate in the range of 27.5–34.8 % and the lowest amount of uronic acid and showed the highest anticoagulant activity by APTT assay in the amounts of 33.0, 24.2, and 26.9 U/mg, respectively, and by PT assay in the amounts of 40.8, 33.2, and 33.0 s, respectively; and, finally, anticoagulant activities performed by TT assay were in the amounts of 72.8, 36.0, and 29.0 s, respectively. In accordance with the previous author, in brown seaweeds, *L. saccharina*, *L. digitata*, and *F. distichus* have been established as the highest anticoagulant activities of 33.0, 24.2, and 26.9 U/mg, respectively. In other brown seaweeds, such as *F. serratus*, *F. evanescens*, *F. spiralis*, and *Ascophyllum nodosum*, lower anticoagulant activities have been determined in the amounts of 19.1, 15.1, 16.6, and 13.4 U/mg, respectively. On the other hand, sulfated polysaccharide of *Cladosiphon okamuranus* with the highest content of 23.4 % of uronic acids and the lowest content of sulfate groups of 15.1 % has showed almost no anticoagulant effect (Cumashi et al. 2007). Analyses of anticoagulant activities of fucoidans have demonstrated that native fucoidans interact with ATIII like heparin and promoted thrombin activation followed by the formation of a complex with thrombin and ATIII. However, in contrast to heparin, they may directly inhibit thrombin (Ushakova et al. 2009). Molecular weight is also important for the anticoagulant activity of fucoidans because of long enough sugar chain, and suitable conformation is needed to bind thrombin. A slight decrease in molecular weight of sulfated fucan dramatically reduces its effect on thrombin inactivation mediated by HCII. Although sulfated fucan with 45 tetrasaccharide repeating units could bind to HCII, it is unable to link efficiently the plasma inhibitor and thrombin because for this action chains with 100 or more tetrasaccharide repeating units are necessary. The template mechanism may predominate over the allosteric effect in the case of linear sulfated fucan inactivation of thrombin heparin cofactor II being present. Linear sulfated fucan requires significantly longer chains than mammalian glycosaminoglycans to achieve the anticoagulant activity (Li et al. 2008; Pomin et al. 2005). Molecular size of the most active fucoidans has been approximately between 50 and 100.000 daltons, whereas fractions with higher molecular weight have exceeded 850.000 Da inclined to lower the anticoagulant activity (Shanmugam and Mody 2000).

Anticoagulant activity of 75 % fucoidan from *U. pinnatifida* consisted of 24.7 % fucose, 20.35 % galactose, no mannose, 29.07 % sulfate, 2.19 % protein, and 7 % bond ions evaluated by APTT, TT, PT, and ATIII assays. It was established that this fucoidan exhibited a very strong hemostasis effect. It prolonged APTT time from 38.8 s for control to 172.5 s at 63 mg/l; TT time was also prolonged at a higher rate in which it was 15.2 s at baseline and went up to 240.1 s at 15.6 mg/l. Further, ATIII decreased with the fucoidan treatment from 108 % for control to 89 % at 10 000 mg/l; and finally, low concentration of fucoidan had no effect on PT until the concentration of 125 mg/l of PT when it began to increase (Irhimeh et al. 2009). Also crude polysaccharide fraction of brown seaweed *Sargassum horneri* consisted of 97 % of polysaccharide and 2 % of protein showed a strong anticoagulant effect exceeding 300 s by APTT assay, whereas anticoagulant active compounds were

mainly concentrated in the fraction with molecular weight higher than 30 kDa (Athucorala et al. 2007). Brown seaweed *Dictyopteris polypodioides* growing on the Lebanese coast was collected in two different seasons in May and July. It was rich in polysaccharides whose main component was alginic acid (11 %) as well as water-soluble polysaccharides, such as fucoidan, laminaran, and mannuronan, whose amounts differed depending on the season between 3.75 % in May and 5.8 % in July. The anticoagulant activities were established in different fractions of polysaccharides composed from fucose and laminaran residues (FL), mixture of fucose, laminaran and mannuronan (FLM), and mannuronan (M). The highest anticoagulant activities of 42.5, 43.1, and 42.1 s by APTT assay in the application doses of 2.5 μg of different fractions FL, FLM, and M were determined, respectively (Karaki et al. 2013).

Laminaran is known as a storage polysaccharide of brown seaweed, and it does not naturally contain sulfate groups. However, laminarin sulfate obtained by sulfation could possess 0.6–2.2 sulfate groups per glucose unit, and its molecule consists of *o*-sulfated β-(1,3)-linked glucose residues. It has been observed that laminarin sulfate with about two sulfate units per glucose exhibits the maximum anticoagulant activity of about 30–40 % of the anticoagulant activity of heparin, and it is therapeutically effective in the prevention and treatment of cerebrovascular diseases (Miao et al. 1995; Adams and Thorpe 1957).

It has been also observed that the degree of sulfation influences the anticoagulant activity of sulfated laminarin, i.e., derivate with 2.31 degree of sulfation is less anticoagulant (26.2 USP-U/mg and 3.1 U/mg by APTT and TT assays, respectively) than derivate of laminarin with a degree of sulfation of 1.98 that shows the highest anticoagulant activity of 36.2 USP-U/mg and 14 U/mg by APTT and TT assays, respectively, while laminarin derivates with 0.3 and 0.64 degree of sulfation exhibits no effect (Hoffmann et al. 1995).

Sulfated β-aminoethyl ether derivates of laminaran, (1,3)-linked glucan of *L. digitata* containing both *o*-sulfated and N-sulfated groups have been found to show anticoagulant activity rather than isolated *o*-sulfated groups. Further, partially oxidized laminaran with a sulfate content of 15.9 % gave the activity of 26 IU/mg in APTT test, while partially reduced sulfated alginic acid with the maximum of sulfate of 13.4 % and the lowest uronic acid content of 20 % gave the highest activity of 55 IU/mg in APTT test than low sulfated one (Shanmugam and Mody 2000). The sargassan obtained from brown seaweed *S. linifolium* was composed of D-glucuronic acid, D-mannose, D-galactose, D-xylose, L-fucose, and a protein moiety. The backbone of sargassan seems to be composed of glucuronic acid, mannose, and galactose residues with partially sulfated side chains consisting of galactose, xylose, and fucose residues, and it exhibited high coagulant activity (Fattah et al. 1974).

Alginic acid is naturally a polymer of D-mannuronic and L-guluronic acids without sulfate contents. Sulfated derivate of alginic acid also shows anticoagulant activity; however, it is much lower and much more toxic than heparin. Also aminated fraction of sulfated alginic acid derivate shows anticoagulant activity in APTT assay by binding to immobilized ATIII (Shanmugam and Mody 2000).

5 Conclusion

High interest in algae is their perspective to be served as food with high nutritional value, as nutraceuticals and medicinal food for their health benefits of bioactive compounds present. Freshwater algae and seaweed are a natural source representing many other interests for medical, therapeutic, and nutritional fields in many applications in the food industry, cosmetic industry, and medicine and pharmaceutical industry by the reason of searching for new natural and less toxic bioactive compounds than synthetic products.

Recently, algal polysaccharides alongside nutritious important compounds, such as proteins, amino acids, minerals, and vitamins, are in the focus of many researches all over the world for their health beneficial activities, such as anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, and antiviral properties, as well as skin protecting and antiaging activities. Heparin has been identified and used for more than fifty years as a commercial anticoagulant. However, some of the side effects of heparin, i.e., thrombocytopenia, hemorrhagic effect, and incapacity to inhibit thrombin bound to fibrin, lead to an increasing interest in discovering new anticoagulants to replace heparin. Algal-derived sulfated polysaccharides have been found to possess anticoagulant activity similar to or higher than heparin. Thus, the results of many research studies suggest that algal sulfated polysaccharides have a promising potential to be used as anticoagulant agents and medication for thrombotic disorders.

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Polysaccharides from Lower Plants: Bryophytes

5

Laura Klavina

Contents

Abstract

Bryophytes, which are considered to be the first land plants, are the second largest taxonomic group in the plant kingdom. Polysaccharides in bryophytes play a key role in the evolution of chemical diversity of vegetation, and they also function as a structural material. Additionally, polysaccharides are believed to be involved in bryophyte stress tolerance and other functions in this plant kingdom group. Despite the recent progress in bryophyte phytochemistry, bryophyte polysaccharides as a group of substances are largely neglected. Still, major differences in the polysaccharide composition in comparison with higher plants can be identified. In *Sphagnum* mosses, a unique polysaccharide – sphagnan – has been found, and its possible applications are on the way. Further, a relatively large group of carbohydrates that form bryophyte polysaccharides has been identified, and their functions in the development of bryophyte stress tolerance are discussed. Study of bryophyte polysaccharides is a challenging and

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prospective field of research, with a potential of providing not only considerable new knowledge about this group of substances, their functions, evolution of chemical compounds and chemical diversity, but also new applications of bryophyte carbohydrates.

Keywords
Polysaccharides • Monosaccharide • Bryophytes • *Sphagnum* • 2-Galacturonic acid • Sphagnan

1 Introduction

The bryophytes are one of the major plant taxonomical groups in the world, and they can be found nearly everywhere globally, growing on differing substrates and in a variety of growth conditions (Goffinet and Shaw 2008; Glime 2007). There have been about 25,000 bryophyte species identified; however, this figure is rapidly changing due to the increasing application of genetic identification of species, supporting better understanding of taxonomical order and differences of their physiology, as well as discoveries of new species (Asakawa et al. 2013; Goffinet and Shaw 2008). Bryophytes are divided in three phyla – Bryophyta (mosses), Marchantiophyta (liverworts), and Anthocerotophyta (hornworts). Taking into account that bryophyte taxonomy is rapidly changing, the taxonomy given in Fig. 1 will be used in this chapter. In respect to the evolution theory, bryophytes are placed between algae and vascular plants and thus considered as the first terrestrial plants (Goffinet and Shaw 2008; Glime 2007).

Bryophyte fossils serve as an indicator of the age of bryophytes, and they date back to the upper Carboniferous. However, the fossil record cannot be considered as a strong indicator, because not many findings are unambiguous, and the possible age estimation differs from sample to sample. The molecular phylogeny of bryophytes suggests that the origin of this division dates back to Ordovician; accordingly, their evolution appears to have begun at least 400 million years ago (Goffinet and Shaw 2008; Glime 2007). Studies of bryophyte phytochemistry and their polysaccharides can be used as a tool to understand the composition of bryophytes and to explain and find new evidence about the genesis of both higher and lower plants, as bryophytes are likely to be a link between them.

The bryophyte polysaccharide studies require understanding of the evolution of chemical diversity during the development from lower plants and microorganisms to higher plants. Furthermore, polysaccharides in bryophytes function as a structural material, demonstrating the importance of cellulose and lignin as cell wall molecules (Popper and Fry 2003; Popper et al. 2004) (absent in bryophytes) in higher plants and revealing how the evolution of chemical structures has supported the biological evolution (Glime 2007). Polysaccharide studies may result not only in identification of new compounds and their combinations, providing new knowledge about this group of substances and their functions, but also in new applications

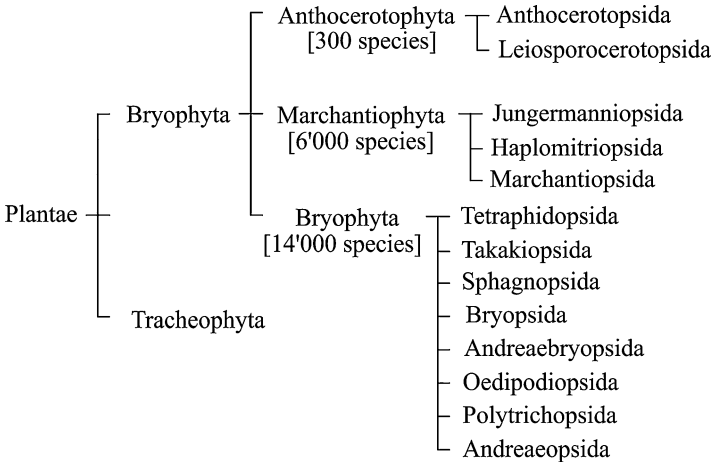


Fig. 1 Taxonomical order of the Bryophyta division (Adapted from Asakawa et al. 2013; Glime 2007)

of bryophyte carbohydrates. Studies of *Sphagnum* polysaccharides as prospective substances for food conservation can be considered exemplary in demonstrating the potential for development in this direction (Balance et al. 2008; Painter 1991).

2 Occurrence/Sources

The bryophyte habitat structure is regulated by the hydrological requirements of the species as well as the requirements in respect to the growth substrate and other aspects. For instance, the compound of 3-hydroxy-β-ionone and momilactone A and B found in bryophytes inhibit the growth of other plants, thereby enabling *Rhynchostegium pallidifolium* and *Hypnum plumaeforme* to form the species-specific dense cover of moss (Kato- Noguchi et al. 2010). Each bryophyte species has its own hydrological requirements that are partly connected with their stress tolerance. Some species are more adapted to cold or extremely cold (such as Arctic) climates, while others are able to survive heat and long-time drought. These unique adaptation skills are the reason of why bryophytes are so widespread in all ecosystems (Glime 2007) and have a wide ecological niche and higher stress tolerance than higher vegetation.

Despite the global abundance, high diversity of species, and unique chemical composition, bryophyte chemistry and their polysaccharides have not been extensively studied. One of the reasons for the low interest in bryophyte chemical composition studies is their relatively small size that usually varies between a couple of millimeters to up to a few centimeters (Zinsmeister and Mues 1990; Glime 2007). The size of bryophytes is limited due to their anatomical and physiological character, as they do not have a vascular system and strong building materials of cell walls, such as cellulose-based polysaccharides and lignin

(Goffinet and Shaw 2008; Popper and Fry 2003). Although in higher plants a significant source of polysaccharides is the reserve polysaccharides, such as starch, this by no means applies to bryophytes, in which polysaccharides can be isolated from the whole plant material and which have a low number of special bodies or structural elements responsible for the accumulation of larger quantities of polysaccharides (Balance et al. 2012; Goffinet and Shaw 2008). Due to their small size, it is complicated to harvest large quantities of sample materials necessary for extensive chemical analysis and identification of individual substances (Goffinet and Shaw 2008; Zinsmeister and Mues 1990). In most cases bryophytes tend to form tight colonies, including other bryophyte species, vascular plants, as well as small living beings, such as fungi, bacteria, ticks, spiders, etc. (Goffinet and Shaw 2008; Glime 2007). One more reason for the slow pace of research in the area of bryophyte polysaccharides that is also partially connected with their size is their complicated identification due to the unclear taxonomy (Asakawa et al. 2013; Goffinet and Shaw 2008; Glime 2007). In some occasions, identification of different bryophyte species can be done only by examining in a microscope the cross section of a leaf that in many cases is about 1 mm or less in size, making the gathering of this kind of species for chemical analysis extremely difficult (Zinsmeister and Mues 1990).

Isolation of polysaccharides from bryophytes usually is done by extensive removal of secondary metabolites by means of exhaustive extraction with low-polarity solvents (hexane, chloroform, dichloromethane, etc.), followed by extractions with more polar solvents (methanol, ethanol, acetone) and extractions with water, acid hydrolysis or autohydrolysis (Balance et al. 2008; Painter 1991). Although the extraction process of bryophyte polysaccharides is well elaborated in respect to *Sphagnum* mosses, however, the suggested approaches include an intermediate step of oxidation with $NaClO_2$, possibly resulting in the oxidation of carbohydrate functional groups (Balance et al. 2008; Painter 1991, 1983), thus potentially giving false results.

3 Brief Phytochemistry

3.1 Bryophyte Phytochemistry

Bryophyte phytochemistry is one of the most challenging and at the same time very rapidly developing field of phytochemistry, and it is closely related to developments in better understanding of the evolution of vegetation. The phytochemistry of bryophytes is also noteworthy as a new field of bioprospecting, and it is important for understanding the ecological functions of bryophytes in nature. The phytochemistry of bryophytes has been analyzed in several review articles and in a recently published book that mainly concentrates on the chemistry of liverworts (Asakawa et al. 2013; Beike et al. 2010). The interest in bryophytes is related to their wide use as medicinal plants to cure burns, bruises, snake bites, tuberculosis, neurasthenia, fractures, convulsions, scald, uropathy, and pneumonia (Asakawa et al. 2013;

Table 1 Major chemical constituent groups of bryophytes (Adapted from Asakawa et al. 2013; Ignat et al. 2011; Üçüncü et al. 2010; Iwashina 2003; Baas et al. 2000; Dembitsky et al. 1993; Zinsmeister et al. 1991; Zinsmeister and Mues 1990; Tutschek 1982)

Chemical constituents	*Anthocerotophyta*	*Marchantiophyta*	*Bryophyta*	Reference
Acetogenins	x		x	Asakawa et al. 2013
Alkaloids		x	x	Zinsmeister et al. 1991
Alkanes		x	x	Baas et al. 2000; Dembitsky et al. 1993
Phenols				
Benzoic acid derivatives		x	x	Zinsmeister and Mues 1990
Bibenzyl derivatives		x		
Cinnamic acid derivatives	x	x	x	
Flavonoids		x	x	Ignat et al. 2011; Iwashina 2003
Epigenine		x		Basile et al. 1999
Sphagnorubin			x	Tutschek 1982
Steroids	x	x	x	
Sterols				Zinsmeister and Mues 1990
Sitosterol	x		x	Asakawa et al. 2013
Fitosterol	x		x	Asakawa et al. 2013
Terpenes				
Monoterpenes		x	x	
Sesquiterpenes	x	x		Üçüncü et al. 2010
Diterpenoids	x	x	x	
Tetraterpenes	x	x	x	
Lipids				Dembitsky et al. 1993

Saboljevic et al. 2010; Singh et al. 2006; Spjut et al. 1986; Hotson 1921). Besides, the presence of biologically active compounds in bryophytes makes them resistant to microorganisms (Asakawa et al. 2013; Üçüncü et al. 2010; Basile et al. 1999). The biological diversity of bryophytes influences their chemical diversity and the close relations between the chemical composition of bryophytes and their taxonomy. The main groups of newly identified substances are terpenoids and steroids, while polyphenols and carbohydrates have also been identified (Table 1).

Only around 10 % of bryophyte species have been investigated to date, identifying more than 400 new substances, many of which are unique and possess biological activity (Asakawa et al. 2013; Beike et al. 2010). A number of different compounds have been found in bryophytes. At first, it should be noted that a high number of lipophilic compounds have been detected in liverworts, especially in

their oil bodies, and similar substances have also been identified in mosses and hornworts. Mono-, sesqui- and diterpenoids, as well as aromatic compounds, such as bibenzyls and bis(bibenzyls), have been found in bryophytes. Flavonoids and terpenoids are ubiquitous components in bryophytes and have been detected in Marchantiophyta and Bryophyta (Asakawa et al. 2013; Ignat et al. 2011; Iwashina 2003). The phytochemical content of bryophytes accounts for their taste (e.g., bitterness) and smell, as well as other characteristics (Asakawa 2007).

4 Carbohydrates in Bryophytes

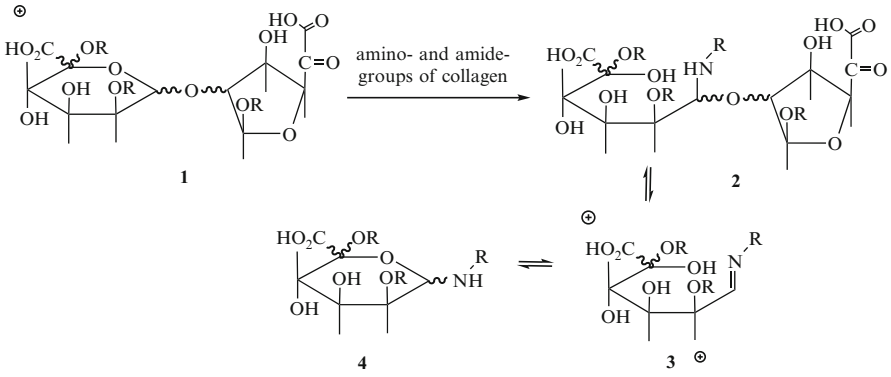
The total amount of carbohydrates and polysaccharides in bryophytes vary in a significant range, largely depending on the studied species and the method used (Judina et al. 1999; Marschall et al. 1998). One of the key groups of bryophyte mosses most abundantly studied to identify the structure-forming polysaccharides is *Sphagnum* moss, which is placed in a separate class – Sphagnopsida – represented by 120 different species and being one of the main species forming northern bogs and wetlands as well as the major precursor of raised bog peat (Glime 2007; Judina et al. 1999). The interest in polysaccharides in S*phagnum* mosses started around the year 1950, when the first short communications on this topic were published and increased after the identification of D-*lyxo*-5-hexopyranuronic acid in the year 1983 (Maass and Craigie 1964; Painter 1983). The first larger-scale investigation was conducted on 14 *Sphagnum* species, and the results showed that glucose, fructose, sucrose, fructosides, as well as short-chain fructans can be found in the water-soluble fraction of bryophytes (Maass and Craigie 1964). In later studies, D-galacturonic acid, glycuronoglycan, L-rhamnose, and D-galactose were identified as the major polysaccharides in *Sphagnum* mosses (Judina et al. 1999; Graham et al. 2010). It was concluded that the cell walls of bryophytes mostly consist of the cross-linking products of cellulosic, hemicellulosic, and pectin-like chains. Pectin-like polysaccharides in *Sphagnum* mosses have a molecular mass of up to 37,000 Da, and they may form up to 20 % of dry-moss weight (Kosonogova et al. 1994). The mentioned studies included intensive extraction, while the most efficient way of treatment was autohydrolysis in distilled water after acid hydrolysis (Painter 1983). The treatment resulted in browning and release of glycuronoglycan in a solution which also contained D-galacturonic acid (25 %), L-rhamnose (19 %), D-galactose (10 %), pentoses (6 %), glucose (7 %), as well as D-mannose (4 %). The remaining 20 % were acid-insoluble polymer-like carbohydrates, but to analyze the rest of the material, further hydrolysis had to be done. The obtained material was porous, with a yield of approximately 40 %, behaving similarly to holocellulose. After the subsequent treatment, residues of D-glucose (33 %) were found, along with other carbohydrates, such as L-fucose (4 %), D-galactose (9 %), D-mannose (23 %), and D-xylose (29 %), and some percentage of unidentified carbohydrates was also evidently present. One of the unknown carbohydrates was identified as D-*lyxo*-5-hexopyranuronic acid, which cross-links cellulose and hemicellulose, as well as other carbohydrates in the *Sphagnum* cell walls (Painter 1983; Hajek

et al. 2011). Sucrose is one of the carbohydrates that can be found in bryophyte content, and its concentration varies from 100 up to 500 μmol/g of dry weight in the *Dicranum majus*, *Hookeria lucens*, *Polytrichum commune*, and *Racomitrium lanuginosum* species (Melick and Seppelt 2002; Smirnoff 1991). Fructose and glucose in bryophytes are found in much lower concentrations than sucrose. During cold weather and in the aging process, the fructose and glucose levels decrease, while sucrose does not change greatly (Smirnoff 1991).

Even if *Sphagnum* mosses is the bryophyte group most frequently chosen for polysaccharide research, there are some studies concentrating on other bryophyte groups, such as liverworts (Suleiman et al. 1978; Marschall et al. 1998; Nagao et al. 2006). Studies on the carbohydrate content of the liverwort *Plagiochila asplenioides* reveal that there are some differences in the carbohydrate content of mosses and liverworts, and the carbohydrate composition can be used as a taxonomic characteristic (Marschall et al. 1998). The water-soluble fraction of *Plagiochila asplenioides* consists of glucose (3 μg * mg of dry weight^{-1}), fructose (6 μg * mg of dry weight^{-1}), sucrose (6 μg * mg of dry weight^{-1}), mannitol (0.93 μg * mg of dry weight^{-1}), and volemitol (10.4 μg * mg of dry weight^{-1}), which have not been found in *Sphagnum* mosses (Suleiman et al. 1978). The major neutral monosaccharide residue sequence in the cell walls of liverworts are glucose > galactose > mannose > xylose ≈ arabinose > fucose ≈ rhamnose (Popper and Fry 2003; Thomas et al. 1984). In the cell walls of liverwort *Riccardia*, residues of mannuronic acid have been found. However, due to slightly different R_f values, galacturonic acid could have been falsely perceived as mannuronic acid. The content of xyloglucan was proved by the HPLC and paper chromatography analysis of Driselase digests of the materials of the primary cell walls. These analyses showed the presence of disaccharide isoprimeverose – a product of Driselase digestion of xyloglucan. The uronic acids are present in primary cell walls as constituents of xylans, pectins, glucuronomannans, and arabinogalactan proteins (Popper and Fry 2003; Popper et al. 2003; Thomas et al. 1984). The major uronic acid found in the tested samples was galacturonic acid. Additionally, in the cell walls of leafy and thalloid liverworts and *Sphagnum*, galacturonic acid was present in a high concentration. 3-*O*-Methylrhamnose has been obtained through purification of acid hydrolysates of the alcohol-insoluble residue of *Anthoceros* (Popper et al. 2003). It has also been found to occur in primary cell walls of other bryophytes (Popper and Fry 2003). A high concentration of mannose residues has been detected in most bryophytes, liverworts, and lycopodiophytes. Despite a relatively low number of studies, the carbohydrates specific for bryophyte polysaccharides can be identified as: (a) xyloglucan, (b) uronic acids, (c) mannose, (d) mixed-linkage glucan, and e) 3-*O*-methylrhamnose (Popper et al. 2003). Xyloglucan can be found only in land plants and not in algae at the same time, while in algae and bryophytes, there are higher concentrations of glucuronic acid than in land plants, thus showing the role of bryophytes in evolution (Popper and Fry 2003). Mixed-linkage glucan is clearly detected in higher plants, while in experiments conducted on bryophytes, it has been detected only in the leafy liverwort *Lophocolea bidentata*, due to licheninase digestion (Popper and Fry

2003). As mentioned above, the primary cell walls of bryophytes contain a large amount of uronic acids, which are mainly represented as galacturonic acid and gallic acid. Bryophyte-specific carbohydrates of the primary cell walls of more advanced (desiccation-tolerant) mosses have lower concentrations of gallic acid. Still, the conclusion from the very few studies on polysaccharides in bryophytes is evident: the polysaccharide composition among bryophytes differs very much, and the few studies dedicated to interspecies comparison have proved this. In liverworts (*Mylia taylorii* and eight others), (–)-L-bornesitol, (–)-D-mannitol, (+)-D-glucose, (+)-sucrose, and (+)-trehalose have been found, and their roles in the cell wall polysaccharides discussed (Matsuo et al. 1986).

Major growth of interest in bryophyte and, specifically, *Sphagnum* moss polysaccharides happened after the discovery that the *Sphagnum* moss polysaccharides can be used for preservation of food articles (Stalheim et al. 2009; Borsheim et al. 2001; Painter 1998, 1991). This concept largely relies on the widespread use of *Sphagnum* mosses for food preservation in traditional cultures of northern countries. In the first publications on the preserving properties of *Sphagnum* mosses, sphagnan – a complex, pectin-like substance found in the living cells of mosses – is mentioned as one of the main substances responsible for this activity. The preserving as well as antimicrobial effects of sphagnan was first noticed in bog bodies, such as the Lindow (2,000 years old) and Tollund (1,400 years old) men, as well as in animal tissues and wooden artifacts, which have been found in bogs. All of the bog bodies found had a distinctive coloring characteristic of tanning caused by the Maillard reaction (Scheme 1) and reactive carbonyl groups in the sphagnan-containing residues. The highly reactive carboxyl groups in the α-ketocarboxylic acids present in sphagnan as a major component of the hyaline cell walls of *Sphagnum* mosses are undergoing the Maillard reaction with ammonia, amino acids, and polypeptides (Painter 1998). The Maillard reaction can happen without hydrolyses of the cell wall polysaccharides because of the reactive carbonyl groups



Scheme 1 Acid-catalyzed reaction of carbonyl group-containing compounds (sphagnan (*1*)) with amino groups in proteins (collagen (*2*)) and amino acids, resulting in production of glucosylamine (*3*) and brown pigment products (*4*), possibly melanoidins (the Maillard reaction)

that can be found in the *Sphagnum* moss cell walls. It is assumed that the antimicrobial activity in peat bogs is partially caused by the specific humic substance that is formed as a result of decay of *Sphagnum* mosses that still contain reactive carbonyl groups able to react with amino acids. As a result, humic substances formed in these conditions are resistant against microbial activity, especially in anoxic conditions (Painter 1991).

The second aspect responsible for antimicrobial activity could be connected with body tanning. This phenomenon is also caused by the Maillard reaction, when collagen reacts with sphagnan, causing inking in these two elements. This process, in turn, causes inactivation of proteolytic enzymes that make nitrogen within animal bodies inaccessible to microorganisms. Some studies claim that the antimicrobial activity of peat in bogs occurs due to the low pH and anoxic conditions that are caused by humic substances, although this argument cannot be considered fully true, as there have been acidophilic, anaerobic bacteria found that could easily survive in this kind of environment (Painter 1991). Nevertheless, some scientists believe that the main reason for the antimicrobial activity of sphagnan is related to its acidic properties that lower the pH level of bog waters (Stalheim et al. 2009). There have also been some investigations of a possibility that when the *Sphagnum* moss cell wall dies, synthesis of hyaline is induced as a result of water consumption in the cell, and the concentration of galacturonic acid increases, resulting in the growth of the inner cell pH levels, eventually causing the antimicrobial effect of moss (Balance et al. 2012). However, it should be noted that this theory applies to acid-sensitive bacteria only (Painter 1991; Stalheim et al. 2009). Although sphagnan has antimicrobial activity, its practical usage in food preservation is uncertain, because, even though the outer layers of products are preserved, inner spoilage is possible for the reason that soluble proteins diffuse from muscles too quickly and neutralize the active carbonyl groups, thereby causing spoilage from inside of the food product (Borsheim et al. 2001; Stalheim et al. 2009). Research conducted in the year 2001 suggests that even though *Sphagnum* moss can be used as a preservation material, there are many aspects that need to be taken into consideration to make it efficient; for example, there cannot be any air bubbles embedded in the surface of the food product, and the "skin" of the product cannot be damaged and needs to be preserved in the moss as soon as possible after the harvesting or gathering of the product (Borsheim et al. 2001).

5 Bryophyte Biology

Bryophytes are especially remarkable for their tolerance to growth conditions. They can be found in both shady and sunny places, even if they usually prefer humid environments. Bryophytes are also adapted to variable temperatures and daylight regimes; therefore, they can be found from Sahara to Arctic regions. One of the most investigated properties of bryophytes is their high tolerance in respect to long drought periods and excessive wetness regimes in comparison with the higher plants (Pence et al. 2005). Their tolerance is one of the reasons why investigation

of bryophytes and their chemical composition has been rapidly growing in the past two decades. The bryophyte cell wall composition reflects changes that happened during plant evolution, at first, events during the colonization of land and vascularization of plants. Cell walls regulate cell expansion and have a crucial role in the growth and development of plants; they also actively participate in defense against microorganisms (Matsunaga et al. 2004). It is suggested that major changes in the cell wall polysaccharide composition happened during these processes. Whereas the cell walls of vascular plants are composed predominantly of cellulose, hemicellulose, and pectin, together with such functional molecules as glycoproteins, mineral substances, enzymes, and lignin, the cell wall composition of bryophytes is much simpler (Carpita and Gibeaut 1993). Changes in the cell wall composition could have been essential for the colonization of increasingly dry land environments. Evidently, not all plants participating in these evolutionary processes are preserved; still, the studies of bryophyte cell wall polysaccharides provide information of the chemistry of land plant evolution. A major step in the evolution of tracheophytes from bryophytes was the development of supporting and conducting tissues with lignified cell walls (Niklas 1997). The attempts to identify lignin as a significant constituent of cell walls in bryophytes failed (Bland et al. 1968; Maksimova et al. 2013), although controversies in this respect exist (Balance et al. 2012). Another key factor differentiating the cell wall structures of bryophytes and vascular plants is the near absence of pectic polysaccharide rhamnogalacturonan II in bryophytes (Matsunaga et al. 2004). However, partially methyl-esterified homogalacturonan has been found in specific water-conducting cells (hydroids) in bryophytes (Ligrone et al. 2002).

One of the key reasons for studying polysaccharides in bryophytes is directly related to their extremely high resistance against many environmental factors, such as drought, heat and cold, and also pollution (Goffinet and Shaw 2008). According to some scientists, the tolerance to freezing, desiccation, and drought is considered to be characteristic mainly of lower plants (Melvin et al. 2005). Many studies have demonstrated that bryophytes can be found under very differing environmental conditions, and they are highly tolerant to cold. A hypothesis has been advanced that the key factor influencing the tolerance of bryophytes is the presence of cell wall polysaccharides and cytoplasmic monosaccharides (Proctor 2000). In contrast with higher plants, in which the desiccation/freezing tolerance is usually restricted to seeds or pollen, the stress tolerance mechanisms of vegetative tissues in bryophytes are common (Oldenhof et al. 2006). Development of freezing tolerance is mostly associated with theanderose and stachyose, the synthesis of which is supposedly promoted by the stress hormone – abscisic acid. The effect of freezing tolerance is mainly due to the carbohydrate accumulation in protonema cells that are located in bryophyte cell walls (Nagao et al. 2006). The resistance of bryophytes to freezing is mainly based on alteration of hormone balance and regulation of osmotic pressure with the help of soluble sugars. Even though it has been shown that abscisic acid increases the synthesis of soluble sugars in cases of environmental stress, such as freezing or desiccation, this account is not true for all bryophyte species. In the case of *Physcomitrella patens*, *Riccia fluitans*, and *Pallavicinia lyellii*, the increase in abscisic acid synthesis does really increase their freezing and desiccation tolerance,

while in the case of *Marchantia polymorpha*, addition of this hormone does not affect its survival. There is an evidence of an abscisic acid-independent cold-signaling pathway that leads to stress-related gene expression that causes freezing tolerance (Minami et al. 2005). As shown in some studies, the stress tolerances in different bryophytes are differing mainly due to their growth environment, and, as there are many species, they are adapted to specific niches (Oliver et al. 2005). The best example is the bryophytes from Antarctic environments, which is able to survive freeze-thaw cycles, reacting by release of sucrose (Melick and Seppelt 2002). The bryophyte freezing stress tolerance and their cold acclimation are associated not only with the expression of stress-related genes but also with the specific composition of their polysaccharides and the release mechanisms of low-molecular carbohydrates during stress conditions (Minami et al. 2005). Thus, it may be concluded that there are more than one way of how bryophytes can cope with environmental stress (Pence et al. 2005; Nagao et al. 2006). In the case of desiccation tolerance, there is an extra water source in comparison with vascular plants. The latter have two water content components – symplast water and apoplast water – while in the case of bryophytes, there is also the third one that needs to be taken in consideration – external capillary water. The importance of water content components in bryophytes varies among species. For example, symplast water dominates in most thalloid liverworts; in contrast to that, in most mosses and leafy liverworts, as well as in nearly all highly desiccation-tolerant species, external capillary water is dominating. External capillary water is the most important aspect that is responsible for the high desiccation tolerance, as the water conduction is mostly external, and although the water content in many cases is near zero, the water potential keeps the inner processes of bryophyte cells intact. Thus, in difference from vascular plants, the main water movement in bryophytes takes place in the capillary spaces between leaves and other external spaces (Proctor 2000). In *Selaginella inaequalifolia*, in turn, the synthesis of trehalose has been related to their high desiccation tolerance (Irudayaray et al. 2010).

Hornworts are thought to be the most primitive land plants (Renzaglia et al. 2000), and, for example, in cell wall material from thalli of the hornwort *Anthoceros caucasicus*, substantial amounts of unusual disaccharide α-D-glucuronosyl(-1→3)L-galactose were isolated (Fig. 2). Notably, it was not detected in other terrestrial plants, including bryophytes, liverworts, and charophytes.

Such polysaccharides could possibly be able to act as mucilaginous substances that minimize desiccation of land plants. Mucilaginous substances are present in simpler bryophytes but absent in other evolutionary more advanced bryophytes. Thus, several unique polysaccharides present in an evolutionary isolated species of bryophytes (*Anthoceros*) represent the unique role of polysaccharides in the development of primary cell walls and the process that happened in plant phylogeny during evolution. There is a consensus among scientists that the presence of such carbohydrates and their composition change due to ecological pressures in bryophytes may have been preadaptive advantages that allowed the colonization of land (Renzaglia et al. 2000).

One of the puzzling issues of the bryophyte carbohydrate research is related to identification of their localization, especially in cell walls. This issue is especially

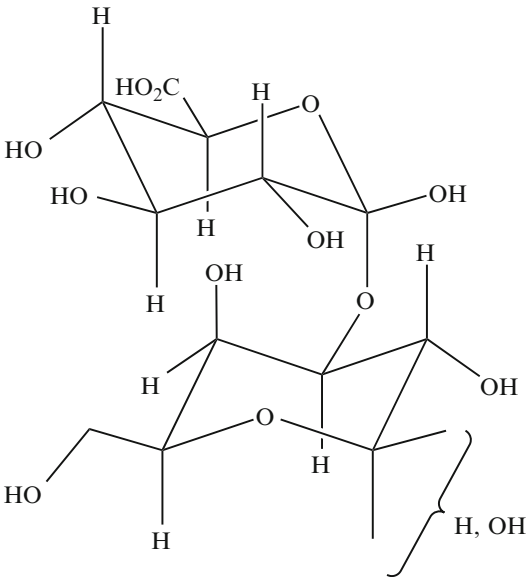


Fig. 2 Polysaccharide α-D-glucuronosyl(-1→3)L-galactose, found in hornworts only, can have an important role in their desiccation tolerance

important in respect to *Sphagnum* mosses, since these bryophytes historically have been intensively used as bandage materials to replace cotton dressings (Painter 2003; Hotson 1921). In recent studies, it has been suggested that *Sphagnum* cell walls are composed of a pectin-like polysaccharide – sphagnan (Stalheim et al. 2009; Painter 2003), which is abundant in hyaline cell walls, fibrils, papillae, chlorophyllous cell walls, and thickenings around hyaline cell pores (Balance et al. 2012). The character of localization of pectin was proved using staining with ruthenium red and by means of transmission electron microscopy of ultrathin cell walls labeled with poly-L-lysine. The presence of reactive pectin in cell walls, in the opinion of Balance et al. (2012), is the main factor influencing the possibilities of using *Sphagnum* mosses for food conservation, food tray pads, and other purposes. Thus, two major roles of polysaccharides could be identified in the constitution of bryophytes: (1) in pectin (or, probably, other similar polysaccharides in other bryophytes), as a major cell wall building material, and (2) functional polysaccharides as substances regulating the interactions of bryophytes with the surrounding environment. However, further research is needed to confirm the relevance of this hypothesis, also in respect to other bryophytes.

6 Biotechnological Approaches of Bryophyte Cultivation

Studies of bryophyte polysaccharides are hampered due to difficulties in acquiring large amounts of bryophyte biomass, considering their tiny size and mostly small populations. Often sites where bryophytes can be found (whether tropics or bogs in colder environments) cannot be easily accessed, and their cultivation cannot be

easily achieved. Nevertheless, the problem of obtaining significant quantities of bryophytes can be solved using in vitro cultures (Zinsmeister et al. 1991). Despite problems related to the identification of growth media, several cultivation experiments proved the efficiency of such an approach and feasibility to obtain cell biomass in sufficient quantities for obtaining bryophyte polysaccharides if they had a potential for application (Duckett et al. 2004). Cultivation approaches are most developed for *Sphagnum* mosses, considering their use for recultivation of bogs and as a media for plant growth (Batra et al. 2003). *Sphagnum* mosses also can be easily cultivated in greenhouses both on peat and water. In this kind of cultivation circumstances moss collection is easy and with high yields; nevertheless, there is still a need for separation of moss species as there is no possibilities to cultivate only one (Graudig et al. 2014).

7 Conclusion/Prospects

The main conclusion in respect to carbohydrates in bryophytes can be made not so much from the existing knowledge on the topic but rather from considering the roles of carbohydrates in the evolution of plant kingdom and bryophyte ecology. Carbohydrates in bryophytes illustrate a significant process in the evolution of chemical diversity and major compounds in the development from lower plants and microorganisms (bacteria, fungi) to carbohydrates in higher plants. Carbohydrates in bryophytes function as a structural material; they demonstrate the importance of cellulose and lignin as cell wall molecules (absent in bryophytes) in higher plants and show how evolution of chemical structures has supported biological evolution. The evolutionary approach also admits major gaps in the knowledge of carbohydrates in bryophytes; the number of studies is rather small, and the results obtained are controversial. For example, the issue concerning major structural carbohydrates in *Sphagnum* mosses (studied relatively much considering significance of this group of bryophytes) is still open. Furthermore, differences in carbohydrate composition among a challenging number of different bryophyte species are neglected: from ~25,000 species, carbohydrates are studied only in ~60–75 species, and the differences found are significant, leaving no doubts that examination of other species will result in finding new compounds and their combinations. Thus, the major conclusion in respect to studies of carbohydrates in bryophytes is that it is a challenging and prospective field of research, with a potential of providing not only considerable new knowledge about this group of substances, their functions, evolution of chemical compounds, and chemical diversity, but also new applications of bryophyte carbohydrates. The recent progress in bryophyte phytochemistry also supports these expectations.

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Microbial Production of Extracellular Polysaccharides from Biomass Sources

6

Emrah Özcan and Ebru Toksoy Öner

Contents

Abstract

The interest in bio-based polymers, especially extracellular polysaccharides (EPSs), has increased considerably in recent years due to their useful physicochemical and rheological properties and diverse functionality. Microbial polysaccharides have many commercial applications in different industrial sectors like chemical, food, petroleum, health, and bionanotechnology. Although microbial EPS production processes are regarded as environmentally friendly and in full compliance with the biorefinery concept, EPSs constitute only a minor fraction of the current polymer market due to their cost-intensive production and recovery. For that reason, much effort has been spent to the development of cost-effective production processes by using cheaper fermentation substrates

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such as low-cost biomass resources. These resources are generally either in liquid form like syrups, molasses, juices, cheese whey, and olive mill wastewater or solid-like lignocellulosic biomass and pomaces. In this chapter, after a brief description of microbial polysaccharides, submerged and solid-state fermentation processes utilizing cheap biomass resources are discussed with a special focus on the microbial production of EPSs with high market value.

Keywords
EPS • Microbial exopolysaccharides • Polysaccharides • Biomass resources • Fermentation

1 Introduction

Since the beginning of the twentieth century, bio-based technologies such as the production of biomolecules like enzymes, antibiotics, metabolites, and polymers have developed to a great extent. Currently, microorganisms are used for commercial production of several products such as pesticides, fertilizers, and feed additives in the agrochemical sector, biopharmaceuticals and therapeutics in the healthcare sector, and biopolymers and biofuels in the energy and environment sectors (Toksoy Oner 2013). Globally, the market for bio-based products has been increasing significantly such that from 2005 to 2010, it increased from 77 to 92 billion € and is expected to increase up to 228 and 515 billion € in 2015 and 2020, respectively (without biofuels and pharmaceuticals) (Fava et al. 2013).

Substrates used by microorganisms for the production of bio-based products are an important cost consideration. Hence, using cheaper substrates such as wastes or by-products of food processing or agro-industry instead of synthetic media leads to a significant decrease in the production costs. In the EU, the annual amount of waste or by-products of agro-industrial, organic household, yard/forestry, and food processing reaches up to 1,000, 200, 550, and 250 million tons, respectively. In 2008, the recovery rate of waste (excluding energy recovery) was 46 % (Fava et al. 2013). Hence the use of wastes or by-products as substrate for microbial production of biotechnologically important molecules like exopolysaccharides (EPSs) has a remarkable potential for not only the recovery of waste but also for reducing the production cost (Kaur et al. 2014).

EPSs are high-molecular-weight polymers that are composed of sugar residues and are secreted by microorganisms to the surrounding environment. Many microorganisms including many species of gram-positive and gram-negative bacteria, archaea, fungi and some algae are known to produce EPSs. These natural, nontoxic, and biodegradable polymers not only protect microorganisms against environmental extremes such as Antarctic ecosystems, saline lakes, geothermal springs, or deep-sea hydrothermal vents but also play important roles in various biological mechanisms such as immune response, adhesion, infection, and signal transduction (Jones et al. 2014; Poli et al. 2011; Kumar et al. 2007; Sutherland 1998) as well as in

biofilm formation, biofouling, and quorum sensing (Garg et al. 2014; Fazli et al. 2014). Some EPSs produced by microorganisms such as cellulose are also produced by higher-order plants. But, due to their slow production rate (3–6 months) as well as their dependence on seasonal conditions and high agricultural land, controlled microbial fermentations are preferred over plants for sustainable and economical production of EPSs at industrial scale (Toksoy Oner 2013).

Due to their useful physicochemical and rheological properties and diverse functionality, the EPSs have been recognized as new biomaterials and been found to have a wide range of applications. They can be used as thickeners, bioadhesives, stabilizers, probiotics, gelling agents, emulsifiers, biosorbents, and bioflocculants not only in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives but also in health and bionanotechnology sectors (Kreyenschulte et al. 2014; Toksoy Oner 2013; Donot et al. 2012; Freitas et al. 2011). However, despite these diverse industrial applications, EPSs still represent only a small fraction of the current polymer market, mostly due to the processes associated with their costly production and recovery (Kreyenschulte et al. 2014). Hence, significant effort has been devoted to the development of efficient downstream processing and cost-effective EPS production processes such as investigating the potential use of biomass resources as cheaper fermentation substrates.

In this chapter, after a brief description of microbial polysaccharides, submerged and solid-state fermentation processes utilizing cheap biomass resources are discussed with a special focus on the microbial production of EPSs with high market value.

2 Microbial Extracellular Polysaccharides

Polysaccharides are high-molecular-weight carbohydrate polymers of sugar residues that are linked by glycosidic bonds. While some polysaccharides like starch or glycogen serve for energy storage, others like chitin or cellulose function as structural support. In microorganisms, they are present either at the outer membrane as lipopolysaccharides (LPS) that mainly determine the immunogenic properties or secreted as capsular polysaccharides (CPSs) forming a discrete surface layer (capsule) associated with the cell surface or excreted as extracellular polysaccharides (EPSs) that are only loosely connected with the cell surface (Cuthbertson et al. 2009). While CPSs are responsible for pathogenicity like resistance to specific and nonspecific host immunity and adherence (Taylor and Roberts 2005), EPSs are related with adhesion, cell-to-cell interactions, biofilm formation (Mann and Wozniak 2012), and cell protection against environmental extremes (Kumar et al. 2007).

These microbial polysaccharides exhibit considerable diversity in their composition and structure. Based on their monomeric composition, they are generally classified as homopolysaccharides and heteropolysaccharides (Sutherland 1982).

Whereas homopolysaccharides consist of one type of monosaccharide connecting each other with either linear chains (pullulan, levan, bacterial cellulose, or curdlan) or ramified chains (dextran), heteropolysaccharides consist of two or more types of monosaccharides being usually present as multiple copies of oligosaccharides, containing three to eight residues (xanthan or gellan) (Purama et al. 2009; Sutherland 2007). Among the EPS producer microorganisms, bacteria are dominant for industrial or technical production. Some bacteria species such as *Xanthomonas*, *Leuconostoc*, *Sphingomonas*, and *Alcaligenes* which produce xanthan, dextran, gellan, and curdlan are the best known and most industrially used (Toksoy Oner 2013).

Dextran, curdlan, and cellulose are neutral bacterial glucans that are homopolysaccharides of glucose monomers. Dextran is produced by *Leuconostoc mesenteroides* cultures (Nasab et al. 2010a) together with levan (Siddiqui et al. 2014). Commercial applications for dextran are generally in the pharmaceutical industry, but new applications are being considered in the food and textile industries (Nasab et al. 2010a; Sarwat et al. 2008). Curdlan produced by the alkaline-tolerant mesophilic bacteria *Alcaligenes faecalis* (Matsushita 1990) can form aqueous suspensions which can form high-set gels upon heating. Curdlan can be used as a gelling agent in food and pharmaceutical industries. Curdlan is also produced by *Cellulomonas flavigena* as an extracellular storage polymer (Kenyon and Buller 2002). On the other hand, bacterial cellulose is produced by many species of bacteria, such as those in the genera *Gluconacetobacter*, *Agrobacterium*, *Aerobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, *Salmonella*, *Enterobacter*, *and Escherichia* and several species of cyanobacteria. Contrary to plant cellulose, bacterial cellulose has many desirable properties such as high purity (free of lignin and hemicelluloses), high crystallinity, high degree of polymerization, a nanostructured work, high wet tensile strength, high water holding capacity, and good biocompatibility (Hungund et al. 2013).

Pullulan from *Aureobasidium pullulans* (Özcan et al. 2014; Singh et al. 2008) and scleroglucan produced by *Sclerotium glucanicum* (Survase et al. 2007) are fungal glucans that are the most known uncharged homopolysaccharides having industrial production for a long time. Whereas the major market for pullulan is still in food industry, there are several reports for its potential applications in pharmaceutical, biomedical, and environmental remediation areas (Özcan et al. 2014). Due to its exceptionally high stability, the first application of scleroglucan was in the oil recovery; however, other applications in the cosmetic, pharmaceutical, and agriculture sectors have also been reported (Schmid et al. 2011; Survase et al. 2007).

Besides these glucan-type EPSs, levan is a linear, fructan-type homopolymer of fructose residues. It is a water-soluble, strongly adhesive, and film-forming EPS, which can be used not only in the food industry as an emulsifying, thickening, and encapsulating agent but also in medicine such as an immunomodulator and a blood plasma substitute (Kang et al. 2009; Silbir et al. 2014). Xanthan, which is after dextran, the second EPS to be approved by the FDA as a safe food ingredient, is an industrially important EPS produced by *Xanthomonas campesteris* through aerobic fermentation (Gunasekar et al. 2014; Palaniraj and Jayaraman 2011). It is a branched, anionic heteropolysaccharide composed of glucose, mannose, and

glucuronic acid monomers. Due to its exceptional rheological properties, xanthan has a considerable market. Another anionic EPS is the gellan gum that is a linear polymer of glucose, rhamnose, and glucuronic acid. Gellan produced by *Sphingomonas paucimobilis* (formerly *Pseudomonas elodea*) is gaining increasing attention due to its novel property of forming thermo-reversible gels and showing good stability over a wide pH range (3.5–8.0). Due to the diversity of its structure and properties, gellan gum has a great commercial potential for food, pharmaceuticals, and predominantly environmental bioremediation (Bajaj et al. 2007; Jin et al. 2003). Hyaluronan, composed of glucuronic acid and acetylglucosamine residues, is widely used in regenerative medicine and cosmetic applications due to its water binding and retention capacity and immune compatibility characteristics (Sutherland 2007). Another heteropolysaccharide is alginate that is an anionic polymer of glucuronic acid and mannuronic acid residues. Alginate produced by species of *Pseudomonas* and *Azotobacter* is widely used as a thickening, stabilizing, and gelifying agent in food, textile, paper, and pharmaceutical industries (Hay et al. 2014, 2009; Gaona et al. 2004).

3 Microbial EPS Production

Microbial EPS production is usually not confined to just one type of EPS as product but rather a mixture of different polymers, each being synthesized by a certain gene cluster. For example, *Pseudomonas aeruginosa* has the genetic capacity to produce three different EPSs, namely, Pel, Psl, and alginate, which are synthesized via different mechanisms (Hay et al. 2014; Franklin et al. 2011). Similarly, *L. mesenteroides* cultures can produce both dextran and levan (Siddiqui et al. 2014). Generally, the availability of the precursors encoded by the related genes has a high impact on the yield and structure of the EPS excreted by the cell (Sutherland 2007). The synthesis of homopolysaccharides is carried out in the extracellular environment through the action of specifically secreted glycoside hydrolase (sucrase) enzymes that act on sucrose and catalyze the transglycosylation reactions forming the polymer chain (Rehm 2009; van Hijum et al. 2006). On the other hand, biosynthetic pathways of heteropolysaccharides are more complex and involve five distinct steps, namely, the uptake of sugar subunits and their activation with a high-energy bond through their conversion into sugar nucleotides, assembly of the repeating monosaccharide unit on an isoprenoid lipid carrier by sequential transfer of monosaccharides from sugar nucleotides by glycosyltransferases, addition of any acyl groups, polymerization of the repeating unit, and secretion of the polysaccharide from the cell membrane into the extracellular environment (Sutherland 2007).

Recent studies on microbial EPS production use systems-based approaches to elucidate the associated biosynthesis mechanisms; to modify physicochemical and/or rheological properties of the biopolymer by changing its composition, length, or degree of branching; and also to improve the microbial productivity via strain improvement strategies. For such an approach, whole genome sequencing (WGS) projects employing next-generation sequencing (NGS) technologies play a

central role by enabling high-throughput genomic data at very high speed with a relatively low cost. Genome data was used in comparative genomic studies of EPS biosynthesis by *Bifidobacterium* (Hidalgo-Cantabrana et al. 2013), *Crocosphaera watsonii* (Bench et al. 2013), and *Salipiger mucosus* DSM 16094^T (Riedel et al. 2014) as well as in functional genomic studies for the xanthan production by *Xanthomonas campestris* (Vorhölter et al. 2008), EPS biosynthesis by *P. aeruginosa* PA01 (Franklin et al. 2011; Hay et al. 2010; Rehm 2005), alginate biosynthesis by *Azotobacter vinelandii* (Kumar et al. 2007), and levan production by the halophilic strain *Halomonas smyrnensis* AAD6^T (Ates et al. 2011, 2013).

4 Microbial Production Processes

Microbial EPS production is significantly affected by fermentation conditions such as temperature, pH, oxygen concentration, bioreactor configuration, and culture medium. Furthermore, the chemical structure of EPS such as molecular weight, monomer composition, and physicochemical and rheological properties of the final product could also change with the type and age of strain (Özcan et al. 2014). Generally, typical microbial EPS fermentations start with the growth phase followed by the production phase (Toksoy Oner 2013). During the cultivations, severe changes in rheological properties of the microbial culture such as highly viscous and non-Newtonian broth may result in serious problems of mixing, heat transfer, oxygen supply, and also instabilities in the quality of the end product (Seviour et al. 2011). Such challenges are encountered in the microbial production of pullulan (Cheng et al. 2011) and xanthan (Palaniraj and Jayaraman 2011) but not in the production of low-viscosity polymers such as levan (Küçükaşık et al. 2011) or in high-temperature processes where thermophiles are utilized as microbial producers (Nicolaus et al. 2010).

Conventional modes of operations for the fermentation processes are batch, fed batch, and continuous. In these operations, different fermenter configurations can be used such as submerged fermentation (SmF) bioreactors including pneumatically or mechanically agitated types as well as solid-state fermentation (SSF) bioreactors. The mode of operation and the fermenter design depend on the microbial system used, and since the production is highly subject to change with the physiological and biochemical requirements of the microbial strain, each process requires a specific design by avoiding generalities (Nicolaus et al. 2010). Hence, to realize the industrial production of EPSs with the desired specifications and standardization, the parameters affecting the process should be well defined via effective optimization methods such as response surface methodology (Özcan et al. 2014).

5 Biomass Resources

Almost 30 % of the cost for a microbial fermentation can be represented by fermentation medium. Using complex media is not attractive due to its high content of expensive nutrients such as yeast extract, peptone, and salts. In order to reach

high production titers at reasonable costs, the fermentation medium should be carefully designed to make the end product compatible with its synthetic petrochemical counterpart. Until the 1990s, studies were concentrated on the recovery and chemical characterization of pure EPSs, and consequently, to avoid impurities, fermentations were preferentially performed under defined culture conditions. However, recently, studies are mainly concerned about the economical aspects of microbial production, and hence a growing number of studies focus on maximizing the cost-effectiveness of the process by replacing synthetic or complex media with appropriate biomass resources as cheaper alternatives (Kaur et al. 2014; Toksoy Oner 2013).

Biomass resources are generally of two physical states, namely, liquid resources like syrups, molasses, juices, cheese whey, and olive mill wastewater and solid resources like lignocellulosic biomass and pomaces. Whereas EPS production from liquid biomass resources is realized through submerged fermentation processes, solid-state fermentation is preferred for the latter state. Furthermore some physical and chemical pretreatment methods can also be applied to biomass resources for removal of heavy metals, colored substances, and other impurities that would interfere with the fermentation or subsequent downstream processing. In Table 1, various biomass resources have been listed for some microbial EPS producers together with the EPS yields obtained after a certain fermentation period.

6 Submerged Fermentation Processes

Submerged fermentation (SmF) processes define fermentations carried out in liquid media. The scale of the process may vary from small scale like shake flasks up to large scale like fermenters. Fermenters designed for SmF can be classified according to their agitation so that they are either pneumatically agitated such as bubble column and airlift bioreactors or mechanically agitated such as stirred tank reactors (Doran 1995). Syrups, molasses, cheese whey, and olive mill wastewater are common biomass resources for microbial production of EPS via SmF.

6.1 Syrups and Molasses

Syrups and molasses have been used as substrates for fermentative production of commercial polysaccharides such as xanthan (Kalogiannis et al. 2003), pullulan (Israilides et al. 1998; Roukas 1998; Lazaridou et al. 2002; Göksungur et al. 2004), dextran (Vedyashkina et al. 2005), levan (Küçükaşık et al. 2011; Han and Watson 1992; Oliveira et al. 2007), scleroglucan (Survase et al. 2007), and gellan (Banik et al. 2007) due to their many advantages like high sucrose and other nutrient contents, low cost, and easy availability. The chemical composition of syrups and molasses includes high ion concentrations such as K^+, Na^+, Fe^{2+}, and Zn^{2+} which could be additional stress factors that trigger the formation of the EPS (Abdel-Aziz et al. 2012a). Not only crude form of syrups and molasses but also pretreated form

Table 1 Biomass resources and applied fermentation types for some microbial EPSs

EPS	Microorganism	Biomass resource	Fermentation type	Yield (time)	Reference
Curdlan	*Agrobacterium* sp. ATCC 31749	Condensed corn solubles	SmF	7.72 gL^{-1} (120 h)	West and Nemmers 2008
Dextran	*L. mesenteroides* NRRL B512	Carob extract	SmF	8.56 gL^{-1} (12 h)	Santos et al. 2005
Dextran	*L. mesenteroides* NRRL B512	Carob extract and cheese whey	SmF	7.23 gL^{-1} (12 h)	Santos et al. 2005
Dextran	*L. mesenteroides* V-2317D	Sugar beet molasses	SmF	50 gL^{-1} (9 days)	Vedyashkina et al. 2005
Dextran	*L. Mesenteroides* NRRL B512	Combination of molasses and cheese whey	SmF	9.51 gL^{-1} (48 h)	Nasab et al. 2010a
Gellan	*S. paucimobilis* ATCC-31461	Sugar beet molasses	SmF	13.81 gL^{-1} (48 h)	Banik et al. 2007
Gellan	*S. paucimobilis* NK2000	Soybean pomace	SmF	7.33 gL^{-1} (3 days)	Jin et al. 2003
Levan	*Halomonas* sp. AAD6	Starch molasses	SmF	12.4 gL^{-1} (210 h)	Küçükaşık et al. 2011
Levan	*Paenibacillus polymyxa* NRRL B-18475	Sugar beet molasses	SmF	38.0 gL^{-1} (5 days)	Han and Watson 1992
Levan	*P. polymyxa* NRRL B-18475	Sugarcane syrup	SmF	19.6 gL^{-1} (5 days)	Han and Watson 1992
Levan	*Zymomonas mobilis* ATCC 31821	Sugarcane molasses	SmF	2.53 gL^{-1} (24 h)	Oliveira et al. 2007
Levan	*Z. mobilis* ATCC 31821	Sugarcane syrup	SmF	15.5 gL^{-1} (24 h)	Oliveira et al. 2007
Pullulan	*Aureobasidium* sp. NRRLY	Condensed corn solubles	SmF	4.5 gL^{-1} (9 days)	Leathers and Gupta 1994
Pullulan	*A. pullulans* SU-M18	Carob extracts	SmF	6.5 gL^{-1} (3 days)	Roukas and Biliaderis 1995
Pullulan	*A. pullulans*	Olive mill wastewater	SmF	8 gL^{-1}	Cormenzana et al. 1995
Pullulan	*A. pullulans* NRRLY-6220	Olive mill wastewater	SmF	10.7 gL^{-1} (7 days)	Israilides et al. 1998

Pullulan	*A. pullulans* NRRLY-6220	Grape pomace	SmF	22.3 gL^{-1} (7 days)	Israilides et al. 1998
Pullulan	*A. pullulans* NRRLY-6220	Sugar beet molasses	SmF	6.0 gL^{-1} (7 days)	Israilides et al. 1998
Pullulan	*A. pullulans*	Sugar beet molasses	SmF	32.0 gL^{-1}	Roukas 1998
Pullulan	*A. pullulans* P 56	Sugar beet molasses	SmF	24 gL^{-1} (144 h)	Lazaridou et al. 2002
Pullulan	*A. pullulans* P 56	Sugar beet molasses	SmF	35 gL^{-1} (96 h)	Göksungur et al. 2004
Pullulan	*A. pullulans* RBF 4A3	Corn steep liquor	SmF	77.92 gL^{-1} (96 h)	Sharma et al. 2013
Pullulan	*A. pullulans* RBF 4A3	De-oiled jatropha seed cake	SmF	83.98 gL^{-1} (120 h)	Choudhury et al. 2012
Scleroglucan	*Sclerotium rolfsii* MTCC 2156	Sugarcane juice	SmF	23.87 gL^{-1} (72 h)	Survase et al. 2007
Scleroglucan	*S. rolfsii* MTCC 2156	Sugarcane molasses	SmF	19.21 gL^{-1} (72 h)	Survase et al. 2007
Scleroglucan	*S. rolfsii* MTCC 2156	Coconut water	SmF	12.58 gL^{-1} (72 h)	Survase et al. 2007
Scleroglucan	*S. rolfsii* MT-6	Waste loquat kernel	SmF	12.08 gL^{-1} (72 h)	Taşkın et al. 2010
Scleroglucan	*S. glucanicum* NRRL 3006	Condensed corn solubles	SmF	14.8 gL^{-1} (144 h)	Fosmer and Gibbons 2011
Xanthan	*Xanthomonas campestris*	Carob extracts	SmF	0.126 gL^{-1} h^{-1}	Roseiro et al. 1992
Xanthan	*X. campestris* PD 656	Apple pomace	SSF	52.1 gL^{-1} (6 days)	Stredansky and Conti 1999
Xanthan	*X. campestris*	Grape pomace	SSF	10 gL^{-1} (6 days)	Stredansky and Conti 1999
Xanthan	*X. campestris* PD 656	Tangerine peels	SSF	32.9 gL^{-1} (6 days)	Stredansky and Conti 1999
Xanthan	*X. campestris* NRRL-B-1459	Sugar beet pulp	SmF	1.19 gL^{-1} (4 days)	Yoo and Harcum 1999

(*continued*)

Table 1 (continued)

EPS	Microorganism	Biomass resource	Fermentation type	Yield (time)	Reference
Xanthan	*X. campestris* NRRL-B-1459	Olive mill wastewater	SmF	4 gL^{-1} (5 days)	Lopez et al. 2001a
Xanthan	*X. campestris* T646	Olive mill wastewater	SmF	7.7 gL^{-1} (5 days)	Lopez et al. 2001b
Xanthan	*X. campestris* EBK-4	Ram horn hydrolysate	SmF	25.6 gL^{-1} (48 h)	Kurbanoglu and Kurbanoglu 2007
Xanthan	*X. campestris* 1182	Cheese whey	SmF	26.35 gL^{-1} (72 h)	Silva et al. 2009
Xanthan	*X. campestris* PTCC 1473	Date syrup	SmF	8.9 gL^{-1} (96 h)	Nasab et al. 2009
Xanthan	*X. campestris* NRRL-B-1459	Sugar beet molasses	SmF	9.02 gL^{-1} (120 h)	Nasab et al. 2010b
Xanthan	*Xanthomonas* sp.	Potato peel	SSF	2.9 g/50 g peel (6 days)	Vidhyalakshmi et al. 2012
Xanthan	*X. campestris* NCIM 2954	Tapioca pulp	SmF	7.1 gL^{-1} (72 h)	Gunasekar et al. 2014
Bacterial cellulose	*Gluconacetobacter hansenii* CGMCC 3917	Waste beer yeast	SmF	7.02 gL^{-1} (10 days)	Lin et al. 2014
Bacterial cellulose	*Gluconacetobacter xylinus* PTCC 1734	Date syrup	SmF	43.5 gL^{-1} (336 h)	Nasab and Yousefi 2011
Bacterial cellulose	*Gluconacetobacter persimmonis*	Watermelon	SmF	5.98 gL^{-1} (14 days)	Hungund et al. 2013
Bacterial cellulose	*G. persimmonis*	Orange juice	SmF	6.18 gL^{-1} (14 days)	Hungund et al. 2013
Bacterial cellulose	*G. Persimmonis*	Muskmelon	SmF	8.08 gL^{-1} (14 days)	Hungund et al. 2013
β-Glucan	*Botryosphaeria rhodina*	Olive mill wastewater	SmF	17.2 gL^{-1} (120 h)	Crognale et al. 2003
EPS	*Paenibacillus jamilae* CECT 5266	Olive mill wastewater	SmF	2.5 gL^{-1} (100 h)	Morillo et al. 2009

EPS	*P. jamilae* CP-38	Olive mill wastewater	SmF	5 gL^{-1} (72 h)	Aguilera et al. 2008
EPS	*Halomonas* sp. AAD6	Sugar beet pulp	SSF	2.22 gL^{-1} (3 days)	Söğütçü et al. 2011
EPS	*Bacillus subtilis*	Sugarcane molasses	SmF	4.86 gL^{-1} (48 h)	Razack et al. 2013
EPS	*P. fluorescens*	Sugarcane molasses	SmF	2.9 gL^{-1} (48 h)	Sirajunnisa et al. 2012
EPS	*Streptococcus Thermophilus* BN1	Skimmed milk	SmF	548 mgL^{-1} (17 h)	Rabha et al. 2012
EPS	*S. Thermophilus* BN1	Whole milk	SmF	325 mgL^{-1} (17 h)	Rabha et al. 2012
EPS	*S. Thermophilus* BN1	Cheese whey	SmF	375 mgL^{-1} (17 h)	Rabha et al. 2012
EPS	*Paenibacillus jamilae* CECT 5266	Two-phase olive mill waste	SmF	2 gL^{-1} (5 days)	Morillo et al. 2006
EPS	*Grifola frondosa* MBFBL 662 and MBFBL 21	Oak sawdust	SSF	3.5 gL^{-1} (45 days)	Mikiashvili et al. 2011
EPS	*Bacillus licheniformis* UD061	Squid processing by-product and maize cob	SSF	14.68 mg/gds	Fang et al. 2013
EPS	*Pleurotus eryngii*	Mushroom hydrolysate powder	SmF and SSF	312 mgL^{-1} (8 days)	Chen et al. 2013
EPS	*Morchella esculenta*	Detoxified loquat kernel extract	SSF	5.2 gL^{-1} (3 days)	Taşkın et al. 2011
EPS	*M. esculenta*	Loquat kernel extract	SSF	4.1 gL^{-1} (3 days)	Taşkın et al. 2011
EPS	*M. esculenta*	Chicken feather hydrolysate	SmF	4.8 gL^{-1}	Taşkın et al. 2012

of syrups and molasses can be used as substrate. Some pretreatment methods for syrups and molasses are acid treatment, pH adjustment (Roukas 1998; Küçükaşık et al. 2011), activated carbon treatment (Lazaridou et al. 2002; Göksungur et al. 2004), ion exchange chromatography (Kalogiannis et al. 2003), and centrifugation followed by filtration (Oliveira et al. 2007).

X. campestris was used for producing xanthan gum using date syrup, prepared from low-quality dates, as a substrate. Fermentation was carried out with date syrup and sucrose syrup. The results showed that EPS concentration increased with an increase in fermentation time with a maximum yield of 8.9 gL^{-1} after 96 h which was much higher than that of the sucrose-containing medium (0.18 g/100 mL) (Nasab et al. 2009). The same group used date syrup, for the production of bacterial cellulose using *Gluconacetobacter xylinus*. Static batch fermentation for the purpose of cellulose production by *G. Xylinus* PTCC 1734 was studied using date syrup and sucrose solution as fermentation media. Results showed that maximum yields of bacterial cellulose after 336 h fermentation were 4.35 and 1.69 g/100 ml of date syrup and sucrose media, respectively (Nasab and Yousefi 2011). Fruit juices are also a good alternative as biomass resource like syrups. For instance, bacterial cellulose was produced by *Gluconacetobacter persimmonis* using some fruit juices as cheaper carbon sources (Hungund et al. 2013). Hungund et al. used various fruit juices including pineapple, pomegranate, muskmelon, water melon, tomato, and orange and also molasses, sugarcane juice, coconut water, and coconut milk as alternative carbon sources for bacterial cellulose production. Out of which, muskmelon juice gave the highest cellulose yield of 8.08 gL^{-1}. Survase et al. (2007) used various dilutions of coconut water, sugarcane molasses, and sugarcane juice which were not subjected to any pretreatment methods before their use for the scleroglucan production by filamentous fungi *S. rolfsii* MTCC2156, and the highest yields (23.87 gL^{-1} in 72 h) were observed using sugarcane juice. Coconut water and sugarcane juice were also used for EPS production by *Lactobacillus confusus* cultures (Seesuriyachan et al. 2011).

Muhammadi (2014) used different carbon sources such as glucose, fructose, sucrose, sugar beet, and sugarcane molasses to produce EPS by *Bacillus* strain CMG1403. The study showed that under optimum culture conditions, sugar beet and sugarcane molasses could be superior and efficient alternatives to synthetic carbon sources providing way for an economical production of EPS. Razack et al. (2013) replaced sucrose with sugarcane molasses in the optimized medium, for an enhanced production of EPS from a soil isolate, *Bacillus subtilis*. Sugarcane molasses at a concentration of 2 % gave higher EPS yield (4.86 gL^{-1}) than that obtained in medium with sucrose (2.98 g EPS/L). Sugarcane molasses was also used to produce EPS from *P. fluorescens*. Sucrose and sugarcane molasses as the carbon substrates at different concentrations (1–7 %) and different incubation times were investigated in this study. Maximum production was obtained in the medium containing 5 % sugarcane molasses and was found to be 2,843 mgL^{-1} at 48 h after which the production decreased. The EPS production using sugarcane molasses gave comparatively a higher yield than sucrose, which could be commercialized for

a cost-effective production of these viscous to plastic polymers (Sirajunnisa et al. 2012). Banik et al. (2007) used response surface methodology to optimize the production of gellan gum by *S. paucimobilis* ATCC-31461 using crude sugarcane molasses and reported a maximum yield of 13.81 gL^{-1} gellan. Abdel-Aziz et al. (2012b) stated that EPS, synthesized by the fungus *Mucor rouxii*, was found to play an important role for the protection of cells against abiotic stress such as extreme pH values or elevated temperature. An acidic pH-shock was found to be the strongest stressor for synthesizing the EPS exploiting beet molasses as an inexpensive carbon source. Beet molasses was also used as a carbon source in other studies to produce xanthan by *X. campesteris* NRRL-B-1459 (Nasab et al. 2010b) and to produce dextran by *L. mesenteroides* (Vedyashkina et al. 2005).

6.2 Cheese Whey

Whey is the major by-product of the dairy products, especially cheese, industry. The nutrient composition of whey is based on the nutrient composition of milk from which it is derived, and it is affected by many factors including how the milk was processed. The major component of whey is lactose which is about 70 % of the total solids of whey. Whey has a rich pool of nutrients and growth factors that have the potential to stimulate the growth of microorganisms. On the other hand, the suitability of whey for EPS production highly depends on the ability of the microorganism to utilize lactose (Toksoy Oner 2013).

Fialho et al. (1999) used the media containing lactose, glucose, and sweet cheese whey as substrates for the production of gellan by *S. paucimobilis* ATCC 31461. The strain was known to grow on lactose and to produce highly viscous gellan directly from lactose (Pollock 1993). Silva et al. (2009) produced xanthan by two strains of *X. campesteris* using cheese whey as carbon source. Although both strains reached comparable yields, the polymers were found to differ in their chemical characteristics such as chemical composition and ionic strength. Nasab et al. (2010a) used a combination of molasses and cheese whey for the production of dextran by *L. mesenteroides*. Results showed that maximum dextran yield was achieved in combination of molasses-whey 10 % (M-W 10 %) and no dextran was produced using only whey. The report showed that the combination of nutrients and minerals in molasses and cheese increased the EPS yield.

Not only crude whey but also partially hydrolyzed whey by protease/peptidase complex can be used for the production of EPS. Fermentation of the hydrolyzed whey using *Lactobacillus delbrueckii* ssp. *bulgaricus* RR (RR, an EPS-producing bacterium) resulted higher EPS yield than fermentation of the unhydrolyzed whey (Briczinski and Roberts 2002). Another pretreatment method for cheese whey is precipitation of protein. Rabha et al. (2012) partially precipitated the residues of milk proteins present in the whey samples in their study which is related to EPS production by *Streptococcus thermophilus* BN1 using skimmed milk, whole milk, and cheese whey as cheap culture media.

6.3 Olive Mill Wastewater

Olive mill wastewater (OMW) is the by-product of the olive oil industry. OMW is a dark-colored juice that consists of a mixture of water from the olive, machinery cooling waters, fruit washings, and remainder of the fruit (Toksoy Oner 2013). About 15 % of OMW is organic material that is composed of carbohydrates, proteins, and lipids as well as a number of other organic compounds including monoaromatic and polyaromatic molecules (Aguilera et al. 2008). OMW also comprises toxic ingredients being mainly derived from its extremely high organic load and the presence of recalcitrant organic compounds such as polyphenols with strong antimicrobial properties. Valorization of OMW produced by the olive oil industry has long been an environmental concern in the Mediterranean countries (Morillo et al. 2009). Among several conventional technological treatment methods applied, biovalorization of OMW to value-added chemicals is considered as the most cost-effective and environmentally compatible alternative (Mantzavinos and Kalogerakis 2005). OMW has been used as a suitable substrate for the production of EPS such as pullulan (Ramos-Cormenzana et al. 1995) and xanthan (Lopez and Ramos-Cormenzana 1996) due to its composition with high carbon-to-nitrogen ratio and other valuable nutrients.

Some pretreatment methods which can be applied before microbial fermentation to reduce the inhibitory effect of phenols in OMW, are filtration, clarification by centrifugation, dilution with water, and saline neutralization (Lopez et al. 2001a; Crognale et al. 2003). Lopez et al. (2001b) used OMW as a substrate to the production of xanthan using four *X. campesteris* strains. Differences among strains were found in the range of tolerance to OMW concentration and the xanthan amount obtained. *X. campestris* NRRL-B-1459 S4LII was chosen by its capability for xanthan production from 50 % to 60 % OMW as the sole nutrient source.

Morillo et al. (2006) investigated the use of a two-phase olive mill waste (TPOMW) which is a thick sludge that contains water and pieces of pit and pulp of the olive fruit as substrate for the production of EPS by *Paenibacillus jamilae* which is able to grow and produce EPS in aqueous extracts of TPOMW as a unique source of carbon. Maximal polymer yield in 100-mL batch-culture experiments (2 gL^{-1}) was obtained in cultures prepared with an aqueous extract of 20 % TPOMW (w/v). The effects of the addition of inorganic nutrients (nitrate, phosphate, and other inorganic nutrients) were also investigated, but the nutrient supplementation did not increase yield.

In addition to EPS production, some microorganisms can be used for bioremediation of OMW. The *Paenibacillus* genus having the high phenol biodegradation ability was not only proposed for the production of a metal-binding EPS that could be used as a biofilter but also for the bioremediation of OMW (Aguilera et al. 2008).

The high amount of phenols which negatively affects the microbial fermentation is the main constraint associated with the use of OMW, and dilution of OMW can be required. On the other hand, dilution of OMW limits the concentration of the used waste as culture medium (Aguilera et al. 2008). For instance, undiluted OMW was found to be a poor substrate for pullulan production by *A. pullulans* (Israilides et al. 1998).

6.4 Others

Carob which is grown on carob tree (*Ceratonia siliqua* L.) in the Mediterranean region has recently found its place in the food industry as a biomass substrate due to its very high sugar content (Turhan et al. 2010). Pretreated carob extracts with 25 gL^{-1} initial sugar content were used for pullulan production by *A. pullulans* SU-M18, and a pullulan productivity of 2.16 gL^{-1}/day was reached (Roukas and Biliaderis 1995). Carob extract can also be used for dextran production. Santos et al. used carob pod residues pretreated by milling and extracting by use of an acetate buffer to get its sugar content. Then carob extracts were used for the production of dextran by *L. mesenteroides* NRRL B512. 8.56 gL^{-1} dextran was produced within 12 h of the fermentation period in this study (Santos et al. 2005).

Waste beer yeast (WBY) being the second major by-product from the brewing industry can be used for bacterial cellulose production by *Gluconacetobacter hansenii* CGMCC 3917. Lin et al. (2014) used pretreated WBY as the only nutrient source for bacterial cellulose production. WBY hydrolysates treated by ultrasonication gave the highest bacterial cellulose yield (7.02 gL^{-1}), almost six times as that from untreated WBY (1.21 gL^{-1}). Tapioca pulp was used as a carbon source for xanthan production by *X. campestris* NCIM 2954. Maximum 7.1 gL^{-1} xanthan was produced using sulfuric acid-pretreated tapioca pulp (Gunasekar et al. 2014). Condensed corn solubles (CCS) containing changing levels of carbohydrates, proteins, vitamins, and nutrients are a by-product of the bioethanol industry (Smith et al. 2008). The use of diluted CCS was reported for the production of scleroglucan by *S. glucanicum* (Fosmer and Gibbons 2011; Fosmer et al. 2010), the poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Rhodospirillum rubrum* (Smith et al. 2008), pullulan by *Aureobasidium* sp. strain NRRL Y-12974 (Leathers and Gupta 1994), and curdlan by *Agrobacterium* sp. ATCC 31749 (West and Nemmers 2008). Mushroom hydrolysate powder (MHP) was used as a nitrogen source for mycelial biomass and EPS productions by *Pleurotus eryngii*. MHP gave higher mycelial biomass growth rate and EPS yield than those of the yeast extract (Chen et al. 2013). Waste loquat kernel is another potential biomass resource for EPS production due to its high protein and carbohydrate content Taşkın et al. 2010).

Pullulan is one of the most producing EPSs using various ranges of biomass resources. Abdel Hafez et al. (2007) produced pullulan by *A. pullulans* ATCC 42023 using molasses, cellulosic wastes, potato starchy waste, glucose syrup, sweet whey, and corn steep liquor. Maximum pullulan concentration (65.3 gL^{-1}) was obtained after 5 days of fermentation at 28 °C using 7 % corn steep liquor as the sole nitrogen source in a medium containing 20 % sucrose. Sharma et al. (2013) also used rice bran oil cake, soya bean oil cake, cotton seed oil cake, and mustard seed oil cake corn steep liquor (CSL) as agro-industrial nitrogen source for the production of pullulan by *A. pullulans* RBF 4A3. CSL was found to be the best production of 77.92 gL^{-1} pullulan under un-optimized conditions. Choudhury et al. (2012) produced pullulan using another nitrogen source, namely, de-oiled jatropha seed cake (DOJSC). Under optimized condition, 8 % DOJSC with 15 % dextrose gave 83.98 gL^{-1} of pullulan which is comparatively a higher yield than current reports.

Some animal wastes such as ram horn hydrolysates and chicken feather have been also reported to be a substrate for EPS production. Taşkın et al. (2012) investigated the usability of chicken feather hydrolysate (chicken feather peptone (CFP)) as substrate for mycelial biomass and EPS production from edible mushroom *Morchella esculenta*. Maximum 15.9 gL^{-1} biomass and 4.8 gL^{-1} EPS were obtained using CFP in this study. Kurbanoglu et al. (Kurbanoglu and Kurbanoglu 2007) used ram horn hydrolysates for xanthan production by *X. campestris* EBK-4 due to their high amino acid and mineral content.

7 Solid-State Fermentation Processes

Solid-state fermentation (SSF) defines bioprocess technologies including the growth of microorganisms on moist solid particles (Aydınoğlu and Sargın 2013). Over the last two decades, SSF has gained significant attention for the development of industrial bioprocesses, particularly due to lower energy requirement associated with higher product yields and cheap and eco-friendly process conditions (Thomas et al. 2013). The substrates used in SSF processes are often the product, by-product, or waste of agro-industrial, forestry, or food processing (Mitchell and Krieger 2006). SSF has been recently explored for the production of biopolymers such as EPS of which yields are comparable to those obtained from conventional submerged cultivation (Thomas et al. 2013). Agro-industrial wastes like lignocellulosic biomass and pomaces are common biomass resource used in SSF for production of EPS.

7.1 Lignocellulosic Biomass

Lignocellulosic biomass is a cheap and abundant substrate for EPS production in SSF, especially for microbial systems with hydrolytic capability via endoglucanases or cellobiose. Otherwise, it is utilized to a limited extend during the fermentation and hence requires pretreatments beforehand (Toksoy Oner 2013). Mikiashvili et al. (2011) investigated 14 strains of *Grifola frondosa* for lignin degradation, ligninolytic enzyme activities, protein accumulation, and EPS production. Experiments were carried out in SSF using oak sawdust as substrate. Among 14 strains, the strains MBFBL 21, MBFBL 662, and MBFBL 638 appeared to be good producers of EPS (3.5, 3.5, and 3.2 gL^{-1}, respectively). *Lentinus squarrosulus* Mont., a high-temperature-tolerant white rot fungus, is attracting attention due to its rapid mycelia growth and lignocellulolytic enzyme activity. Isikhuemhen et al. used cornstalks as carbon source to evaluate lignocellulolytic enzyme activity and EPS production of *L. squarrosulus* MBFBL 201 in SSF. The results showed that *L. squarrosulus* was able to degrade cornstalks significantly and maximum 5.13 gL^{-1} EPS could be recovered from the fermentation media (Isikhuemhen et al. 2012). Chowdhury et al. (2012) used lignocellulosic fibers of jute with 58–63 % cellulose content as a carbon source for EPS production by *Bacillus*

megaterium RB-05 cells with known cellulase activity in SSF. Considerable cellulase activity and maximum polymer yield of 0.297 g g^{-1} substrate were found after 72 h fermentation in this study.

7.2 Pomace

Pomace is also a common substrate for microorganism fermentation in SSF due to its content of pectin, crude fiber, and minerals such as K, Mg, Fe, and Mn (Shalini and Gupta 2010). Globally, about 10 million tons of grape pomace (seeds, skin, and stem) and 1 million tons of apple pomace are produced each year (Toksoy Oner 2013; Shalini and Gupta 2010). Stredansky and Conti (1999) produced xanthan by *X. campesteris* strains in SSF using agro-industry wastes or by-products, including spent malt grains, apple pomace, grape pomace, and citrus peels as solid substrate. Yields of the xanthan ranging from 32.9 to 57.1 gL^{-1} revealed a composition consistent with those of commercial xanthan analyzed by NMR spectroscopy. Xanthan was also produced by *X. campesteris* strains in SSF using potato peel as substrate in another study (Vidhyalakshmi et al. 2012). The ammonium nitrate is a nitrogen source for the production of gellan gum by *S. paucimobilis* NK2000. The production of gellan gum by *S. paucimobilis* NK2000 significantly increased using soybean pomace as a nitrogen source instead of ammonium nitrate (Jin et al. 2003). Using soybean pomace as nitrogen source also increased pullulan production by *A. pullulans* HP-2001 compared to using yeast extract as a nitrogen source (Seo et al. 2004).

7.3 Others

Moussa and Khalil (2012) used SSF for the production of dextran by *Saccharomyces cerevisiae* using date seeds. Different concentrations of date seeds were investigated, and the highest dextran production was achieved at 6 g/flask. The purified dextran was comparable to commercial ones. Seesuriyachan et al. (2010) compared the production of EPS by a lactic acid bacteria, *Lactobacillus confuses*, in SSF and SmF using coconut water and sugarcane juice as renewable wastes with agar medium. High concentrations of EPS (62 and 18 gL^{-1} of sugarcane juice and coconut water with agar, respectively) were obtained in SSF. Fang et al. (2013) used response surface methodology to optimize physical and nutritional variables for the production of antioxidant EPSs by *Bacillus licheniformis* UD061 in SSF using squid processing by-products and maize cobs as a carbon and nitrogen source. Succinoglycan is also produced using SSF. Various solid substrates such as spent malt grains, ivory nut shaving, and grated carrots were used for the production of succinoglycan by *Agrobacterium tumefaciens* in SSF. The highest succinoglycan yield in SSF was 30 g EPS/kg solid in this study (Stredansky and Conti 1999). Taşkın et al. (2010) used detoxified loquat kernel extract (DLKE) and neutralized loquat kernel extract (LKE) prepared from waste loquat kernels as main carbon

sources in the submerged and solid cultures of *M. esculenta* for the production of EPS having various biologic and pharmacologic activities, including antitumor, immunostimulating, and hypoglycemic activities. Maximum EPS concentrations using DLKE and KLE were 5.2 and 4.1 gL^{-1}, respectively, in this study.

Whereas liquid sugar beet molasses is commonly used in SmF, solid sugar beet pulp (SBP) which is another by-product of the sugar beet industry can be used in SSF as biomass resource for EPS production. SBP is the fibrous material left over after the sugar is extracted from sugar beets and is mainly composed of cellulose, hemicellulose, and pectin (Toksoy Oner 2013). Yoo and Harcum investigated the feasibility of using pretreated SBP as a supplemental substrate for xanthan gum production from *X. campestris*, and they reported a production yield of 0.89 g xanthan per gram of SBP in 4 days of fermentation time (Yoo and Harcum 1999). Söğütçü et al. (2011) used SBP to produce EPS by halophilic *Halomonas* sp. AAD6 cultures. Some pretreatment methods such as milling, dialyzing, and autoclaving were reported to increase the EPS yields in this study.

8 Conclusion/Prospects

The constant increase in population and industrialization has created enormous quantities of industrial waste biomass and associated environmental and health hazards. Hence biomass management via biorefinery approach has become an important issue for sustainable development and economic competitiveness. The utilization of biomass requires intensive research activities for the development of feasible pretreatment, fermentation, and downstream processing techniques. The most challenging part in the whole process is to combine the production stream with the suitable waste stream.

Microbial EPS production is strictly dependent on the nutritional and environmental requirements of the microbial culture. In this respect, omics technologies and systems biology tools provide ample knowledge on the genetic capabilities of the microbial producer cultures; hence, by functional and comparative genomics, the most appropriate biomass resource leading to high polymer titers can be identified. Such systems-based approaches are expected to have an ever-increasing importance in microbial EPS production and in general industrial biotechnology.

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Tree Gum: Gum Kondagogu

7

Rao Beedu Sashidhar, D. Raju, and Rasineni Karuna

Contents

Abstract

Tree gums are obtained as the natural exudates of different tree species and exhibit unique properties with a wide variety of applications. Commercially, gums have a separate niche in the world market as a commodity of international trade. These biopolymers are abundant, come from renewable sources, are relatively inexpensive and nontoxic, and are amenable to both chemical and biochemical modifications that find widespread and extensive food and nonfood applications. Most gums are heterogeneous polysaccharides with complicated structures and extremely high molecular masses. Major tree exudate gums include gum arabic, gum tragacanth, gum karaya, ghatti, and gum kondagogu. Exudate gums possess a unique combination of functionalities and properties that can never be matched by any other alternative synthetic polymers, which makes their complete substitution impossible. Importantly, these biopolymers

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are eco-friendly as they are biodeteriorable. Gum kondagogu is a naturally occurring nontoxic polysaccharide derived as an exudate from the bark of *Cochlospermum gossypium* (Bixaceae family), a native tree of India. To exploit its potential commercial applications, its morphological, structural, physico-chemical, compositional, solution, conformational, rheological, emulsifying, and metal-biosorption properties have been elucidated. Gum kondagogu is an acidic gum with high content of uronic acid and the major functional groups identified in the gum are hydroxyl, acetyl, carbonyl, and carboxylic groups. The primary structure of this biopolymer contains sugars, such as arabinose, rhamnose, glucose, galactose, mannose, glucuronic acid, and galacturonic acid. Based on the spectroscopic categorization, the probable structural feature consigned to gum kondagogu is (1 → 2) *β*-D-Gal *p*, (1 → 6) *β*-D-Gal *p*, (1 → 4) *β*-D-Glc *p* A, 4-0-Me-*α*-D-Glc *p* A, (1 → 2) *α*-L-Rha, and (1 → 4) *α*-D-Gal *p* A.

The outcome of the experimental studies carried out with gum kondagogu has established its efficacy as a proficient biopolymer for (i) bioremediation of toxic metals, (ii) green synthesis of metal nanoparticles and magnetic iron oxide nanoparticles (MNP), (iii) mercury biosensor, and (iv) nanosilver-based antibacterial agent for medical applications. Additionally, appropriate chemical modification of the functional groups present in gum kondagogu may lead to the development of novel technologies for applications in pharmaceutical and food and biotechnology industries.

Keywords

Gum kondagogu • Biosorption • Bioremediation • Nanoparticles • ICP-MS • SEM • TEM • Metals

Abbreviations

AFM	Atomic force microscopy
Da	Dalton
EDAX	Energy-dispersive X-ray analysis
FTIR	Fourier transform infrared spectroscopy
GCC	Girijan Co-operative Corporation
GK	Gum kondagogu
h	Hour
ICP-MS	Inductively coupled plasma-mass spectrometry
kg	Kilogram
M^+	Metal ion
mg g^{-1}	Milligram per gram
MNP	Magnetic nanoparticles
MW	Molecular weight
nm	Nanometer
NMR	Nuclear magnetic resonance spectroscopy
NPs	Nanoparticles
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

UV	Ultraviolet
XRD	X-ray diffraction
μL	Microliter

1 Introduction

The term *gum* is applied to water-soluble substances and refers to natural non-starch polysaccharides and their structurally modified derivatives**.** Gums are transparent or translucent substances produced for the protection of plant when injured**.** Gums are either soluble in water or absorb water and swell to form an adhesive viscous solution. Gums are insoluble in alcohol and other organic solvents. Many trees and shrubs produce exudate gums as a means of natural defense. Injury to plant's bark causes the exudation of aqueous gum that seals the wound, averting infection and dehydration of the plant. The solution dries in contact with air and sunlight, to form hard, glass-like lumps, which can easily be collected. Gums are not normal products of plant metabolism but are produced under abnormal or unfavorable conditions. Hence, gums are called as abnormal or pathological products and the process of exudation of gum is called as *gummosis*. Gums may be harvested in nature from trees, seeds, and marine weeds, or they may be synthesized biologically. Further, gums are also considered as hydrocolloids, which are hydrophilic in nature and are found in almost every biosphere on earth. They contain a large number of hydroxyl groups arranged usually in a fairly regular manner along the backbone of the molecule, which allows for the chelation of mono- and divalent cations, thereby cross-linking the hydrocolloid chains together and forming complex macrostructures. Hydrocolloids are some of the most well-known polysaccharides with complex structures with glycosidic bonding. Most gums are heterogeneous polysaccharides with complicated structures and have extremely high molecular masses. A broad classification for gums based on their functions has been earlier described (Mocak et al. 1998; Hall 2009). The simplest interactions of hydrocolloids are with water and it is this interaction that makes these gums applicable to their use in foodstuffs. The gum hydrocolloids are effective water adsorbents and to a greater and lesser degree will be solubilized by water. Owing to the high number of hydroxyl groups, water is held within the molecular structure by hydrogen bonding and also within the voids created by the complex molecular configuration (Hall 2009). Exudate gums are among the oldest natural products used by mankind. They were being used as thickening and stabilizing agents for centuries because they provide excellent water-binding capacity. Some gums possess a natural biological activity related to the glycoproteins they contain, which results in a particular role of gums in the pharmaceutical industry.

Gums may be harvested in nature from trees, plant seeds, and marine weeds, or they may be synthesized by microbial fermentation. Major tree exudate gums include arabic, tragacanth, karaya, ghatti, and kondagogu, which are polysaccharides that contain salts of hexuronic acids and a number of neutral monosaccharides that are often esterified in highly branched structures (Roberts et al. 1998).

Table 1 Tree gums commonly used in food industries

Food	Percent used	Function
Frozen dairy desserts and mixes	0.3	Formulation aid, stabilizer/thickener
Milk products	0.02	Stabilizer/thickener
Soft candy emulsifier	0.9	Emulsifier salt, stabilizer/thickener
Other foods	0.002	Formulation aid, stabilizer/thickener

The presence of hexuronic acids or salts favors their water solubility. The contents of uronic acids in these gums vary, e.g., karaya contains 37 % of uronic acid residues, while ghatti consists of 10 % of D-glucuronic acids (Budavari 1989). The molecular weight (MW) of these gums generally ranges from 100 to 1,000 kilodalton (kDa). In addition, the exudate gums exhibit compositional heterogeneity combined with branched linkages. Gums commonly used in food industry are presented in Table. 1.

Commercially, the exudate gums have been used in food, pharmaceutical, adhesive, paper, textile, and other industries (Phillips and Williams 2001; Verbeken et al. 2003).

2 Exudate Tree Gums

2.1 Gum Arabic, Gum Tragacanth, Gum Karaya, Ghatti, and Gum Kondagogu

Compared to the gums from marine sources, the gums in this group are structurally more complex. Galacturonic acid is known to be one of the constituents of all these exudate gums. None of these gums is produced in industrialized countries. They must be imported from third-world countries. The chemical composition of these gums is complex and varies to some extent depending on the source and its age. Therefore, it is not possible to provide defined structural formulas of these biopolymers. Exudate gums are used in an overwhelming number of applications, especially in the arena of food industry and also considerably in nonfood applications (Table 2).

2.1.1 Gum Arabic

It is also known as gum arabic or Indian gum. Gum arabic is the oldest and best known of all natural gums. It is the dried gummy exudates obtained from the stem and branches of *Acacia arabica* belonging to the family Leguminosae. Gum arabic is a branched, neutral or slightly acidic, complex polysaccharide obtained as a mixed calcium, magnesium, and potassium salts of arabic acid. Arabic acid on hydrolysis gives L-arabinose, L-rhamnose, D-galactose, and D-glucuronic acid. The backbone of the polymer consists of 1,3-linked β-D-galactopyranosyl units. It is identified as a heterogeneous gum (Anderson and Stoddart 1966).

Table 2 Nonfood applications of gum

S. no.	Industry	Uses
1	Pharmaceutical	Suspending agent, emulsifier, adhesive, and binder in tabletting and in demulcent syrups
2	Cosmetic	Stabilizer in lotions and protective creams, where it increases viscosity, imparts spreading properties, and provides a protective coating and a smooth feel
3	Soap	Adhesive agent in blusher and as a foam stabilizer in liquid soaps
4	Lithography industry	Etching and plating solutions
5	Paints and insecticidal	Pigments and active components uniformly distributed throughout the product
6	Textile industry	Thickening agent in printing pastes
7	Pet food	Canned meat-containing pet foods
8	Other applications	Inks, polishes

2.1.2 Gum Tragacanth

It is the dried gummy exudation obtained by incision from stems and branches of *Astragalus gummifer* and other species of *Astragalus* belonging to the family Leguminosae. It contains two fractions of which one is soluble in water. The water-soluble portion is known as tragacanthin, constituting about 8–10 % of the gum. Water-insoluble portion is known as bassorin (60–70 %). Tragacanth on hydrolysis yields galacturonic acid, D-galactopyranose, L-arabino-rhamnose, and D-xylopyranose. It is also used as an emulsifying, suspending and thickening, and binding agent (Verbeken et al. 2003).

2.1.3 Gum Karaya

It is also known as Indian tragacanth or *Sterculia* gum. It is as dried gum exudates obtained from the tree *Sterculia urens*, *Sterculia villosa*, and *Sterculia tragacantha* belonging to the family Sterculiaceae. Karaya gum is composed of galacturonic acid, β-D-galactose, glucuronic acid, L-rhamnose, and other residues. Karaya gum has unique features such as high swelling, water retention capacity, high viscosity, inherent nature of antimicrobial activity, and abundant availability. It is neither digested nor absorbed by the body and hence it is a good bulk laxative (Anderson et al. 1982).

2.1.4 Gum Ghatti

Gum ghatti is a naturally occurring water-soluble, complex polysaccharide derived as an exudate from the bark of *Anogeissus latifolia* (Combretaceae family), a native tree of the Indian subcontinent. The gum is light yellow to amber in color, has a glassy fracture, and often occurs in rounded tears and larger vermiform masses. The gum is almost odorless, only about 80–90 % of the gum dissolves in water and forms a colloidal solution. The name, gum ghatti, has originated from its transportation through mountain passes or *ghats*. This biopolymer is a high-arabinose,

protein-rich, acidic heteropolysaccharide, occurring in nature as mixed calcium, magnesium, potassium, and sodium salt. The primary structure of this gum is composed of sugars such as L-arabinose, D-galactose, D-mannose, D-xylose, and D-glucuronic acid in a molar ratio of 48:29:10:5:10 and <1 % of rhamnose, which is present as nonreducing end groups (Kaur et al. 2009).

3 Gum Collection and Processing

Although natural exudates are sometimes harvested, virtually all exudate gum is tapped from the trees. When acacia trees lose their leaves and become dormant at the beginning of the dry season, usually by the end of October or beginning of November, superficial incisions are made in the branches and bands of bark are stripped off. After 5 weeks, gum is manually collected as partially dried tears. This collection is repeated at 15-day intervals for up to five or six collections in total, depending on the weather conditions and the health of the tree (Imeson 1992). After collection, the gum is cleaned and graded. This is traditionally done by women, who manually sort the gum according to the size of the lumps and remove foreign matter (FAO 1995). Since the 1990s, cleaning has also been performed mechanically using conveyor belts and sieving machines. Since 1995, the gum from *Acacia seyal* (talha) has been divided into three grades: super, standard clean, and siftings.

Gum karaya exudation begins immediately after wounding the *Sterculia* tree and is particularly extensive during the first days. The exudates are allowed to solidify on the tree and are then removed as large, irregular tears. The yield of gum from a mature tree is 1–5 kg per season. India is the world's leading producer of gum karaya. The best quality gum is collected from April to June, before the monsoon season, as the temperature increases. Collection may be repeated in September, although in this period gum may be less viscous and darker, due to the presence of higher amounts of foreign matter. In Senegal, the biggest African producer of gum karaya, harvesting is done from September to January and from March to July. After collection, the gum is manually or mechanically cleaned and sorted. Commercial Indian gum karaya is available in five different grades: handpicked selected, superior no. 1 and no. 2, fair average quality, and siftings (FAO 1995). The higher grades contain less foreign matter and have a lighter color. Senegalese gum is sorted in two grades, handpicked and standard, and is generally inferior to Indian gum.

Gum tragacanth exudes from wounds in *Astragalus* trees and hardens as curled ribbons or flakes, which can be collected after a few weeks. The primary source of the gum is the rather large taproots of the bush, inside of which the gum is contained in a central gum cylinder. Incisions are also made in the bark of the branches, although this usually yields gum of an inferior quality. The plants require a plenitude of rainfall prior to tapping, but dry conditions during harvesting. Rain and wind during the gum exudation may wash some gum off and lead to a decrease in quality. Tapping and collecting is carried out in the dry summer months from July to September. After collection, the gum is sorted manually into various grades

Table 3 Major suppliers of exudate gums all over the world

S. no.	Company	Products
1	Gum Arabic Co., Sudan	Gum arabic
2	Victoria Agro Exports Ltd., Nigeria	Gum arabic
3	Importers Service Corp., USA	Gum arabic, karaya, tragacanth
4	TIC Gums, USA	Gum arabic
5	Colloides Naturels International, France	Gum arabic
6	Alok International, India	Gum arabic, karaya, tragacanth
7	Natural Colloids, Singapore	Gum arabic, tragacanth
8	AEP Colloids, USA	Gum arabic, karaya, tragacanth
9	Agri products, USA	Gum arabic
10	KIC Chemicals Inc., USA	Gum arabic
11	Girijan Co-operative Corporation (GCC), AP, India	Gum karaya, gum kondagogu, gum babul

of ribbon and flake. Iranian tragacanth ribbons are sorted into five grades, while flakes are sold in seven different grades (FAO 1995). After arrival in the importing country, the gum is usually ground into powder, with particle size varying according to the desired viscosity. A list of the major producers/suppliers of exudate gums all over the world is given in Table 3.

Gum arabic is the oldest and best known of all natural gums. Its use can be traced back to the third millennium B.C., the time of the ancient Egyptians. Early Egyptian fleets shipped gum arabic as an article of commerce. It was used as a pigment binder and adhesive in paints for making hieroglyphs, and ancient inscriptions refer to it as *kami*.

Furthermore, it was used as a binder in cosmetics and inks and as an adhering agent to make flaxen wrappings for embalming mummies. Introduced in Europe through various Arabian ports, it was called gum arabic after its place of origin. During the middle age, gum arabic trade was controlled by the Turkish Empire, giving rise to the name turkey gum. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) defines gum arabic as "a dried exudates obtained from the stems and branches of *Acacia senegal* (L.) Willdenow or *Acacia seyal* (Fam. *Leguminosae*)" (FAO 1999). In a wider sense, the name gum arabic is also used to denominate gums produced by other *Acacia* species, like *A. karroo*, and is sometimes referred to as gum acacia (FAO 1995).

4 Physical Properties of Gums

Physical properties of gums are the most important factors in determining their use and commercial value. Viscosity, gel-forming tendency, colloidal nature, and adhesiveness are some of the important physical properties of gums. The physical properties of various gums vary depending on botanical origin, climatic conditions, harvesting period, age of the tree, post-harvesting practices, and storage conditions. The following are details of physical properties of gums.

4.1 Viscosity

Viscosity or the "thickness" of a solution that a gum forms with water is of paramount importance in determining the quality of gum. It is said that the higher the viscosity, the better the gum particularly in the case of medicinal use.

4.2 Shape

Gums are available in two shapes, majority of them are tear shaped, excepting few, which are globular.

4.3 Color

It is a very important factor in the commercial valuation of gums. It is believed that color is mainly due to the presence of impurities. However the concrete reasons have not been researched properly. But as per the traders, good market is always there for light-colored gums. The darker the color, the commercial value decreases. Majority of the good gums are almost water white with traces of yellow. The other colors that are found among gums are amber, orange, dark brown, black, and brownish.

4.4 Colloidal Nature

This property makes gums valuable in manufacturing processes, notably in the textile, cosmetic, pharmaceuticals, and food industry. Colloidal nature exhibits swelling pressures and forms gel structures at very low concentrations and over a wide range of concentrations. They have low surface tension, do not crystallize, and act as protective colloids and stabilizing agents. In effect they prevent agglomeration.

4.5 Taste and Smell

The true gums are nearly odorless and in this sense differ markedly from some of the resins and oleoresins that are so distinctive in smell. They may be tasteless and are in fact generally devoid of any characteristic taste. But some are slightly sweet or bitter according to botanical origin. In some gums there is distinctively bitter taste which is a serious disadvantage in their application for edible purposes.

4.6 Hardness

Gums vary in hardness. Hardness is obviously governed partly by the amount of moisture present. Density also provides variable in one and the same gum according

to the amount of air that may have been incorporated in it when it was formed. Most gums break with a clear glassy fracture when properly dried and may be readily pulverized.

5 Gum Kondagogu Occurrence

Indian forests are a major source of large number of non-wood forest products. One such product is an exudate tree gum, regionally called as gum kondagogu (*Cochlospermum gossypium*) (Fig. 1), belonging to the family Bixaceae. This gum is an important non-wood forest natural product collected by tribals in the state of Andhra Pradesh and is commercially marketed by Girijan Co-operative Corporation (GCC), Hyderabad, Andhra Pradesh, India (Table 4). Three grades of gum are marketed as grades I, II, and III. The production in the state of Andhra Pradesh is in the range of 0.5–1.5 tons of gum per season. This gum is also produced

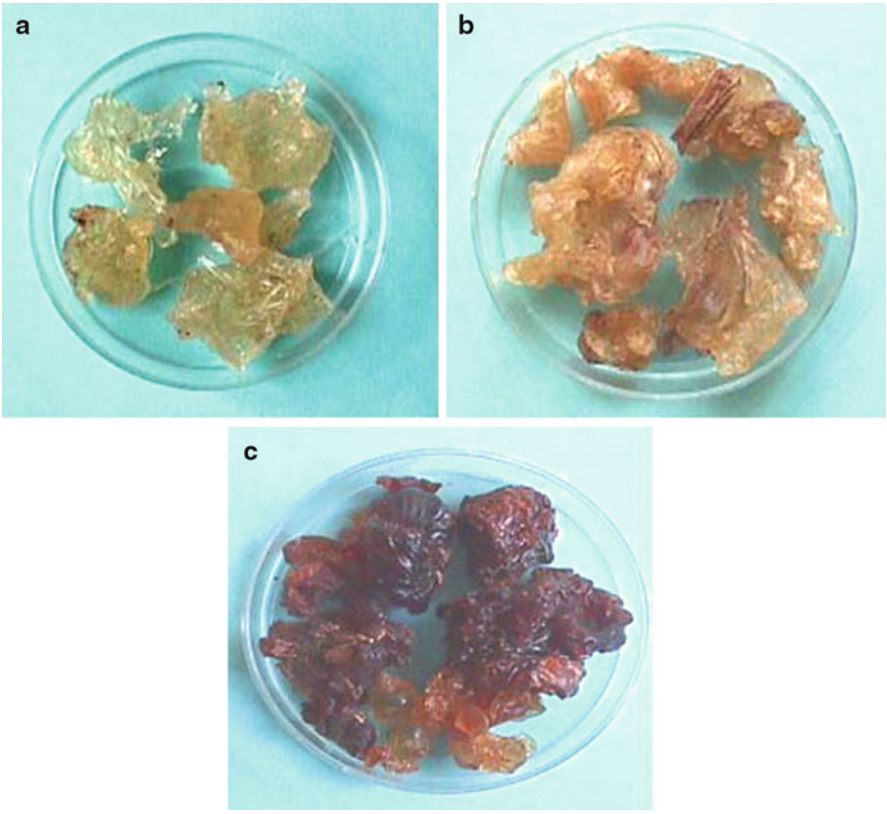


Fig. 1 Specimens of different grades of gum kondagogu. (**a**) Grade I. (**b**) Grade II. (**c**) Grade III

Table 4 Gum kondagogu collection in the state of Andhra Pradesh, India, and its export sale value

Year	Quantity in quintals	Value in rupees (in lakhs)
2000–2001	887	84.37
2001–2002	1,321	143.16
2002–2003	1,150	116.07
2003–2004	1,222	136.06
2004–2005	1,136	126.69
2005–2006	1,158	135.67
2006–2007	1,165	145.89
2007–2008	1,175	155.09

Source: Girijan Co-operative Corporation, Andhra Pradesh, India

and marketed by other Indian states, namely, Rajasthan, Madhya Pradesh, and Chhattisgarh.

Gum kondagogu is trapped from the tree by blazing or stripping of the bark of the tree. The maximum amount of the gum is produced within the first 24 h of blazing and continued for several days. The gum is solidified in the form of "tears."The best quality of the gum is produced during April to June. Janaki and Sashidhar (1998) pioneered the studies on gum kondagogu in relation to its physicochemical properties and as a potential new food additive. Their investigation provided a separate identity to this gum as compared to other established tree gum such as gum arabic, gum tragacanth, and gum karaya. The physicochemical analysis revealed that gum kondagogu differs from gum karaya with respect to fiber, protein, tannin, minerals, sugar compositions, intrinsic viscosity, water-binding capacity, and pH (Janaki and Sashidhar 1998). The toxicological assessment of gum kondagogu established this gum to be nontoxic. The toxicological evaluation of gum kondagogu fed to Wistar rats did not show any adverse effects on feed consumption and utilization or clinical condition, as revealed by hematological, blood chemistry, enzymatic profile, urine analysis, organ mass, and histopathological examination, suggesting that gum kondagogu can be used in the food and pharmaceutical preparations (Janaki and Sashidhar 2000). Later, comprehensive experimental investigations were carried out in our laboratory to elucidate the morphological features, physicochemical, structural, rheological, solution, and conformational properties of this novel gum (Vinod et al. 2008a, b; Vinod and Sashidhar 2009).

5.1 Morphological Studies

Morphological analysis of gum kondagogu was carried out using scanning electron microscopy (SEM), atomic force microscopy (AFM), and transmission electron microscopy (TEM). Microscopy (SEM, TEM, and AFM) was used to provide visual evidence for the formation of multilayer interfaces around colloidal particles.

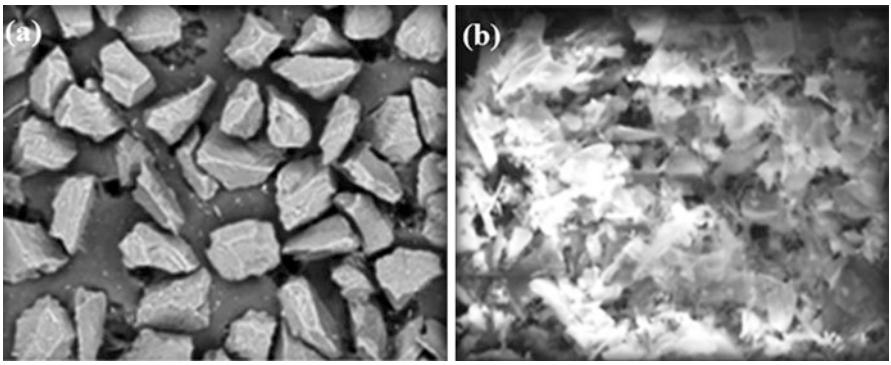


Fig. 2 (**a**) Scanning electron microscopy (SEM) of native gum shows that irregular particle size and (**b**) deacetylated gum was found to be fibrillar, indicating the loss in particulate morphology that was observed in the native form of the gum, suggesting that acetyl groups are essential for its structural integrity

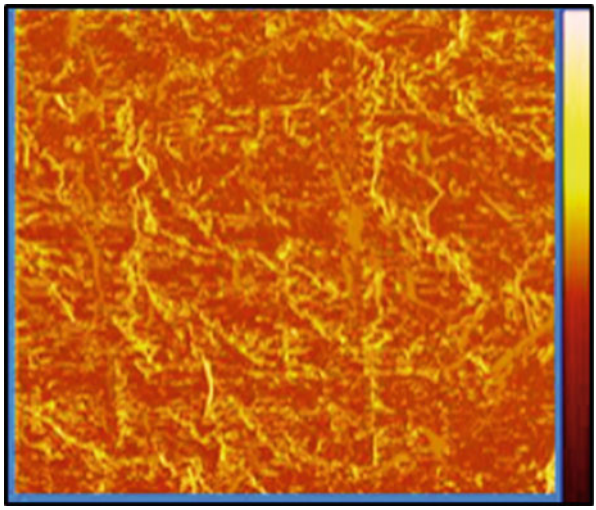
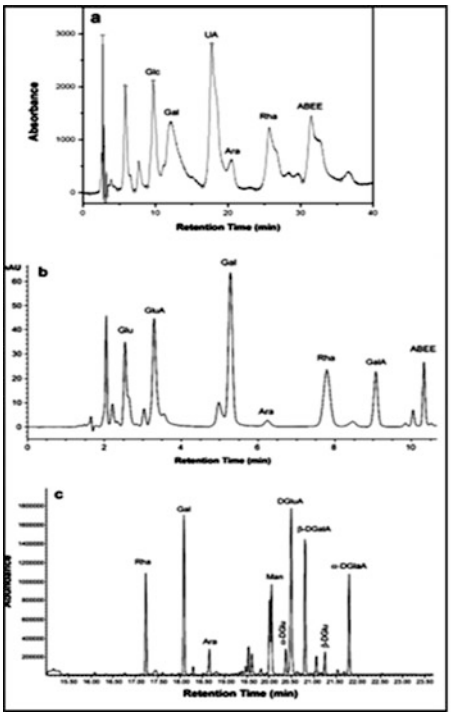


Fig. 3 Topographical phase of native gum kondagogu determined by AFM (phase image) analysis suggesting an irregular network-shaped structure for gum kondagogu

Scanning electron microscopy (SEM) of native and deacetylated gum showed that the native form of the gum had irregular particle size (Fig. 2a). Deacetylated gum was found to be fibrillar, indicating the loss in particulate morphology that was observed in the native form of the gum, suggesting that acetyl groups are essential in the structural integrity of the gum for particulate appearance in the native form (Fig. 2b). Topographic image of gum kondagogu based on AFM analysis suggested an irregularly shaped network structure, indicating the involvement of inter- and/or intramolecular aggregations (Fig. 3). The TEM analysis of gum kondagogu showed elongated chain structures with extensive branching (Vinod and Sashidhar 2010).



Sugar residue	Composition (mol %)
Rhamnose	12.85
Galactose	18.95
Arabinose	2.52
Mannose	8.30
α-D-glucose	2.48
D-glucuronic acid	**19.26**
β-D-galacturonic acid	**13.22**
β-D-glucose	2.52
α-D-galacturonic acid	**11.02**

Fig. 4 Compositional analysis of gum kondagogu: sugar profile of gum kondagogu as determined by (**a**) HPLC, (**b**) LC-MS, and (**c**) GC-MS analysis

5.2 Phytochemistry

Essentially, gum kondagogu is a biopolymer of rhamnose, galacturonic acid, glucuronic acid, β-D-galactopyranose, α-D-glucosopyranose, galactose, arabinose, mannose, and fructose with average molecular mass of 7.23×10^6 g mol^{-1} determined by static light scattering method and Berry plots. Gum kondagogu is also composed of higher uronic acid content, cruse protein, tannin, and soluble fibers.

5.3 Compositional Analysis

Composition of gum kondagogu was deciphered based on High performance liquid chromatography (HPLC), Liquid chromatography mass spectrometry (LC-MS), and Gas chromatography mass spectrometry (GC-MS) analysis. The analytical data on sugar composition suggested that gum kondagogu contains very high concentration of uronic acids [D-glucuronic acid (19.26 mol %), β-D-galacturonic acid (13.22 mol %), and α-D-galacturonic acid (11.22 mol %)] (Vinod et al. 2008a, b) (Fig. 4). The amino acids present in gum kondagogu include alanine (32.25 ± 1.44 μg g^{-1}), glycine (5.05 ± 0.55 μg g^{-1}), valine (7.24 ± 0.60 μg g^{-1}),

leucine (3.85 ± 0.22 μg g^{-1}), proline (42.45 ± 2.56 μg g^{-1}), methionine (44.24 ± 2.25 μg g^{-1}), aspartic acid (72.85 ± 3.45 μg g^{-1}), threonine (30.45 ± 1.54 μg g^{-1}), glutamic acid (34.25 ± 1.56 μg g^{-1}), tyrosine (32.89 ± 1.85 μg g^{-1}), and tryptophan (10.88 ± 0.84 μg g^{-1}). Aspartic acid, methionine, proline, glutamic acid, tyrosine, and threonine were observed to be the major amino acids in gum kondagogu. The fatty acids identified in gum kondagogu included: lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), γ-linolenic acid ($C_{18:3}$), arachidic acid ($C_{20:0}$), behenic acid ($C_{22:0}$), erucic acid ($C_{22:1}$), and lignoceric acid ($C_{24:0}$). Gum kondagogu contains 84.4 % of saturated fatty acid and 15.6 % of unsaturated fatty acids. Stearic acid ($C_{18:0}$) (37.25 %) was the major fatty acids present in the gum. The unsaturated fatty acid detected includes linoleic acid (3.45 %) and γ-linolenic acid (0.54 %), oleic acid (0.50 %), palmitoleic acid (5.92 %), and erucic acid (6.02 %) (Vinod and Sashidhar 2010; Vinod et al. 2010a). Gum kondagogu is an acidic gum with high content of uronic acid, and the major functional groups identified in the gum are hydroxyl, acetyl, carbonyl, and carboxylic groups.

5.4 Structural Analysis

The structural feature of gum kondagogu was elucidated by spectroscopic methods. The purified gum kondagogu sample was studied by X-ray diffraction analysis (XRD), Fourier transform infrared spectroscopy (FTIR) (Fig. 5a, b), and nuclear magnetic resonance spectroscopy (NMR) [by1-D (^{1}H and ^{13}C) and 2-D (DQF COSY, NOESY, TOCSY, HMBC, and HSQC)]. Based on these spectroscopic studies, the probable structural feature that can be assigned to gum kondagogu was a backbone and linkages with (1 → 2) *α*-L-Rha*p* (1 → 4) α-D-Gal A (1 → 2) and pendent residue of *β*-D-Gal (1 → 4) *α*-L-Rha*p* and *β*-D-Glu A (1 → 3) *α*-D-Gal A, with branched structures composed of galactose, arabinose, rhamnose, mannose, glucose, *β*-D-glucuronic acid, *β*-D-galacturonic acid residues, and 4-*O*-methyl derivatives (Fig. 6). The backbone structure of the gum kondagogu was also assigned based on Smith degradation analysis (Vinod et al. 2008a, b; Vinod and Sashidhar 2010). Table 5 depicts the summary of proximate and physicochemical properties of gum kondagogu.

6 Biotechnological Applications of Gum Kondagogu

6.1 Bioremediation of Toxic Metal Ions from Industrial Effluents

In recent years, the presence of heavy metals in industrial wastewater and effluents is an important environmental problem due to their more rigorous discharge norms, their increased usage, and their high toxicity. One of the methods of decontamination includes green approach, which utilizes the available natural products that have low operating cost, improved selectivity for specific metals of interest, and removal of heavy metals from effluents. Gum kondagogu, a natural biopolymer, has been exploited as an efficient, environmentally friendly matrix for removal of toxic metal

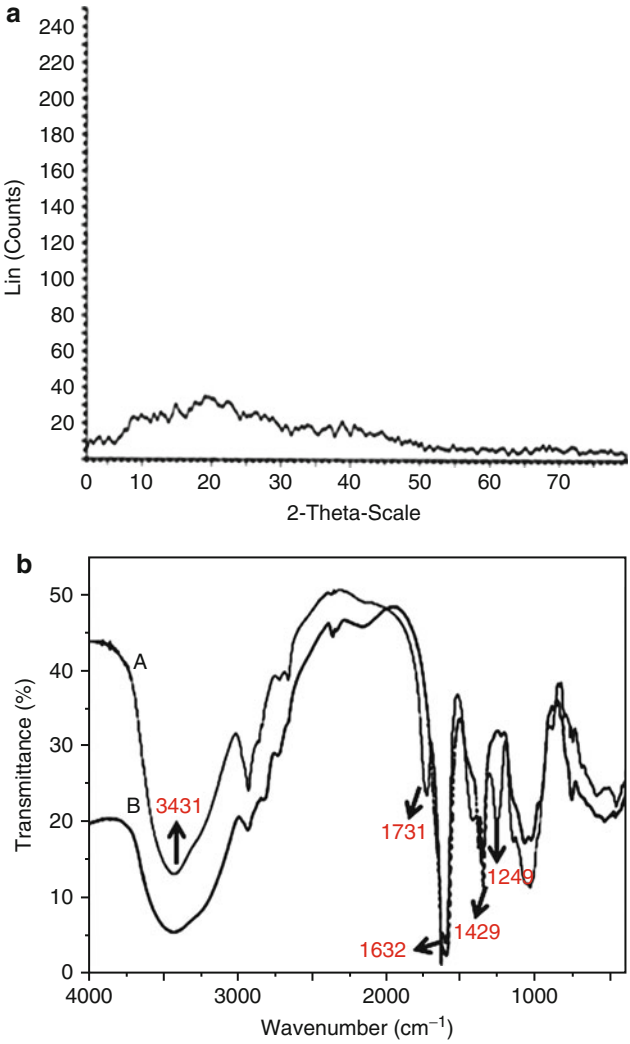


Fig. 5 (**a**) The XRD pattern of the native gum indicates a completely amorphous structure. (**b**) FTIR analysis of native and deacetylated gum kondagogu. The major functional groups identified from FTIR spectrum include 3,431 cm^{-1} (–OH), 1,731 cm^{-1} (CH_3CO–), 1,632 cm^{-1} (–COO–), 1,429 cm^{-1} (–COO), and 1,249 cm^{-1} (–CH_3CO). Absence of peaks at 1,736 and 1,227 cm^{-1} were attributed to acetyl groups; these groups were absent in deacetylated gum

pollutants from industrial effluent discharges. The biosorption of metals from wastewaters using biomass byproducts is a viable and cost-effective technology that should be included in process evaluations. The utility of this novel natural biopolymer in the bioremediation of toxic metal ions [Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and Cr (total)] has been investigated for bioremediation of toxic metal ions present in industrial effluents.

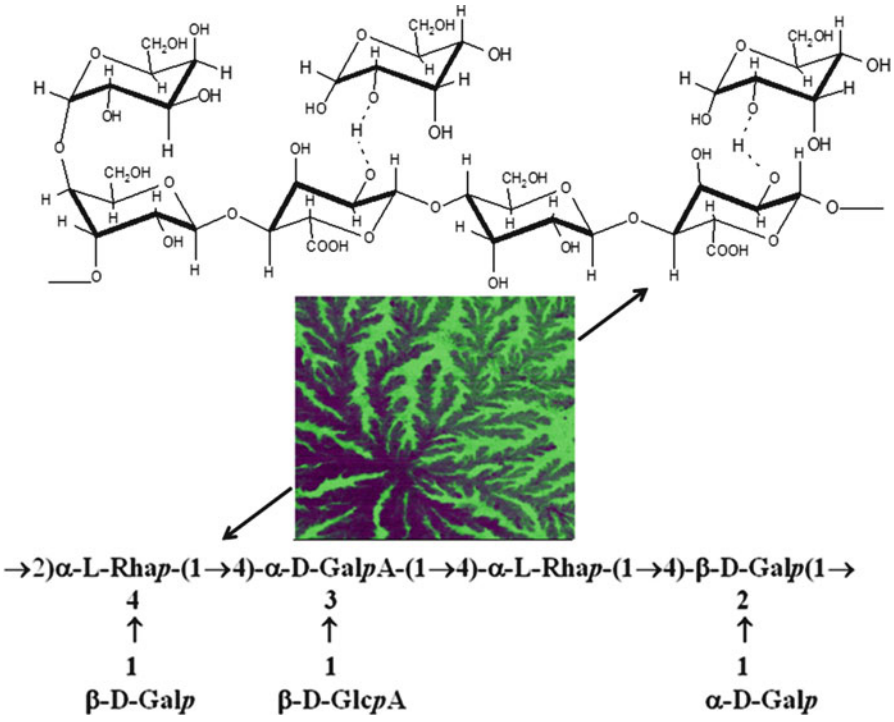


Fig. 6 The assigned structure of the gum kondagogu by NMR and GC-MS analysis. Structural assignment was carried out using acid hydrolysis, Smith degradation, and NMR studies [^{1}H, ^{13}C, 2-D NMR (TOCSY and NOESY)]. TEM analysis of native gum kondagogu showing backbone and branched network structures (Scale bar 100 nm)

The continuously increasing demand for the commodities produced by chemical industries has generated heavy metal accumulation in the ecosystem. Biosorption is a potentially attractive technology for removal of toxic heavy metals from industrial effluents. A number of technologies have been developed over the years to remove toxic metal ions from water. Newer technologies are required that can reduce heavy metal concentrations to environmentally acceptable levels at reasonable cost. One of the methods of decontamination includes green approach, which utilizes the available natural products that have low operating cost, improved selectivity for specific metals of interest, and removal of heavy metals from effluents. Among the toxic metal ions, Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Hg^{2+}, and Cr are harmful wastes produced by industry that pose a risk of contaminating groundwater and other water resources, impacting the water quality.

The study was performed on five different industrial locations (Nacharam, Cherlapally, Tank Bund, Balanagar, and Kattedan) in city zone of Hyderabad (Telangana, India). Biosorption of toxic metals was quantitatively determined by ICP-MS. The FTIR spectra were obtained using an IR spectrophotometer.

Table 5 Summary of proximate and physicochemical properties of gum kondagogu

Proximate	
Moisture (g%)	15.2 ± 1.18
Crude protein (g%)	6.3 ± 1.11
Lipid content (g%)	2.2 ± 0.14
Total fiber (g%)	80.0 ± 20.2
Volatile acidity (g%)	15.9 ± 0.35
Water-binding capacity (mL g^{-1})	35.1 ± 1.96
pH	4.9 ± 0.2
Ash (g%)	7.3 ± 0.33
Tannin (g%)	0.072 ± 0.008
Values, mean ± SD	
Physicochemical properties	
Morphology	Branched with network structures
Functional groups	–OH, –COOH, –CO, $–CH_3CO$
Structural components	Rich in rhamnose, galactose, and uronic acid (Rhamnogalacturonan type gum)
Molecular mass (Da)	$7.23 \pm 0.15 \times 10^6$
Intrinsic viscosity (dL g^{-1})	32.68
Nature of gum	Amorphous
Glass transition temperature	34.5°C
Rheological	Newtonian behavior

IR spectra of gum kondagogu and gum kondagogu–metal complexes were obtained in the spectral region of 4,000–400 cm^{-1} using resolutions of 4 cm^{-1} and 64 co-added scans.

The Langmuir isotherms of Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and total Cr ions adsorbed on the gum kondagogu are comparatively illustrated in Fig. 7. The amount of metal ions adsorbed at equilibrium per gram of gum kondagogu (q) is represented as a function of the equilibrium metal ion concentration (C_e) as shown in Fig. 7. The uptake of metal ions by gum kondagogu varies in the range of $Cd^{2+} > Cu^{2+} > Fe^{2+} > Pb^{2+} > Hg^{2+} >$ total Cr $> Ni^{2+} > Zn^{2+}$ over the whole range of metal ion initial concentrations examined.

The effluent samples were analyzed for the bioremediation property of gum kondagogu for removal of toxic metal ions contaminants. Gum kondagogu was capable of competitively adsorbing eight metal ions from the samples of industrial effluents tested, which was observed to be in the following order: $Cd^{2+} > Cu^{2+} > Fe^{2+} > Pb^{2+} > Hg^{2+} >$ total Cr $> Ni^{2+} > Zn^{2+}$. The adsorption capacity ($\boldsymbol{q}$) of metals by gum kondagogu varied in the range of 31–37 mg g^{-1} for Fe^{2+} and a minimum of 5.5–9.3 mg g^{-1} for Hg^{2+}, in the tested effluent samples. The competitive biosorption capacity of gum kondagogu for removal of metal ions was best effective at pH 5 (Fig. 8)

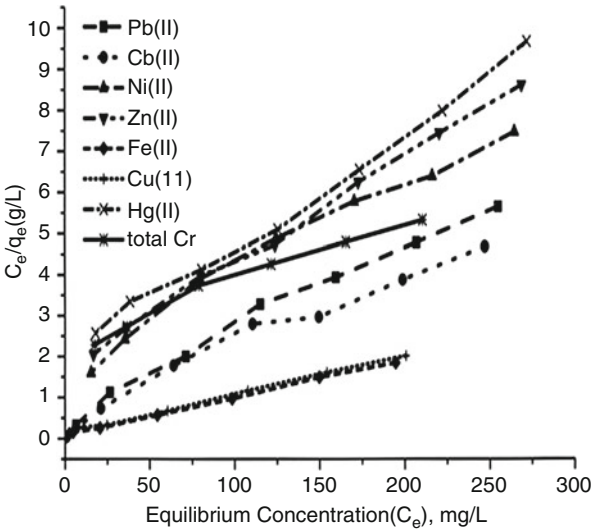


Fig. 7 Langmuir plots for metal ion (Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and total Cr) biosorption by gum kondagogu. Conditions: initial metal ion concentrations (25–300 mg L^{-1}); gum kondagogu dose (1 mg mL^{-1}); pH 5; temperature, 25 °C; time of contact, 2 h

6.2 Nature of Metal Interaction with Gum: Analysis by FTIR

The interactions between metal ions (Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and total Cr) and functional groups on the gum kondagogu were confirmed by FTIR analysis (Fig. 9i). The FTIR result indicates that bioremediation of metal ions by gum kondagogu occurred as a result of electrostatic interaction as well as complexation with hydroxyl, carboxylic, carbonyl, alcoholic, ester, amino, acetyl groups, and high amount of uronic acid of the biopolymer. Gum kondagogu biopolymer possesses functional groups, which can adsorb heavy metals. The major functional groups of the type include carboxylate anion (-COO), hydroxyl (-OH), and others (–C–N, –C–O, –C–H, –C = O, –NH). The wide band with maxima at 3,405 cm^{-1} was assigned to the stretching of O-H group of macromolecular association, the band at 2,925 cm^{-1} was assigned to stretching of CH_2 bond of methylene groups, and the sharp band observed at 1,708 cm^{-1} was assigned to a C-O bond of carboxylic acid. The strong peak that appears at 1,622 cm^{-1} can be attributed to C-O stretching vibration of a carboxylic acid that exists because of intermolecular hydrogen bond. The bands at 1,125 cm^{-1} and 1,127 cm^{-1} were due to the –C–O stretching of ether groups. The bands at 1,072 cm^{-1} and 1,062 cm^{-1} were assigned to the –C–O stretching of alcoholic groups. These band changes result in the absorbance band at 1,380 cm^{-1} became stronger. The C = C stretching in aromatic ring at 1,610 cm^{-1} shifts to 1,601 cm^{-1} after Cd^{2+} biosorption. The –OH and (–COO) groups in gum kondagogu were involved in Cd^{2+} biosorption. The FTIR spectra of unloaded and lead-loaded gum kondagogu in the range of 400–4,000 cm^{-1} were studied to find out which functional groups are responsible for the biosorption. The FTIR spectrum of lead-loaded gum kondagogu indicated that the peaks around 1,455 and 1,280 cm^{-1} gradually disappeared with increase of Pb^{2+} ions concentration

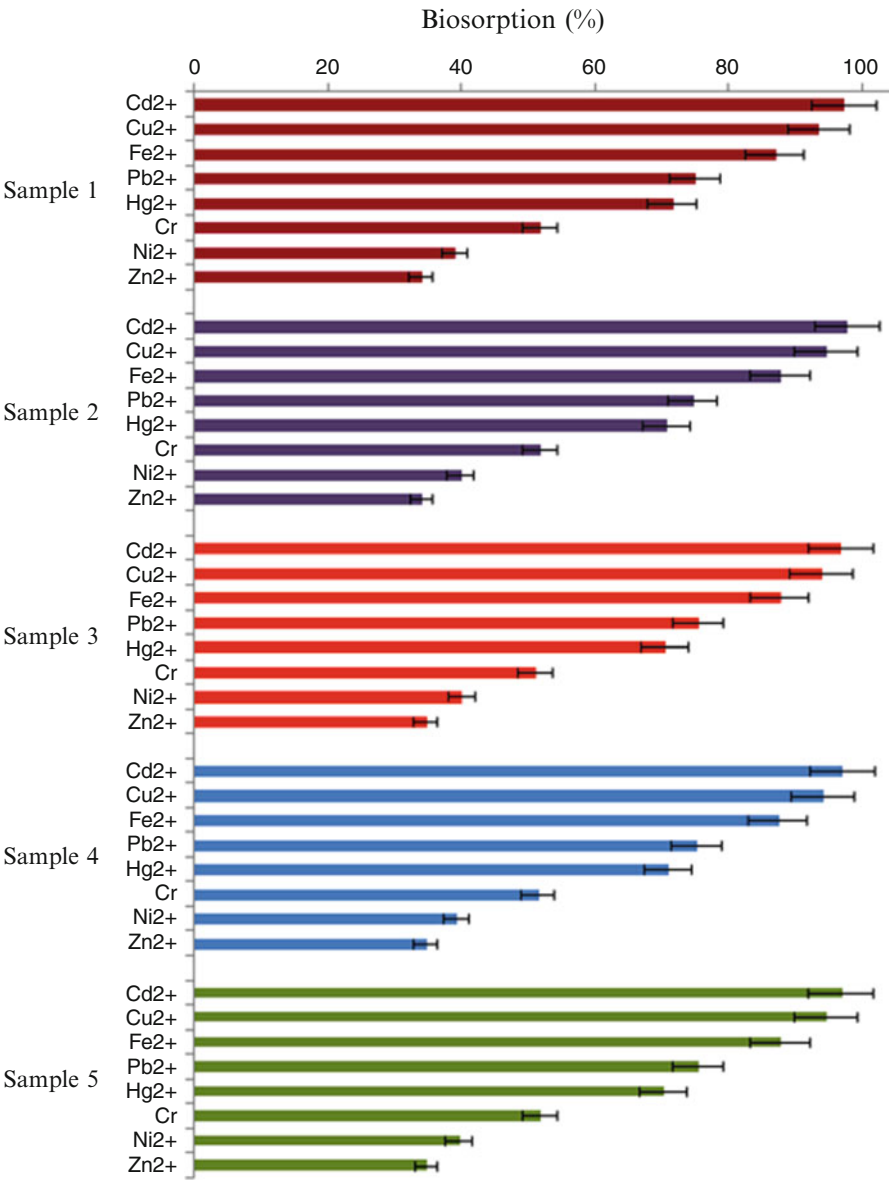


Fig. 8 Metal biosorption (%) by gum kondagogu as determined by ICP-MS in industrial effluent samples (1 Nacharam, 2 Cherlapally, 3 Tank bund, 4 Balanagar, and 5 Kattedan) Hyderabad (Telangana, India)

in solution, while the peak around 1,380 cm^{-1} was found to increase. The shift of peak at 1,455 cm^{-1} transposing to lower frequencies up to disappearance is due to the complexation of amino and hydroxyl groups with Pb^{2+}. Another shift was observed at 1,280 cm^{-1} to higher frequencies corresponding to complexation of

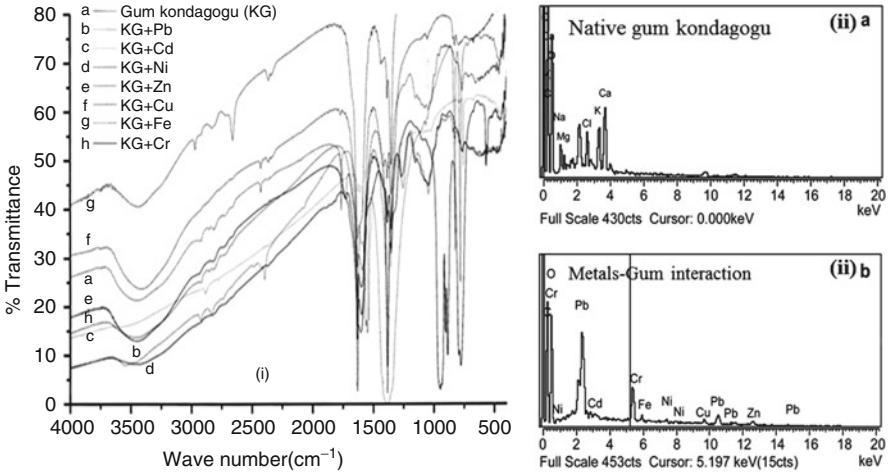


Fig. 9 (**i**) FTIR spectra of (*a*) gum kondagogu before metal biosorption, (*b*) Pb^{2+} loaded, (*c*) Cd^{2+} loaded, (*d*) Ni^{2+} loaded, (*e*) Zn^{2+} loaded, (*f*) Cu^{2+} loaded, (*g*) Fe^{2+} loaded, and (*h*) Cr loaded. (**ii**) The energy-dispersive X-ray analysis (EDXA) spectrum of native gum kondagogu presented shows that the (*a*) major elements present in gum kondagogu are Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, C, and O, respectively. (*b*) Depicts the interactions between gum kondagogu with Pb, Cd, Ni, Cr, Fe, Cu, and Zn elements respectively, suggesting the interaction of metal ions with hydroxyl, carboxyl, acetyl, and carbonyl functional groups present in gum kondagogu

oxygen from the carbonyl –C–O bond. The functional groups involved in bivalent metal biosorption included hydroxyl, carboxyl, ether, and alcoholic groups. A comparison of the spectra for native gum kondagogu with that of lead-loaded gum reveals characteristic changes of the hydroxyl groups and the acetyl groups, which shift from 3,415 cm^{-1} (–OH), amide (1,654 cm^{-1}) before lead adsorption to 3,427 cm^{-1} (–OH) and amide (1,649 cm^{-1}) after lead adsorption. Lead–gum kondagogu interaction is more evident at 3,415, 1,654, 1,550, and 1,250 cm^{-1} because of –OH, –C = O, –COOH, and –C–C vibrations. The broad stretching absorption band at 3,407 cm^{-1} shifted to 3,414 cm^{-1} after Ni^{2+} biosorption. The band intensity at 1,725 cm^{-1} clearly decreased after Ni^{2+} biosorption, i.e., there would be an interaction of Ni^{2+} with carboxylate groups. The band at 1,384 cm^{-1} appears only in the spectra at 1,082 cm^{-1}, which could be assigned to C = O from polysaccharide, while the band at 1,787 cm^{-1} was found to disappear after metal interaction. On the other hand, the band at 1,488 cm^{-1} would be attributed to the C–H bending shifted to 1,468 cm^{-1}. The appearance of new bands at 1,535 and 1,240 cm^{-1} could be assigned to –NH and C = O, respectively. The peaks at 1,725 cm^{-1} turned to a shoulder after Ni^{2+} biosorption, and this may indicate a possible Ni^{2+} interaction with C = O. A band at 1,385 cm^{-1} appears after Ni^{2+} biosorption, which could be attributed to the bending mode of C–O–H that would occur in an alcoholic group or a protonated alcoholic group or a protonated ether group. The shift of absorbance peak after Ni^{2+} biosorption provided the evidence those alcoholic groups would be one of the biosorption sites for removing Ni^{2+} metal ion.

The spectral analysis before and after metal adsorption indicated that especially the bonded –OH groups and C–O stretching were involved in Ni^{2+} biosorption. The C–O band absorption peak is observed to shift to 1,047 cm^{-1} when gum kondagogu was loaded with chromium. A broad adsorption band was observed at 3,307 cm^{-1}, which can be attributed to the bonded –OH groups present in the structure. The other prominent peaks are due to $–OCH_3$ group and aromatic compounds. However, in case of gum kondagogu, there is a remarkable shift in position and shape of the –OH group indicative of chromium binding mostly with –OH groups. These changes in FTIR spectra confirm the band shifts indicating the bonded –OH groups and/or –NH stretching and carboxyl groups (C = O) may especially play a major role in chromium biosorption on gum kondagogu. Biosorption of metal ions (Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, and total Cr) by gum kondagogu occurs as a result of electrostatic interaction and complexation with hydroxyl, carboxylic, carbonyl, alcoholic, ester, amino, uronic acid, and acetyl groups present in the biopolymer that are amenable for the interaction with metal ions at pH 5 (Vinod et al. 2010b; Vinod and Sashidhar 2011). Figure 9ii (a, b) shows the energy-dispersive X-ray analysis (EDXA) spectrum of native gum kondagogu, which contains elements such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, C, and O, and its interaction with Pb, Cd, Ni, Cr, Fe, Cu, and Zn metal ions, respectively, suggesting the role of hydroxyl, carboxyl, acetyl, and carbonyl functional groups in binding of metal ions.

The combination of biodegradability, high metal uptake capacity, and high uronic acid content contributes to make gum kondagogu a suitable material for toxic, heavy metal remediation by the biosorption process. The adsorption of metal ions by gum kondagogu occurs as a result of electrostatic interaction as well as complexation with hydroxyl, carboxylic, carbonyl, alcoholic, ester, amino, acetyl functional groups, and high content of uronic acid of the biopolymer. Gum kondagogu being a polyanionic polysaccharide was successfully employed as metal binder for nucleation and precipitation of heavy metals present in effluents. A schematic diagram showing the interaction of metal cation with gum kondagogu is presented in Fig. 10.

6.3 Metals Biosorption by Gum from Mixture of Toxic Metal Ions

The potential of gum kondagogu for the biosorption of toxic metals from mixture of metal in solutions was qualitatively assessed by inductively coupled plasma-mass spectrometry (ICP-MS). The aqueous mixtures of heavy metals were used for the determination of competitive metal-biosorption capacity of gum kondagogu, at three different pH (4.0, 5.0, and 6.0) points. The adsorption capacity (mg g^{-1}) of metals by gum kondagogu is presented in Fig. 11. The percent biosorption of metal ions (Cr, Fe, Ni, Co, Cu, Zn, As, Se, Cd, and Pb) by gum kondagogu from the mixed metal solutions occurred at pH 5.0 ± 0.1, as compared to other two pH values (pH 4.0 ± 0.1 and 6.0 ± 0.1). The percent biosorption of metal ions by gum kondagogu observed was highest

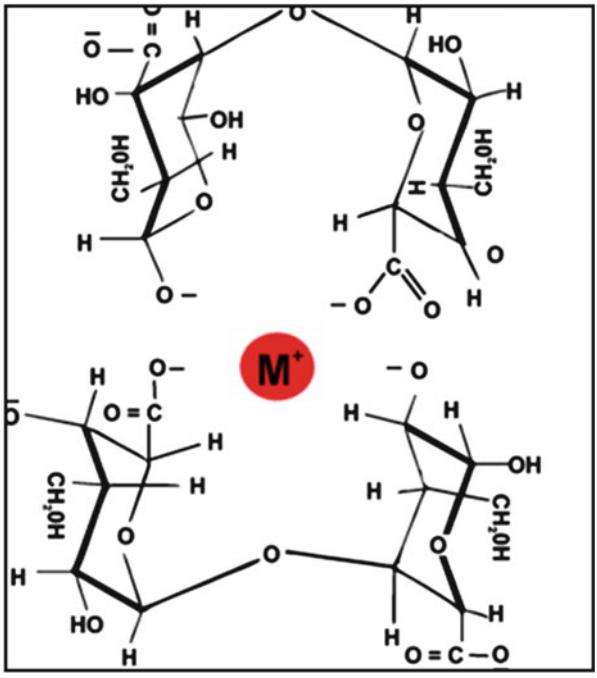


Fig. 10 Schematic diagram showing the interaction of metal cation with gum kondagogu (M^+ = metal ion)

for Cd^{2+} (97.30 ± 1.44 %) and the least for As^{2+} (16.73 ± 0.16 %) from the metal solutions.

The percent biosorption and adsorption capacity of metals by gum kondagogu follows the order $Cd^{2+} > Cu^{2+} > Fe^{2+} > Se^{2+} > Pb^{2+} >$ total Cr $> Ni^{2+} > Zn^{2+} > Co^{2+} > As^{2+}$ at pH 5.0 ± 0.1 and temperature 25 ± 2 °C. Further, to remove metal ions at pH 7.0 from wastewater, chitosan was used as a biosorbent (Gamage and Shahidi 2007). Previous studies on heavy metal biosorption showed that pH was an important factor affecting the biosorption process. The interaction of metal ions with the electron-rich functional groups located on the biopolymer may be strongly sensitive to the pH values of the environment. At very low pH values (pH 1–2), metal uptake was negligible. The optimum initial pH values for biosorption of metal ions were 5.0 ± 0.1. The pH of the adsorption medium affects the solubility of metal ions and the ionization state of the functional groups on the surface of biosorbent. The biosorption capacity of metal cations strongly depends on pH value (Yin et al. 1999; Saeed et al. 2005a). Electrostatic attraction to negatively charged functional groups might be one of the specific biosorption mechanisms. At pH 4.5–5.0, phosphate, carboxyl, and sulfate groups are active. The pH value of solution strongly influences not only the site dissociation of the biomass surface but also the solution chemistry of the heavy metals such as hydrolysis, complexation by organic or inorganic ligands, redox reactions, precipitation, and the speciation and the biosorption availability of the heavy metals (Esposito et al. 2002). The biosorption capacity of metal cations increases with increasing pH of the sorption

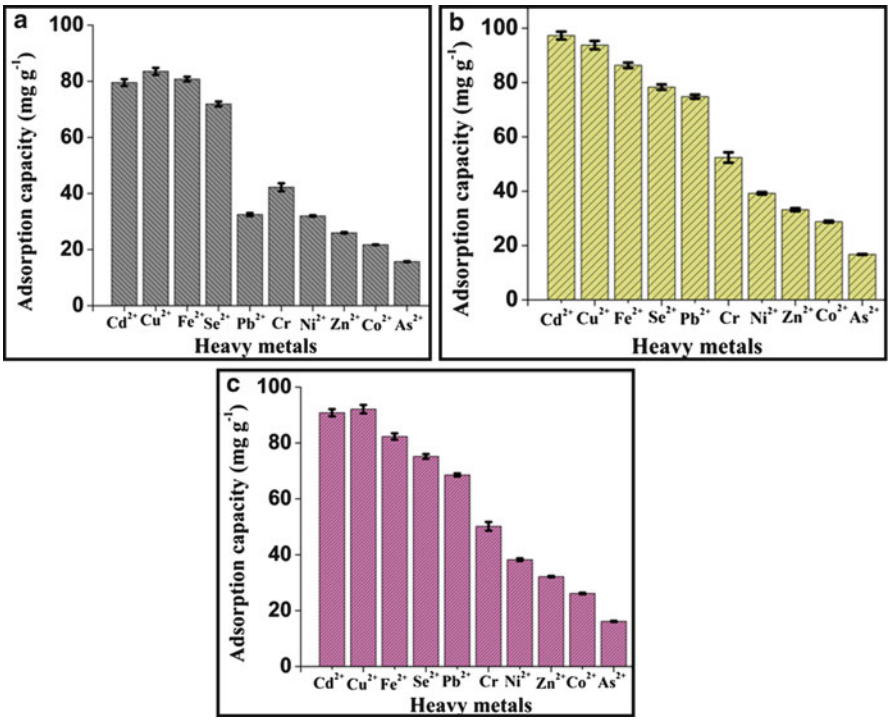


Fig. 11 Heavy metal adsorption capacity (mg g^{-1}) of gum kondagogu as determined by ICP-MS at different pH values: (**a**) pH 4.0, (**b**) pH 5.0, and (**c**) pH 6.0

system, but not in a linear relationship. At lower pH values, biomass functional groups (binding sites) would be closely associated with the hydronium ions and restrict access to binding sites by metal cations as a result of repulsive forces (Malkoc 2006). On the other hand, too high pH value can cause the formation of the metal hydroxides. The acidic groups present in gum kondagogu are responsible for metal binding. In addition, the gum also contains tannin, fibers, and proteins. Further, the pH of gum kondagogu in aqueous solution was reported to be 4.9–5.0 (Janaki and Sashidhar 1998; Vinod et al. 2008a, b). At low pH value, most of the carboxylic groups present in the gum kondagogu are protonated. With increasing pH (optimal pH 5.0), these groups get de-protonated and thus the negatively charged site formed on the gum enhances metal binding. Similar pH values (pH 5.0) were also observed for maximum biosorption of toxic metals (Cr, Fe, Ni, Co, Cu, Zn, As, Se, Cd, and Pb) by cellulose/chitin and black gram husk (Zhou et al. 2004; Saeed et al. 2005b). The high content of uronic acid (glucuronic and galacturonic) present in gum kondagogu is also responsible for metal biosorption due to negatively charged functional groups. Similar results were observed earlier, in epiphytic moss and lichen, wherein galacturonic acid was the main sugar residue involved in metal binding (Basile et al. 2008).

The native pH of gum kondagogu being 4.9–5.0 can be exploited as a natural ion exchanger to remove toxic metal from the metal-contaminated water. The observed maximum decontamination of toxic metals removed by gum kondagogu was at pH 5.0 ± 0.1 (Fig. 11). The percent adsorption of metal uptake by gum kondagogu follows the order $Cd^{2+} > Cu^{2+} > Fe^{2+} > Se^{2+} > Pb^{2+} >$ total Cr $> Ni^{2+} > Zn^{2+} > Co^{2+} > As^{2+}$ at pH 5.0 ± 0.1 and at room temperature 25 ± 2 °C.

7 Gum Kondagogu and Green Nanotechnology

7.1 Eco-friendly Synthesis of Metal Nanoparticles by Using Gum Kondagogu

There has been a remarkable research interest in the area of nanotechnology to develop reliable processes for the synthesis and stabilization of metal nanoparticles, especially noble metals owing to their potential applications in the field of biosensing and biomedical devices (Shipway et al. 2000; Yi et al. 1994; Rao et al. 2000; Sandeep Kumar et al. 2012). Numerous methodologies have been formulated in the past, to synthesize noble metal nanoparticles, such as Ag, Au, and Pt, with different compositions, sizes, and controlled monodispersity. A general method for the preparation of noble metal nanoparticles involves the treatment of metal salts with appropriate reducing agents, such as citrates, borohydrides, and other organic compounds. Many of these reducing agents have associated environmental toxicity or biological hazards. With an increasing interest in minimization or total elimination of waste and implementation of sustainable processes through the adoption of the fundamental principles of green chemistry, the development of biological and biomimetic approaches for the preparation of nanomaterials is a desirable attribute (Raveendran et al. 2003; Rao et al. 2002).

The interesting chemical composition of gum kondagogu motivated us to use this biopolymer as a template for the synthesis and stabilization of metal nanoparticles due to its (i) natural availability and low cost; (ii) nontoxicity and potential as food additive; (iii) abundance in hydroxyl, acetyl, carbonyl, and carboxylic functional groups; and (iv) metal-biosorption properties. The green route for the synthesis of metal nanoparticles using a nontoxic, renewable natural plant, gum kondagogu, as both the reducing and stabilizing agent was successfully developed. Being a natural polymer, gum kondagogu is amenable for biodegradation. The synthesis was carried out in aqueous medium without the requirement of any added chemical reducing agent, which is completely biogenic. The synthesis of metal nanoparticles by gum kondagogu is listed in Table 6 and depicted in Fig. 12.

The gum kondagogu reduced the Ag^{+} ions into Ag^{0} nanoparticles at a pH of 5.0, and mild heating (45 °C) was adequate for the complete reduction of Ag^{+} ions. In the case of $HAuCl_4$, reduction of Au^{3+} ions to Au^{0} ions occurs at a pH of 10.0 and a temperature of about 75 °C. The solution remained colorless initially for 1 h. Following the addition of 10 μL of 1 M NaOH, the solution turned into wine red color indicating the formation of Au^{0} nanoparticles. The addition of NaOH

Table 6 List of metal nanoparticles synthesized with gum kondagogu

S. no.	Metals	GK–NP size (nm)
1	Au	5.5 ± 2.5
2	Ag	7.8 ± 2.3
3	Fe	5–15
4	Pt	2.4 ± 0.7

Values, mean ± SD

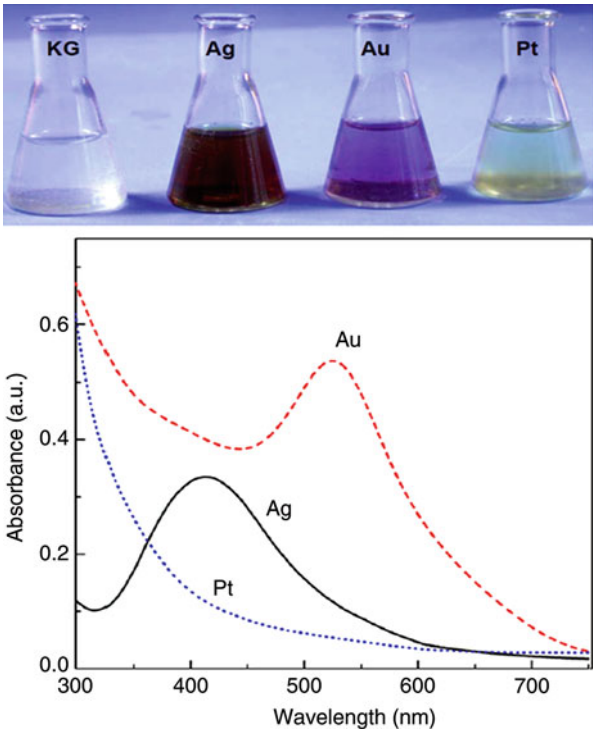


Fig. 12 Formation of striking colors of gum kondagogu – Au, Ag, and Pt nanoparticles and their specific absorption spectra

facilitates the opening of the glucose ring by the abstraction of the α-proton of the sugar ring oxygen and metal ions oxidize glucose to gluconic acid (Raveendran et al. 2003). In the case of Pt, the reduction and formation of nanoparticles occurs at a pH of 8.0 and at a temperature of 120 °C (15 psi, autoclaving).

Gum kondagogu, a natural carbohydrate biopolymer, was used for the synthesis of Ag, Au, and Pt nanoparticles in an aqueous medium. The hydroxyl functional group present in the gum network was found to be responsible for the formation of noble metal nanoparticles. The colloidal suspensions of Ag, Au, and Pt nanoparticles stabilized with the gum templates were highly stable and do not show any sign of aggregation even after storage for a period of 6 months. The Ag and Au nanoparticles formed were in the average size range of 5.5 ± 2.5 nm and 7.8 ± 2.3 nm, while Pt nanoparticles were considerably smaller in their size (2.4 ± 0.7 nm) as compared to Ag and Au nanoparticles.

7.2 Removal of Toxic Metals by Magnetic Nanoparticles Synthesized by Using Gum Kondagogu

Nanoparticles of metal oxides are proven to be a candidate material for providing better kinetics for the adsorption of metal ions from aqueous solutions (Zhong et al. 2006; Hu et al. 2008). However, for such an application, it is necessary to use a method of purification that does not generate secondary waste and involves materials that can be recycled and easily used on an industrial scale. Magnetic separation has been shown to be a very promising method for solid–liquid phase separation technique (Yantasee et al. 2007). To facilitate the recovery and manipulation of nanoparticles, magnetic nanoparticles are used as an excellent candidate for combining metal-binding and selective adsorption properties with ease of phase separation. By this means, they have the capability to treat large amounts of wastewater within a short time and can be conveniently separated from wastewater; moreover, they could be tailored by using functionalized polymers and novel molecules to impart surface reactivity. In this context, a novel magnetic nano-adsorbent based on the surface modification of magnetic iron oxide nanoparticles with GK was developed for the adsorption of toxic metal ions which is a natural, harmless, and environment-friendly polymer (Saravanan et al. 2012).

The effect of pH of the solution on the biosorption of metal cations by the GK − MNP has been investigated at pH values of 1 − 8, and the results are depicted in Fig. 13. The biosorption (%) values tend to increase with the increase in pH up to 5, and then they tend to decrease with the further increase of pH, i.e., beyond 6. The decrease in biosorption (%) at higher pH values (>6) could be probably due to the formation of metal hydroxides. A maximum biosorption of metal cations was observed at pH 5.0 ± 0.1. TEM image of the surface-modified MNPs that were synthesized by gum kondagogu is shown in Fig. 14a. The adsorption (%) of various metal cations by the GK − MNP is listed in Fig. 14b. A maximum biosorption (%) of 98.25 ± 1.42 was recorded for the Cd^{2+} ions, and a minimum biosorption of 28.5 ± 0.25 % was observed for the Hg^{2+} ions. The percent biosorption and adsorption capacity of metal cations by the GK − MNP follows the order $Cd^{2+} > Cu^{2+} > Pb^{2+} > Ni^{2+} > Zn^{2+} > Hg^{2+}$ at pH 5.0 ± 0.1 (Table 7). GK − MNP developed in the present study was found to be quite efficient in the rapid biosorption of toxic heavy metal ions as compared to the native form of GK. The biosorption (%), adsorption capacity (mg/g), and equilibrium biosorption time of the GK − MNP are found to be superior, as compared to that of the native gum (Vinod et al. 2009, 2010c).

7.3 Gum Kondagogu–Silver Nanoparticles as a Biosensor for Detection of Mercury Ions

The gum kondagogu–silver nanoparticles have potential applications as biosensors and antimicrobial agents, which have been successfully exploited in our laboratory (Kora et al. 2010; Rastogi et al. 2014). Mercury is a naturally occurring element that is found in air, water, and soil. It exists in several forms: elemental or metallic

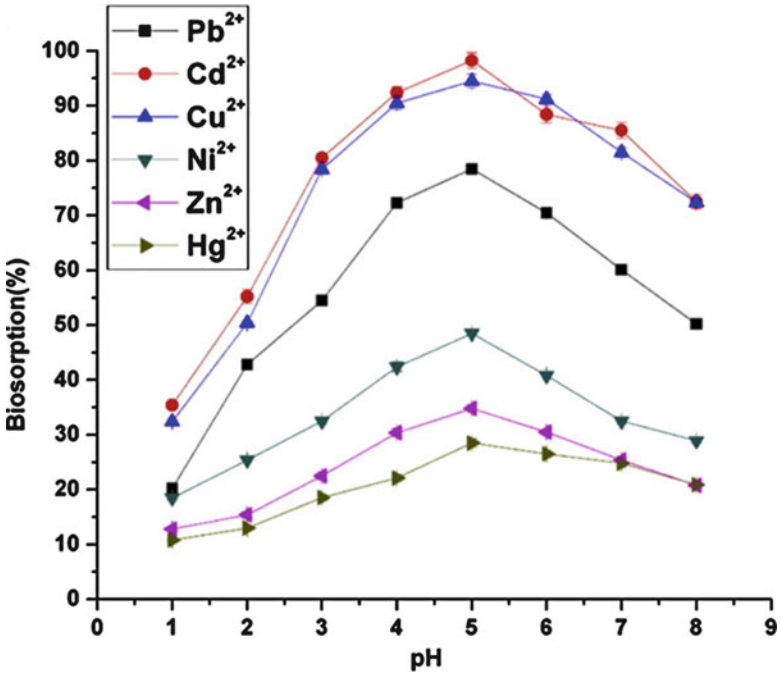


Fig. 13 The effect of pH of the solution on the biosorption of metal cations by the GK − MNP has been investigated at pH values of 1 − 8

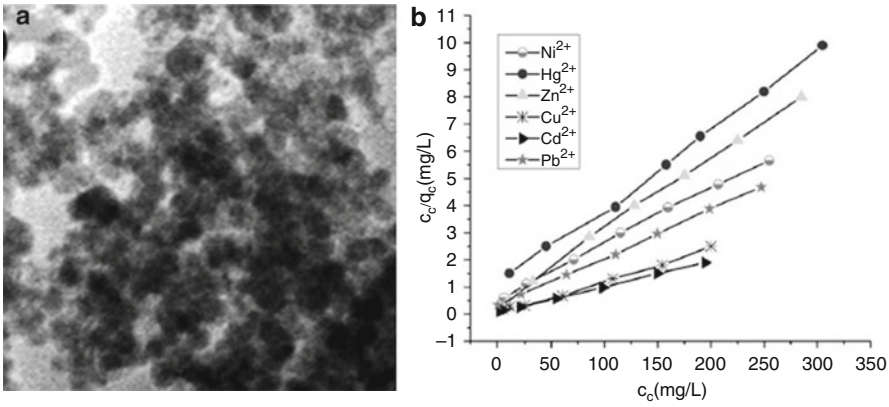


Fig. 14 (**a**) Typical TEM image (scale: 50 nm) of gum kondagogu-magnetic nanoparticles and (**b**) Langmuir isotherm plot of various metal ions illustrating the linear relationship between ratio of equilibrium concentration of metal ions and unit mass of gum kondagogu - magnetic nanoparticles

Table 7 Adsorption (%) of various metal cations by the GK − MNP

Heavy metal cations	Adsorption (%)[a,b]
Cd^{2+}	98.2 ± 1.42
Cu^{2+}	94.5 ± 0.98
Pb^{2+}	78.4 ± 0.54
Ni^{2+}	48.5 ± 0.54
Zn^{2+}	43.8 ± 0.28
Hg^{2+}	28.5 ± 0.25

Conditions: metal ion concentration, 100 mg/L; adsorbent dosage, 0.1 g/100 mL; agitation speed, 200 rpm; temperature, 25 ± 2 °C
[a]Value = mean ± S.D; n = 3
[b]Adsorption$(\%) = \frac{C_i - C_f}{C_i} \times 100$, where C_i and C_f are initial and final metal ion concentration, respectively

mercury, inorganic mercury compounds, and organic mercury compounds (Kim et al. 2011). Major source of mercury emissions are thermal power plant emissions, which are of great environmental hazard because their exposure can affect the human nervous system and harm the brain, heart, kidneys, lungs, and immune system (Bernhoft 2012).

Nanoparticle-based colorimetric probes have attracted lots of attention in the recent past because of their high extinction coefficients (108–1,010 M^{-1} cm^{-1}) often several orders of higher magnitude than those of organic dyes (Otsuka et al. 2001). Most of the nanoparticle-based colorimetric probes were designed by rational modification of nanoparticle surfaces with specific ligands which upon interaction with analyte can change the dispersion/aggregation state of nanoparticles accompanied by color change (Chauhan et al. 2011; Hamaguchi et al. 2010; Radwan and Azzazy 2009; Sugunan et al. 2005). Various colorimetric sensors based on functionalized gold nanoparticles have been developed in the recent past for the selective and sensitive detection of Hg^{2+} (Huang and Chang 2007; Huang et al. 2007; Lin et al. 2010; Sheng et al. 2011; Tanaka et al. 2007; Xue et al. 2008). Although these methods have led to successful development of mercury ion sensors, they suffer from the drawback of high cost of ligands (oligonucleotide probes/fluorescent dyes/selective organic molecule) used and long detection times. Apart from this, Ag NPs are preferred over Au NPs of same size due to their higher extinction coefficient (Lee et al. 2007). A simple ligand-free colorimetric protocol was developed for the facile detection of Hg^{2+} by silver nanoparticles synthesized by gum kondagogu (Rastogi et al. 2014).

A highly sensitive and selective method has been developed for the colorimetric detection of Hg^{2+} in aqueous system by using label-free silver nanoparticles (Ag NPs). Ag NPs used in this method were synthesized by gum kondagogu (GK) which acted as both a reducing and a stabilizing agent. The average size of the GK–Ag NPs was found to be 5.072.8 nm as revealed by TEM analysis, and the nanoparticles were stable at various pH conditions (pH 4–11) and salt concentrations (5–100 mM). The GK reduced/stabilized Ag NPs (GK–Ag NPs) were directly used for the selective colorimetric reaction with Hg^{2+} without any further modification. The bright yellow color of Ag NPs was found to fade in a

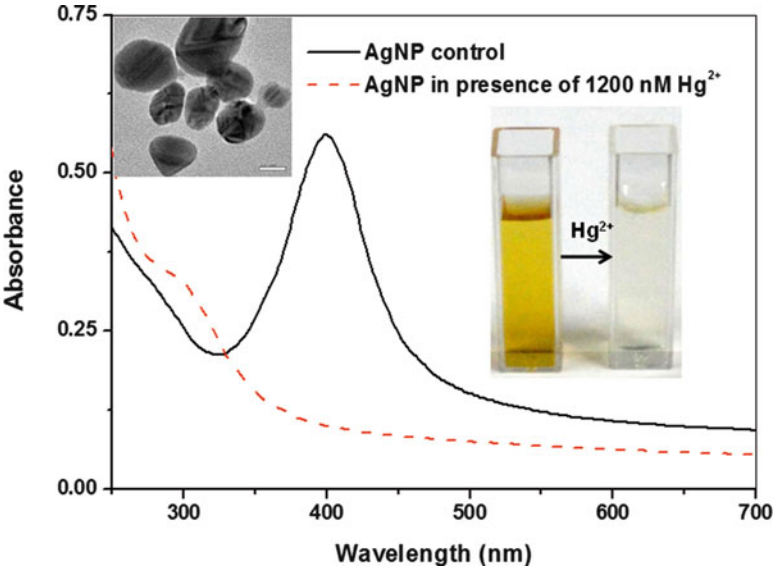


Fig. 15 UV-visible spectra of as synthesized GK–Ag NPs and Ag NPs in the presence of 1,200 nM of Hg^{2+} ions. *Inset*: TEM image of silver nanoparticles (scale: 20 nm) and photograph showing color change of Ag NPs from *yellow* to colorless after Hg^{2+} addition

concentration-dependent manner with the added Hg^{2+} ions. The fading response was directly correlated with increasing concentration of Hg^{2+}. More importantly, this response was found to be highly selective for Hg^{2+} as the absorption spectra were found to be unaffected by the presence of other ions like: Na^{2+}, K^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, As^{2+}, Fe^{2+}, and Cd^{2+} (Fig. 15). The metal-sensing mechanism is explained based on the turbidimetric and XRD analysis of GK–Ag NPs with Hg^{2+}. The proposed method was successfully applied for the determination of Hg^{2+} in various groundwater samples. The reported method can be effectively used for the quantification of total Hg^{2+} in samples, wherein the organic mercury is first oxidized to inorganic form by ultraviolet (UV) irradiation. The limit of quantification for Hg^{2+} using the proposed method was as low as 4.9×10^{-8} mol L^{-1} (50 nM). The proposed method has potential application for on-field qualitative detection of Hg^{2+} in aqueous environmental samples.

8 Gum Kondagogu–Silver Nanoparticles as an Antimicrobial Agent

The methodology developed in the synthesis of gum kondagogu–silver nanoparticles has been successfully exploited for its biomedical applications in our laboratory. Silver nanoparticles are of considerable interest owing to their distinctive electrical, electronic, thermal, optical, magnetic, catalytic, sensing, and

antimicrobial functionalities compared to bulk metal (Wei and Qian 2008). For antimicrobial applications, gum kondagogu–silver nanoparticles were synthesized by autoclaving 0.5 % (w/v) gum along with silver nitrate solutions (1 mM) at 121 °C under 15 psi pressure for 20 min. Under the influence of temperature and pressure, this biopolymer expands and becomes more accessible for the silver ions to interact with the available functional groups of the gum. The large number of hydroxyl and carboxyl groups on this biopolymer facilitates the complexation of silver ions. Subsequently, these silver ions oxidize the hydroxyl groups to carbonyl groups, during which the silver ions are reduced to elemental nanosilver. We have also demonstrated the potential antibacterial activity of the prepared gum nanoparticles on both Gram-positive and Gram-negative bacteria (Kora et al. 2010). As the silver nanoparticles are encapsulated with functional group rich gum kondagogu, they can be easily integrated for various medical and environmental applications.

9 Conclusion

Gum kondagogu is a dried gum exudates obtained from the tree *Cochlospermum gossypium* and other species of *Cochlospermum* belonging to the family Bixaceae. Basically it is a polymer of rhamnose, galacturonic acid, glucuronic acid, β-D-galactopyranose, α-D-glucopyranose, β-D-glucopyranose, galactose, arabinose, mannose, and fructose with sugar linkage of (1 → 2) β-D-Gal*p*, (1 → 6)-β-D-Gal *p*, (1 → 4) β-D-Glc *p* A, 4-0-Me-α-D-Glc *p* A, (1 → 2) α-L-Rha*p*, with average molecular mass of 7.23×10^6 g mol^{-1}. GK is composed of higher uronic acid content, protein, tannin, and soluble fibers. The continuously increasing demand for the commodities produced by chemical industries has generated heavy metal accumulation in the ecosystem. Biosorption is a potentially attractive technology for removal of toxic heavy metals from industrial effluents.

Gum kondagogu (*Cochlospermum gossypium*), a naturally occurring tree biopolymer, is exploited as a biosorbent to remove metal ions from aqueous solutions. The native pH of gum kondagogu being 4.9–5.0 can be exploited as a natural ion exchanger to remove toxic metal from the metal-contaminated water. The removal efficiency of toxic metals by gum kondagogu was determined quantitatively in the order $Cd^{2+} > Cu^{2+}$ $Fe^{2+} > Se^{2+} > Pb^{2+} >$ total Cr $> Ni^{2+} > Zn^{2+} > Co^{2+} > As^{2+}$ at pH 5.0 ± 0.1 and temperature 25 ± 2 °C. The biosorption (%) of various metal ions tested was found to be in the range of 97.3–16.7 %, at pH 5.0. Further, the utility of a novel natural biopolymer (gum kondagogu) obtained as an exudate gum from the tree *Cochlospermum gossypium* in the bioremediation of toxic metal ions [Cd^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+},Ni^{2+}, Zn^{2+}, Hg^{2+}, and Cr (total)] from industrial effluents supports green chemistry principle.

Gum kondagogu has also been explored in conversion of metal ions to metal nanoparticles. The green process for the synthesis of nanoparticles using a nontoxic, renewable natural plant polymer, gum kondagogu, has been used as a reducing and a stabilizing agent for different nanoparticles like Au, Ag, Cu, Pt, Fe, etc. Being a

natural polymer, gum kondagogu is amenable for biodegradation. The synthesis was carried out in aqueous medium without the requirement of any added chemical reducing or capping agent. The gum-based magnetic nanoparticles were successfully used in biosorption of toxic metals. The biosorption of toxic metals enhanced with increasing concentration of toxic metals by using magnetic nanoparticles as compared to that of the native gum. A highly sensitive and selective method has been developed for the colorimetric detection of Hg^{2+} in aqueous system by using label-free silver nanoparticles (Ag NPs). The GK reduced/stabilized Ag NPs (GK–Ag NPs) were directly used for the selective colorimetric reaction with Hg^{2+} without any further modification. The bright yellow color of Ag NPs was found to fade in a concentration-dependent manner with the added Hg^{2+} ions. The fading response was directly correlated with increasing concentration of Hg^{2+}. This method was successfully applied for the determination of Hg^{2+} in various groundwater samples. In addition, the gum nanosilver composite material as a potential antibacterial agent was also explored, and its impeding relevance needs to be further investigated. Their efficacies in biomedical and environmental applications are waiting to be exploited.

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Chitosan

8

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Contents

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Abstract

Chitin is the most abundant natural amino polysaccharide and is next to cellulose in abundance on the planet. Chitosan is obtained by deacetylation of chitin. Chitosan is being researched by academic and industrial scientists as an underutilized resource and as a new functional material of high potential in various fields. The purpose of this chapter is to give an overview of chitosan production, characterization, modification, and applications.

Keywords

Chitin • Chitosan • Polysaccharide • Deacetylation • Antimicrobial • Gel formation • Biopolymer • Active packaging • Metal chelation • Food additive • Biomedical • Biosorbent • Biodegradability

1 Introduction

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages and on hydrolysis give the constituent monosaccharides or oligosaccharides. They have linear to highly branched structure. Their major role in organism is to store energy or to give structural support. Starch and glycogen are examples of storage polysaccharide and cellulose and chitin are examples of structural polysaccharides.

2 Chitin

Henri Braconnot, a French professor, discovered chitin in 1811 and named it fungine. In 1823, Odier found the same material in insects and plants and named it chitin. After cellulose, chitin is the most abundant natural polysaccharide available on the planet. Chitin is similar to cellulose in chemical structure (Fig. 1) and in biological function. Both polymers mainly serve as structural components supporting cell and body surfaces: cellulose strengthens the cell wall of plant cells, whereas chitin contributes to the mechanical strength of fungal cell walls and exoskeletons of arthropods (Rudall and Kenchington 1973).

It has been estimated that at least 1.1×10^{13} kg of chitin is present in the biosphere. However, its use has been limited because it is insoluble in most solvents and relatively difficult to isolate from natural sources in pure form under economically viable conditions. Chitin is a white, hard, inelastic, nitrogenous polysaccharide found in the exoskeleton as well as in the internal structures of invertebrates. It is a linear cationic polymer of *N*-acetylglucosamine residues with β-1,4-linkage. Chitin occurs in three polymorphic forms, α, β, and γ, but α-chitin is the most abundant (Khoushab and Yamabhai 2010). The arrangement of the chains is found to depend on the origin of the chitin. α-Chitin is present in fungal and yeast cell walls, insect cuticles, egg shells of nematodes and rotifers,

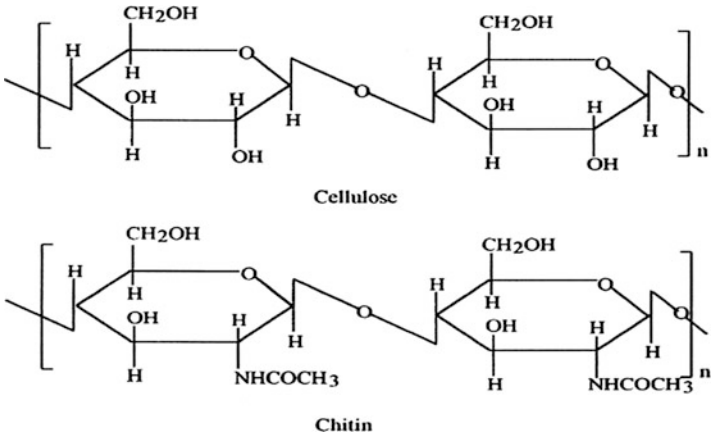

Fig. 1 Chemical structure of cellulose and chitin

the radulae of mollusks, and cuticles of arthropods. This form of chitin is also present in krill, lobster, crab tendons and shells, and shrimp shells, as well as in other marine organisms such as the harpoons of cone snails and the filaments ejected by *Phacocystis* seaweed. β-Chitin is found in the pen and cuticle of squid and the diatom *Thalassiosira fluviatilis*. In α-chitin, sheets are formed by intermolecular hydrogen bonding in parallel chains. Interchain hydrogen bonding occurs between sheets in different directions. There is also intermolecular hydrogen bonding between CH_2OH groups, which is believed to be the cause for the lack of swelling of α-chitin in water. β-Chitin has a monoclinic unit cell with polysaccharide chains attached in a parallel manner (Gardner and Blackwell 1975). In β-chitin, hydrogen bonding occurs only within sheets, not between sheets as in α-chitin. This is thought to be responsible for the swelling of β-chitin, as water can be included between the sheets. γ-Chitin is said to be a combination of α and β structure rather than a third polymorph (Robert 1992). Chitin has <10 % degree of acetylation, 7 % nitrogen content, nitrogen/carbon ratio of 0.146, and molecular weight of 1–2.5 $\times$ 10^6 Da corresponding to a degree of polymerization of ca. 5,000–10,000, which differ in the arrangement of their molecular chains.

During biosynthesis of chitin, monomers of *N*-acetylglucosamine are joined in a reaction catalyzed by the membrane-integral enzyme chitin synthase, a member of the family of glycosyltransferases. The polymerization requires UDP–*N*-acetylglucosamine as a substrate and divalent cations as cofactors. Chitin formation can be divided into three distinct steps. In the first step, the catalytic domain of chitin synthase facing the cytoplasmic site forms the polymer. The second step involves the translocation of the nascent polymer across the membrane and its release into the extracellular space. The third step completes the process as single polymers spontaneously assemble to form crystalline microfibrils of varying diameter and length (Merzendorfer 2006).

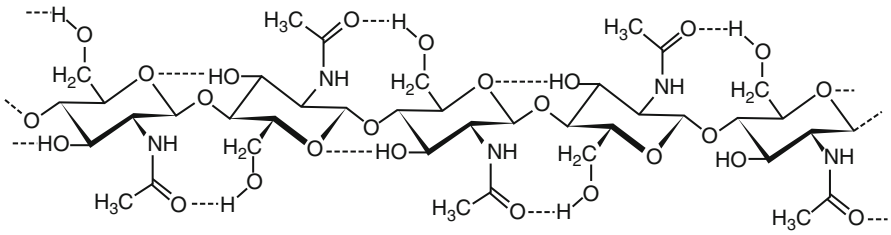

Fig. 2 Chemical structure of chitin shown with its intramolecular hydrogen bonds (*dotted lines*)

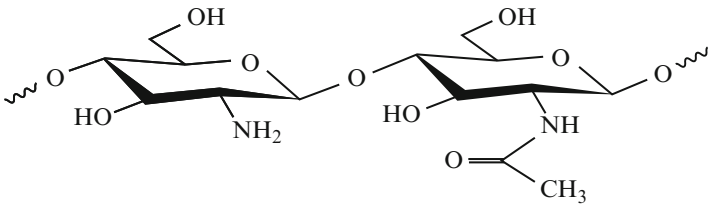

Fig. 3 Chemical structure of chitosan

In the chitin crystal structure, the chains form hydrogen-bonded sheets linked by C=O and H-N-groups. In addition, each chain has intramolecular hydrogen bonds between the neighboring sugar rings: the carbonyl group bonds to the hydroxyl group on C6. There is also a second hydrogen bond between the OH-group on C3 and the ring oxygen, similar to that in cellulose (Minke and Blackwel 1978). This extensive hydrogen bonding shown in Fig. 2 enhances the stiffness of the chitin chain.

With only one known exception, the chitin of diatoms, chitin is found in nature cross-linked to other structural components. The chitin microfibrils combine with other sugars, proteins, glycoproteins, and proteoglycans to form fungal septa and cell walls as well as arthropod cuticles and peritrophic matrices, notably in crustaceans and insects (Kozloff 1990). In animals, chitin is associated with proteins, while in fungal cell wall it is associated with glucans, mannans, or other polysaccharides. In fungal walls, it is found covalently bound to glucans, either directly or via peptide bridges (Roberts 1992). In insects and other invertebrates, the chitin is always associated with specific proteins, with both covalent and non-covalent bonding, to produce the observed ordered structures.

3 Chitosan

Chitosan is obtained by deacetylation of chitin. It consists of D-glucosamine linked to *N*-acetyl D-glucosamine by β-1,4-glycosidic bond (Fig. 3). The distribution of these subunits depends on the method of preparation of chitosan. In chitosan, degree of deacetylation ranges from 40 % to 98 % and the molecular weight ranges between 5×10^4 Da and 2×10^6 Da.

Intense research and development work is being carried out on chitosan as it is considered to be a material of great futuristic potential with immense possibilities for structural modifications to impart desired properties and functions. The presence of reactive amino groups at C2 atom and the hydroxyl group at atom C3 and C6 on chitosan is useful in a wide application in various industries. The positive attributes of excellent biocompatibility and admirable biodegradability with ecological safety and low toxicity with versatile biological activities such as antimicrobial activity and low immunogenicity have provided ample opportunities for further development.

4 Sources

Chitosan can be extracted from insects, yeast, mushroom, cell wall of fungi, and marine shellfish such as crab, lobster, krill, cuttlefish, shrimp, and squid pens (Table 1). In shellfish, chitin forms the outer protective coating as a covalently bound network with proteins and some metals and carotenoids. Shrimps are in general sold headless and

Table 1 Contents of chitin in different commercially important organism

Organism	W (chitin)%
Cancer (crab)	72.1[a]
Carcinus (crab)	64.2[b]
Paralithodes (king crab)	35.0[b]
Callinectes (blue crab)	14.0[c]
Crangon and *Pandalus* (shrimp)	17–40
Alaska shrimp	28.0[d]
Nephro (lobster)	69.8[a]
Homarus (lobster)	60–75[a]
Lepas (goose barnacle)	58.3[a]
Bombyx (silk worm)	44.2[a]
Mollusks	
Clam	6.1
Shell oysters	3.6
Squid pen	41.0
Krill, deproteinized shells	40.2
Fungi	
Penicillium notatum	18.5[e]
Penicillium chrysogenum	20.1[e]
Mucor rouxii	44.5
Lactarius vellereus	19.0

Adapted from Jo et al. (2011) and Kurita (2006)
[a]Based on the mass of the organic cuticle
[b]With respect to the body dry mass
[c]Compared to the body fresh mass
[d]Compared to the total mass of the cuticle
[e]Relative to the dry mass of the cell wall

often peeled of the outer shells and tail. Crustacean shells consist of 30–40 % proteins, 30–50 % calcium carbonate, and 20–30 % chitin and also contain pigments (astaxanthin, canthaxanthin, lutein, and β-carotene). These proportions vary with species and with seasons. Shrimp, prawn, and crab wastes are the principal source of commercial chitin and chitosan production. The increase in consumption of shellfish and the expansion of aquaculture have led to a tremendous increase in the quantity of shrimp and prawn being processed and hence in the amount of waste available for chitin/chitosan production. Using mycelium waste from fermentation processes as a source of chitin and chitosan still remains a vast and as yet untapped potential source.

5 Production of Chitosan

Majority of chitosan available globally is produced by the USA, Japan, Norway, Thailand, India, Australia, and Poland. The production of chitosan involves various steps such as preparation of the chitin from the biological material followed by the deacetylation that would result in chitosan. Thus, typical production of chitosan from crustacean shell generally consists of four basic steps: demineralization, deproteinization, decoloration, and deacetylation. Demineralization and deproteinization steps are interchangeable in terms of order. The exoskeleton of crustacean is a major starting material used for commercial production of chitosan. Typical flow chart for manufacture of chitosan is given below (Fig. 4).

5.1 Demineralization

The mineral content in the exoskeleton of crustacean is not the same for all species of crustaceans. Demineralization is generally carried out using acids such as hydrochloric acid, nitric acid, acetic acid, or formic acid (up to 10 %) at room temperature with agitation to dissolve calcium carbonate as calcium chloride. However, hydrochloric acid is the preferred acid and is used at a concentration of 0.2–2 M for 1–48 h at temperatures varying from 0 °C to 100 °C. Demineralization for 1–3 h using dilute (1–8 %) hydrochloric acid at room temperature produces appreciable amounts of calcium chloride. A solid-to-solvent ratio of 1:15 (w/v) is usually used. The ash content of the demineralized shell is an indicator of the effectiveness of the demineralization process.

5.2 Deproteinization

Chitin occurs naturally in association with protein. The protein is bound by covalent bonds to chitin through aspartyl or histidyl residues, or both, thus forming stable complexes (Attwood and Zola 1967). Deproteinization of chitin is usually carried out by alkaline treatment. The shells are treated with sodium or potassium hydroxide at 65–100 °C at a minimum shell-to-alkali ratio of 1:4 for periods ranging from 1 to 12 h. Under these conditions, the protein becomes detached from the solid

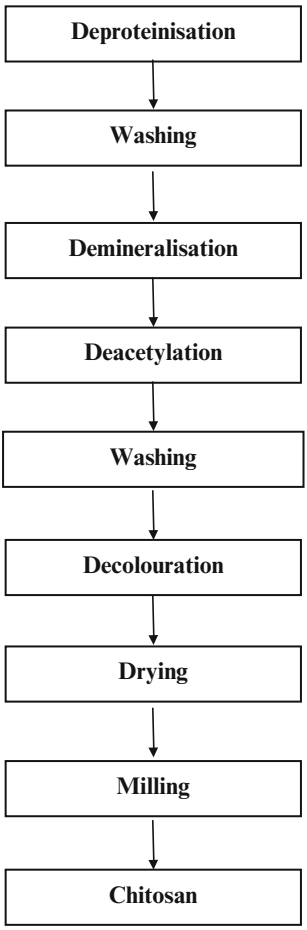

Fig. 4 Flow chart for chitosan production

component of the shrimp waste. Relatively high ratios of solid-to-alkali solution of 1/10 or 1/20 with proper agitation are used to increase the deproteinization efficiency. To prevent oxidation of the products, the process is usually carried out in a nitrogen atmosphere and in the presence of sodium borohydride ($NaBH_4$). After completion of deproteinization step, the protein hydrolysate is removed easily by separation of the solids from the protein slurry by filtration. Prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation.

5.3 Decoloration

Chitin obtained after the demineralization and deproteinization of shell waste is a colored product. For commercial acceptability, the chitin needs to be decolorized or bleached to yield cream white chitin powder (No et al. 1989). The pigment in the

crustacean shells forms complexes with chitin. Fox (1973) found one 4-keto-ß-carotene and three 4, 4′-diketo-ß-carotene derivatives firmly bound to the exoskeletal chitin of red kelp crab. The level of association of chitin and pigments varies from species to species among crustacean. The residues are decolorized using solvents and/or oxidants (Acosta et al. 1993). During the process of decoloration, the chemical used should not affect the physicochemical or functional properties of chitin and chitosan. No et al. (1989) were able to prepare a near-white-colored crawfish chitin by extraction with acetone, which was dried for 2 h at ambient temperature, followed by bleaching with 0.315 % (v/v) sodium hypochloride solution (containing 5.25 % available chlorine) for 5 min with a solid-to-solvent ratio of 1:10 (w/v), based on dry shell.

5.4 Deacetylation

Deacetylation is the process to convert chitin to chitosan by removal of acetyl group. There are several critical factors that affect the extent of deacetylation including temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, atmosphere (air or nitrogen), ratio of chitin to alkali solution, density of chitin, and the particle size. Considering all these as necessary conditions, the ideal process condition of deacetylation should yield a chitosan that is not degraded and is soluble in dilute acetic acid in minimal time (Muzzarelli et al. 1980). The *N*-acetyl groups cannot be removed by acidic reagents without hydrolysis of the polysaccharide, thus, alkaline methods must be employed for *N*-deacetylation (Muzzarelli 1977). Severe alkaline hydrolysis treatments are required due to the resistance of groups imposed by the trans arrangement of the C2-C3 substituents in the sugar ring. It is generally achieved by treatment with concentrated sodium or potassium hydroxide solution (40–60 %) usually at 80–140 °C for 30 min or longer using a solid-to-solvent ratio of 1:10 (w/v) to remove some or all of the acetyl groups from the polymer (No and Meyers 1989). Sodium hydroxide is the preferred alkali. After deacetylation, the chitosan is washed to completely remove alkali and is dried to give flakes. The material should be low in protein and ash. Production of chitosan by chemical processes has several disadvantages such as environmental pollution, inconsistent molecular weights, and degree of acetylation.

6 Novel Methods for Preparation of Chitosan

The conventional harsh conditions used for extraction could adversely affect the quality of the chitin. Novel methods are being developed to replace conventional demineralization and deproteinization to extract chitin from crustacean waste. The use of enzymes in the deproteinization step has been extensively studied. Shrimp waste deproteinized using *Aspergillus niger*, washed, dried, and then demineralized using acetic or lactic acid produced by fermentation from low cost biomass such as

cheese whey, has been reported (Rinaudo 2006). A number of microorganisms such as *Bacillus subtilis, Lactobacillus helveticus, Pseudomonas aeruginosa, Lactobacillus paracasei, Lecanicillium fungicola*, and *Penicillium chrysogenum* have been utilized for demineralization (Choorit et al. 2008; Oh et al. 2008). These microorganisms are responsible for the precipitation of organic salts such as calcium lactate, which is easily removed from media by wash out. Deproteinization is also carried out with the aid of proteolytic activities of some microorganisms. The calcium, magnesium, and potassium acetates obtained as by-products are suggested as possible de-iceing agents, while the calcium and potassium lactates could find applications as food preservatives. Enzymatic deacetylation by using fungal chitin deacetylase also has commercial potential.

7 Characterization of Prepared Chitosan and Its Properties

7.1 Molecular Weight

One of the most fundamental parameters characterizing a macromolecule is its molecular weight. Knowledge of the molecular weight of polysaccharides is of fundamental importance for the understanding of their applications and their role in living systems. The molecular weight of chitosan depends largely on the conditions of deacetylation and can be determined by methods such as chromatography (Bough et al. 1978), light scattering (Muzzarelli 1977), and viscometry (Maghami and Roberts 1988). Viscometry is the simplest and most popular method to determine molecular weight of chitosan. The method however has the disadvantage of not being absolute because it relies on the correlation between the values of intrinsic viscosity with those of molecular weight. Chitosan is available commercially with molecular weight ranging from 10,000 to 1,000,000 Da.

7.2 Viscosity

Viscosity of chitosan increases with increase in its molecular weight and concentration. Increasing the degree of deacetylation also increases the viscosity (Skaugrud 1991). This can be explained by the fact that high and low deacetylated chitosan have different conformations in aqueous solution. Chitosan has an extended conformation with a more flexible chain when it is highly deacetylated because of the charge repulsion in the molecule. However, the chitosan molecule has a rod-like shape or coiled shape at low degree of deacetylation due to the low charge density in polymer chain. The viscosity of chitosan solution is also affected by factors such as concentration and temperature. As the chitosan concentration increases and the temperature decreases, the viscosity increases. Chitosan viscosity decreases with an increased time of demineralization due to depolymerization (Moorjani et al. 1975). Similarly, No et al. (1999) demonstrated that chitosan viscosity is considerably affected by physical (grinding, heating, autoclaving,

ultrasonication) and chemical (ozone) treatments. Viscosity of chitosan solution stored at 4 °C is found to be relatively stable.

7.3 Solubility

Solubility characteristics of chitosan are based on its degree of deacetylation. High degree of deacetylation shows higher solubility, and low degree of deacetylation shows poor solubility (Heux et al. 2000). It has swelling characteristics due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains. Chitosan solubility depends on the amount of protonated amino groups in the polymeric chain and, therefore, on the proportion of acetylated and non-acetylated D-glucosamine units. Its cationic nature is unique relative to other neutral or negatively charged polysaccharides. Chitosan is a strong base possessing primary amino group with a pKa value of 6.3. The pH of solution substantially alters the charged state and properties of chitosan (Yi et al. 2005). At low pH, the amines get protonated and become positively charged and that makes chitosan a water-soluble cationic polyelectrolyte. On the other hand, as the pH increases above 6, chitosan amines become deprotonated, and the polymer loses its charge and becomes insoluble. At higher pH, precipitation or gelation tends to occur, and the chitosan solution forms poly-ion complex with anionic hydrocolloid resulting in gel formation (Kurita 1998). The soluble–insoluble transition occurs at its pKa value around pH between 6 and 6.5. Chitosan can easily form quaternary nitrogen salts at low pH values. So, organic acids such as acetic, formic, and lactic acids can dissolve chitosan. The most commonly used solvent for chitosan is 1 % acetic acid at about pH 4.0 (Rinaudo et al. 1999). Chitosan is also soluble in 1 % hydrochloric acid and dilute nitric acid but insoluble in sulfuric and phosphoric acids. Thus, solubility of chitosan is related to the degree of deacetylation, the ionic concentration, pH, the nature of the acid used for protonation, and the distribution of acetyl groups along the chain, as well as the conditions of isolation and drying of the polysaccharide. The high molecular weight of chitosan, which results in poor solubility at neutral pH and its high solution viscosity, limits its use in the food, cosmetics, agriculture, and health industry (Xia et al. 2011).

7.4 Degree of Deacetylation

Degree of deacetylation (DD) has often been cited as an important parameter that determines many physiochemical and biological properties of chitosans such as crystallinity, hydrophilicity, degradation, and cell response. Degree of deacetylation of chitosan is generally controlled by processing of the native polymer with alkali and with increasing time and temperature to obtain the highest degree of deacetylation (>90) materials. During the deacetylation reaction, the acetyl group of the chitin reacts with NaOH and produces an amine group. This is a reversible reaction, and when NaOH concentration is increased, the reaction is biased toward the forward

direction by producing more chitosan. As a result, deacetylation will increase. In the deacetylation process, acetyl groups are removed from the polymers randomly, resulting in a final polymer that has a random distribution of acetyl glucosamine and glucosamine units. The biopolymer is characterized as either chitin or chitosan according to the deacetylation which is determined by the proportion of D-glucosamine and *N*-acetyl D-glucosamine. Various methods have been reported for the determination of the degree of deacetylation of chitosan such as (1) spectroscopy (infrared, ultraviolet, or 1H, 13C, 15N nuclear magnetic resonance), (2) conventional methods (various types of titration, conductometry, potentiometry, ninhydrin assay, adsorption of free amino groups of chitosan by picric acid), and (3) destructive methods (elemental analysis or acid or enzymatic hydrolysis of chitin or chitosan) followed by colorimetric methods or high-performance liquid chromatography, pyrolysis gas chromatography, and thermal analysis using differential scanning calorimetry. Of these, 1H NMR has been found to be simple, rapid, and more precise than many of the other methods (Rinaudo 2006).

7.5 Crystallinity

One of the major physical characteristics that determine the functional properties of chitosan is the crystallinity (Trang et al. 2006). Crystallinity has been found to have an effect on metal sorption. Piron et al. (1997) found that the crystallinity of chitosan controlled the sorption rate and total uptake of uranyl, concluding that sorption was only possible in the amorphous domains and not in the crystalline domains. The crystallinity of the polymer can also control the accessibility of the amine groups (Guibal 2004). The crystallinity of chitosan is determined by X-ray diffraction (XRD) in which the pattern produced by the diffraction of X-rays through the closely spaced lattice of atoms in a crystal is recorded and then analyzed to reveal the nature of the lattice.

7.6 Complex Formation with Metals

Chitosan exhibits superior metal ion sequestering ability than chitin. It has reactive amino group and hydroxyl group and chelates many transition metal ions. Chelation is related to the amino content as well as to the distribution of the amino group. The nature of the cation is very important in the mechanism of interaction (Rhazi et al. 2002). Various processes such as adsorption, ion exchange, and chelation have been considered as the mechanisms responsible for complex formation between metal ions and chitosan. The type of interaction prevailing depends on the metal, its chemistry, and the pH. Under heterogenous conditions, at pH less than 6, chitosan acts as a poly(monodentate) ligand, while at a higher pH, it behaves as a poly(bidentate) ligand forming chelates. However, in solution, the formation of complexes in which two amino groups belonging to the same chain or different chains coordinated to the same metal ion can also take place.

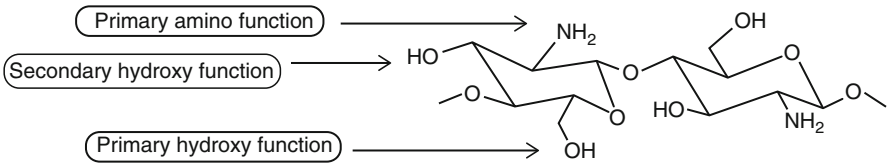

Fig. 5 Functional groups in chitosan that can be modified

8 Modifications of Chitosan

Chitosan can be modified to improve its physicochemical properties to suit various applications. Modification of chitosan is possible due to the presence of several functional groups in the polymer (Fig. 5). It has both reactive amino and hydroxyl groups that can be used to chemically alter its properties under mild reaction conditions The main goals of modifying chitosan are to provide derivatives that are soluble at neutral and basic pH values; to control hydrophobic, cationic, and anionic properties; as well as to attach various functional groups and ligands (Mourya and Inamdar 2008). Strong intramolecular and intermolecular hydrogen bonds exist in chitosan to form random orientations. The dissociation and reorganization of these hydrogen bonds by chemical modification facilitate the production of novel molecular conformations in the forms of solutions, hydrogels, fibers, films, and sponges (Tokura et al. 1996).

8.1 Acylation

A variety of acylation reactions are possible with chitosan. Acylation with long chain aliphatic carboxylic acid chlorides such as hexanoyl, dodecanoyl, and tetradecanoyl chlorides give derivatives with a high degree of acylation. *N*-acylation of chitosan with fatty acid (C6–C16) chlorides increased its hydrophobic character. Such acylated products are soluble in chloroform (Fujii et al. 1980). Chitosan with a higher degree of deacetylation is more susceptible for acylation owing to a decrease in hydrogen bonding. *N*-acyl chitosan has the ability for longer retention in body and resistance to digestible enzymes like lysozyme and chitinase and is more biocompatible than native chitosan (Hirano and Yagi 1980).

8.2 Graft Copolymerization

Graft copolymerization reaction introduces side chains and makes various molecular designs possible, thus affording novel types of tailored hybrid materials composed of chitosan and synthetic polymers. The properties of the graft copolymers can be controlled by molecular structure, length, and number of side chains attached. Grafting of chitosan allows the formation of functional derivatives by

covalent binding of a molecule, the graft, onto the chitosan backbone. The swelling behavior of chitosan at different pH has been improved by graft polymerization of vinylic monomers such as acrylic acid, acrylamide, and acrylonitrile onto chitosan (Borzacchiello et al. 2001; Mahdavinia et al. 2004). Super absorbents (absorb aqueous solutions up to hundreds of times their own dry weight) have been prepared by grafting these resins with chitosan (Nge et al. 2004) and have possible applications in infant diapers, feminine hygiene products, agriculture, and other specialized areas (Dutkiewicz 2002). Different types of chitosan graft copolymers have been prepared for use as flocculants, paper-binder strengtheners, and slow-release drug carrier. Polyethylene glycol (PEG) has been grafted onto chitosan to prepare water-soluble chitosan derivatives that have been used as carrier of anticancer drugs. Phosphorylated chitosan synthesized by grafting mono(2-methacryloyl oxyethyl) acid phosphate onto chitosan improved antimicrobial activities (Jung et al. 1999).

8.3 Carboxymethyl Chitosans

It is an amphoteric polymer, is a derivative of chitosan, and is prepared under controlled reaction conditions. It can be synthesized by reductive alkylation wherein the amino group of chitosan is reacted with the carbonyl group of aldehyde glyoxylic acid and then hydrogenated by reaction with $NaBH_4$ or $NaCNBH_3$ to give carboxymethyl chitosans. It can also be prepared by direct alkylation using monohalocarboxylic acids such as monochloroacetic acid in alkaline medium. Carboxymethyl chitosans have enhanced biological and physicochemical properties compared to chitosan and hence have promising biomedical applications (Mohan et al. 2012).

8.4 *N*-methylene Phosphonic Chitosans

These are anionic derivatives with amphoteric character and are synthesized under various conditions and proved to have good complexing efficiency for cations such as Ca^{2+} and those of transition metals (Cu (II), Cd (II), Zn (II), etc.) (Heras et al. 2001). The complexation provides corrosion protection for metal surfaces. These derivatives are also modified and grafted with alkyl chains to obtain amphiphilic properties that have potential applications in cosmetics.

8.5 Carbohydrate-Branched Chitosan

Carbohydrates can be grafted on the chitosan backbone at the C2 position by reductive alkylation: disaccharides such as cellobiose and lactose (having a reducing end group) are introduced, in the presence of a reductant, on chitosan in the open chain form. These derivatives are water soluble. Carbohydrates can also be

introduced without ring opening on the C6 position. These derivatives are important as they are recognized by the corresponding specific lectins and thus could be used for drug targeting (Morimoto et al. 2001).

8.6 Alkylated Chitosans

Alkylated chitosans are very important as amphiphilic polymers based on polysaccharides. They exhibit surface activity and increase considerably the viscosity of aqueous solution due to hydrophobic interchain interactions. Alkyl chitosans are compatible with neutral and cationic surfactants (Yang et al. 2002).

9 Chitosan Depolymerization

The main limitations in the use of chitosan in several applications are its high viscosity and low solubility at neutral pH. Low molecular weight chitosans and oligomers can be prepared by hydrolysis of the polymer chains. For some specific applications, these smaller molecules have been found to be much more useful (Rege and Block 1999). Chitosan depolymerization can be carried out chemically, enzymatically, or physically.

9.1 Chemical Depolymerization

It is mainly carried out by acid hydrolysis using HCl or by oxidative reaction using HNO_2 and H_2O_2 (Prashanth and Tharanathan 2007). It has been found to be specific in the sense that HNO_2 attacks the amino group of D-units, with subsequent cleavage of the adjacent glycosidic linkage.

9.2 Enzymatic Depolymerization

In the case of enzymatic depolymerization, low molecular weight chitosan with high water solubility is produced by several enzymes such as chitinase, chitosanase, gluconase, and some proteases (Cabrera and Cutsem 2005). Nonspecific enzymes including lysozyme, cellulase, lipase, amylase, and pectinase that are capable of depolymerizing chitosan are also used. Enzymatic methods for the hydrolysis of chitosan are performed in gentle conditions, and the molecular weight distribution of the product can be controlled (Jeon et al. 2001).

9.3 Physical Depolymerization

Physical depolymerization yielding dimers, trimers, and tetramers has been carried out by radiation (Co-60 gamma rays) but low yields have been achieved. High-pressure homogenization is a novel method employed for the depolymerization of

chitosan (Mistry et al. 2012). Chitosan has been physically modified in a variety of ways, resulting in conditioned forms such as powders, nanoparticles, gel beads, gels, fibers, and sponge (Denkbas 2006).

10 Applications of Chitosan

A lot of research is being carried out by both academic and industrial scientists on applications of chitosan. This can be seen by a number of relevant research papers and patents on the subject. Chitosan and its derivatives have varied applications in agriculture, food processing, biotechnology, chemistry, cosmetics, dentistry, medicine, textiles, veterinary medicine, and environmental sciences. The polyelectrolyte nature and the presence of reactive functional groups are responsible for the gel-forming ability, high adsorption capacity, biodegradability, and antimicrobial properties of chitosan which in turn are essential for its commercial applications.

11 Antimicrobial Activity

Chitosan displays a broad-spectrum antimicrobial activity against bacteria, molds, and yeasts. It is effective against both Gram-positive and Gram-negative foodborne microorganisms, including *Aeromonas hydrophila*, *Bacillus cereus*, *B. licheniformis*, *B. subtilis*, *Clostridium perfringens*, *Brochothrix* spp., *Enterobacter sakazakii*, *Lactobacillus* spp., *Listeria monocytogenes*, *Pseudomonas* spp., *Salmonella typhimurium*, *S. enteritidis*, *Serratia liquefaciens*, *Staphylococcus aureus*, and *Escherichia coli* O157H7; the yeasts *Candida*, *Saccharomyces*, and *Rhodotorula*; and the molds *Aspergillus*, *Penicillium*, and *Rhizopus*. The chitosan and its derivatives are effective against plant pathogenic bacteria such as *A. tumefaciens, C. fascians, E. amylovora, E. carotovora, P. solanacearum*, and *S. lutea* and fungi *A. alternata*, *B. fabae*, *F. oxysporum*, *P. digitatum*, *P. debaryanum*, and *R. solani* (Vishnukumar et al. 2005; Venugopal 2011).

The exact mechanism of antibacterial activity of chitosan is not fully understood and several factors contribute toward this. Three models have been proposed, to explain the antimicrobial action of chitosan. The most satisfactory model suggests that the antimicrobial effect of chitosan is due to its polycationic nature. In an acid environment, the NH_2 groups in the C2 position of chitosan protonates to yield NH_{3+}, which binds to negatively charged carboxylate (–COO–) groups located on the surface of the bacterial and fungal cell surfaces, causing disruption of the barrier properties of the outer membranes of the microorganisms followed by leakage of cell components (Tsai and Su 1999). This hypothesis is supported by electron microscopy studies that show binding of chitosan to outer membrane of bacteria (Raafat et al. 2008). The pH of the microenvironment in which chitosan functions determines the relative concentrations (ratios) of unprotonated and protonated amino groups. At a pH ~ pKa, 50 % of amino group are protonated. At pH 5.5, the positively charged amino group contributes 90 %, and at pH 4.5, 99 %.

The antimicrobial effectiveness of chitosan appears to be highest below pH 6.0, where the protonated form predominates and where chitosan is most soluble.

Second proposed mechanism is based on ability of chitosan to bind with microbial DNA, leading to inhibition of the mRNA and protein synthesis (Sebti et al. 2005). In this hypothesis, chitosan molecules are assumed to be able to pass through the bacterial cell wall, composed of multilayers of cross-linked murein, and reach the plasma membrane. This theory is supported by confocal laser scanning microscopy where the presence of chitosan oligomers (a chain with few number of monomer units) inside *E. coli* exposed to chitosan under different conditions has been demonstrated (Lui et al. 2001).

The third mechanism is based on ability of chitosan to chelate metals. It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation; this results in reduced microbial growth and toxin synthesis (Goy et al. 2009). This mechanism is likely to be more efficient at high pH values where positive ions are bounded to chitosan, since the amine groups are unprotonated and the electron pair on the amine nitrogen is available for donation to metal ions.

The ability of chitosan to form gas-impermeable coating interferes with fungal growth. It inhibits different developmental stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors (El- Ghaouth et al. 1992).

The derivatives of chitosan, such as *N*-trimethyl, sulfonated chitosan, and chitose oligomers, have been reported to demonstrate antibacterial activities against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, and *Proteus vulgaris* to different extents (Venugopal 2011).

11.1 Factors Affecting Antimicrobial Activity

The antimicrobial activity of chitosan depends on its molecular weight, degree of deacylation, pH of solution, and, of course, the target organism.

Molecular weight: The antimicrobial activity of chitosan increases as the molecular weight increases. However, it is difficult to find a clear correlation between molecular weight and antimicrobial activity of chitosan when comparisons are between different studies. This is mainly attributed to the fact that many investigators have used an uncertain term for low MW (LMW) and high MW (HMW) chitosan without indicating exactly its MW. There are reports that conclude positive, negative, and neutral effects of MW on antimicrobial activity of chitosan (Badawy and Rabea 2011).

Degree of deacetylation: The antimicrobial activity of chitosan is directly proportional to the degree of deacetylation of chitosan. The increase in degree of deacetylation means the increased number of amino groups on chitosan. As a result, chitosan has an increased number of protonated amino groups in an acidic condition

and dissolves in water completely, which leads to an increased chance of interaction between chitosan and negatively charged cell walls of microorganisms (Sekiguchi et al. 1994).

The pH: The antimicrobial activity of chitosan is strongly affected by the pH. At lower pH, there is an increase in the number of protonated amino groups on chitosan in addition to the "hurdle effect" of inflicting acid stress on the target organisms (Badawy and Rabea 2011).

Temperature: The incubation temperature also has an effect on the antimicrobial activity of chitosan. Higher temperature (37 °C) has been shown to enhance its antimicrobial activity compared to refrigeration temperatures (Kong et al. 2010).

Cations: Antimicrobial action of chitosan is inhibited by divalent cations in the order of $Ba^{+2} > Ca^{+2} > Mg^{+2.}$ It is proposed that the cations form complexes with chitosan and consequently the reduced available amino groups of chitosan lead to the reduced bactericidal effect (Badawy and Rabea 2011).

Chitosan posses a number of characteristics that make it a suitable antimicrobial polymer for various industrial applications. These include the following: (1) easy and abundant availability, (2) long-term storage stability at the temperature of its intended application, (3) it does not decompose to and/or emit toxic products, (4) it is not toxic or irritating to handlers, and (5) it is biocidal to a broad-spectrum of pathogenic microorganisms.

12 Antioxidant Activity

Chitosan and its derivatives have been reported to have strong antioxidant activity. They control lipid oxidation by scavenging free radicals, which can be attributed to their ability to chelate metals. The antioxidant effects of chitin and chitosan are dependent on their molecular weight, viscosity, and degree of deacetylation (Venugopal 2011).

13 Chitosan as Edible Coating for Fruits and Vegetables

The edible films and coatings are used to extend shelf life and improve quality of food products. At present, edible films based on cellulose and proteins are being used for the purpose. They provide good reduction of O_2 and CO_2 partial pressure but are not so good for moisture transfer between food and the surrounding environment. Chitosan forms tough, long-lasting, flexible, semipermeable films that can be used as food wraps for extending their shelf life.

Fruits and vegetables undergo a number of physiological changes during postharvest storage. These include tissue softening, increase in sugar levels, degradation of chlorophyll, and synthesis and degradation of volatile flavor compounds. Controlling respiration rate significantly improves the storability and shelf life of fresh produce, as a certain level of respiration activity is required to prevent plant tissues from senescing and dying. In minimally processed agricultural

products, the most important quality attributes contributing to marketability are appearance, color, texture, flavor, nutritional content, and microbial quality. The marketability of these products, therefore, demands efficient control of these quality changes. Due to its barrier properties, chitosan film can prevent moisture loss and drip formation, retain color and flavor attributes, and improve microbial quality, thereby extending the shelf life of a variety of fruits and vegetables. Rather than packaging produce within a chitosan film, dipping the produce in a dilute solution of chitosan and dilute acetic acid can be performed. The technique also allows the incorporation of additives such as vitamin E, rosemary, oleoresin, calcium, and potassium to enhance the efficiency of treatment (Aider 2010). The efficacy of treatment is demonstrated in strawberries, bell peppers, cucumbers, peaches, pears, and kiwifruit (Bautista-Banosa et al. 2006).

14 Chitosan as Functional Additive in Muscle Foods

Chitosan is used as an additive in flesh foods to control flavor loss, microbial growth, and oxidation resulting in extended shelf life. When cooked flesh foods are stored, a "warmed-over" flavor develops which is perceived as loss of freshness. Chitosan is capable of preventing this flavor deterioration due to its antioxidant activity (No et al. 2007; Venugopal 2011). *N*-carboxymethyl chitosan (NCMC) and its lactate and acetate derivatives are effective in controlling the oxidation and off-flavor development in cooked meat at refrigerated temperatures. Research by the US Department of Agriculture has revealed that NCMC is useful as preservative in flesh foods. It can be sprinkled on gravies or meat products. NCMC is very useful in preserving microwavable or quickly prepared foods as well as in preventing development of the "warmed-over" flavor of institutional foods. It is advantageous to use as it is itself tasteless, blends well with foods as a colorless ingredient, and is nontoxic and nonallergenic. It is used as a glazing compound prior to flash-freezing of many flesh foods to inhibit surface oxidation and enhance shelf life. Meat and poultry processors use NCMC as a post-slaughter perfusion and as a long-term flavor and storage preservative (Flick and Martin 2000). Textural properties of surimi products can also be improved by addition of chitosan in combination with other additives (Benjakul et al. 2001; Gomez-Guillien et al. 2005).

Chitosan as coating for eggs: Chitosan coating of eggs can provide a protective barrier against moisture and CO_2 transfer from the albumen through the egg shell, thus extending the shelf life of eggs. It prevents weight loss and enhances Haugh unit and yolk index values, indicating improved albumen and yolk quality of eggs, respectively. The coated eggs can be preserved for up to 5 weeks at 25 °C, which is at least 3 weeks longer than that observed for control, uncoated eggs. Overall consumer acceptability of coated eggs did not differ from that for control and commercial eggs (Bhale et al. 2003).

Chitosan as additive in bakery and dairy products: Chitosan and chitin can be used as food additives in cookies, noodles, and bread to improve their texture. These effects are due to the ability of chitosan to control starch retrogradation.

Microcrystalline chitin has a positive effect on emulsion stability, in addition to increasing the specific loaf volume of white bread and protein-fortified breads (No et al. 2007). Maillard reaction products (MRPs) prepared from chitosan and xylose extend the shelf life of fresh noodles (Huang et al. 2007). Chitosan–lysozyme (CL) film is reported to prevent growth of *Listeria monocytogenes*, *Escherichia coli*, or *Pseudomonas fluorescens* in pre-inoculated mozzarella cheese (Duan et al. 2007).

15 Chitosan as Clearing Agent in Wines and Vinegars

Browning due to oxidation is one of the most common defects affecting white wines. It can be minimized by using adsorbents to reduce phenolic compounds. Chitosan is useful for the clarification of wine and vinegars. It exhibits high affinity to a number of phenolic compounds, particularly cinnamic acid, and prevents browning in a variety of white wines (Spagna et al. 1996).

16 Chitin as Feed Additive

Chitin has a growth-promoting effect on broiler chickens. It increases average live weight and dressed weight and decreases wastage during dressing in broiler chickens. The use of chitin as a source of dietary fiber in chicken feed promotes the growth of bifidobacteria in the guts (Hirano et al. 1990). Similarly, feeds containing chitin and glucosamine could also be used in aquaculture for improved growth of cultured fish (Kono et al. 1987). Chitin hydrolysates produced through the digestion of crustacean waste by chitinases are used as a carbon source for the cultivation of yeast that can convert chitin oligosaccharides into single-cell proteins (Carroad and Tom 1978). The yeast could be utilized as feed component.

17 Chitosan as Lipid-Lowering Agent

Chitosan is used as a dietary ingredient due to its ability to reduce serum cholesterol. It reduces lipid absorption by binding neutral lipids, such as cholesterol and other sterols, by means of hydrophobic interactions. Because of this inhibitory activity on fat absorption, chitosan acts as fat scavenger in the digestive tract and eliminates fat and cholesterol via excretion (Luo and Wang 2013). Chitosan satisfies the requirements of dietary fiber, including non-digestibility in the upper GI tract, high viscosity, and high water-binding ability in the lower GI tract. From a physiological standpoint, the prime function of a dietary fiber is to lower cholesterol levels and to promote the loss of body weight through a reduction of intestinal lipid absorption. It differs from other dietary fibers in that it possesses a positive ionic charge, which has the ability to bond chemically with the negatively charged lipids, fats, and bile acids. It is desirable that its prolonged use as fiber in diets should be

monitored to ensure that it does not disturb the intestinal flora or interfere in the absorption of micronutrients, particularly lipid-soluble vitamins and minerals, and that it does not have any other negative effects. Chitosan shows an LD_{50} (median lethal dose) of around 16 g/kg, comparable to the salt and glucose values ensuring safety for long-term use (Singla and Chawla 2001).

18 Biomedical Applications

Chitosan due its polyelectrolyte nature, gel-forming capability, biodegradability, biocompatibility, nontoxicity to living tissues, and antimicrobial and antitumor properties has extensive applications in medicine. It is used in hemodialysis membranes, artificial skin, hemostatic agents, and drug delivery systems. The property of chitosan to form gels at a slightly acid pH gives chitosan its antacid and antiulcer activities. Chitin and chitosan oligosaccharides, when intravenously injected, enhance antitumor activity by activating macrophages.

Chitosan as control release system: Chitosan has an advantage of forming covalent or ionic bonds with the cross-linking agents, building a sort of network, where the active substance is retained. Consequently, these bonds carry advantages in terms of controlled release (Estevinho et al. 2013). Depending on the cross-linker, the major interactions involved in the formation of the network are covalent or ionic bonds. Covalent cross-linking leads to the formation of hydrogels or microparticles with a permanent network structure, because irreversible chemical bonds are formed. This type of linkage allows absorption of water and/or bioactive compounds without dissolution and allows its release by diffusion. The addition of a second polymer as encapsulating agent makes possible the pH-controlled delivery (Berger et al. 2004). Cross-linking compounds used to create covalent bonds are molecules that have at least two reactive functional groups that allow the formation of linkage between polymeric chains. The most common cross-linkers used with chitosan are dialdehydes, such as glyoxal and in particular glutaraldehyde. But they are known to be toxic. For example, glutaraldehyde is known to be neurotoxic and glyoxal is known to be mutagenic. Hence, even if microparticles are purified before usage, the presence of free unreacted dialdehydes cannot be completely excluded and will induce toxic effects (Estevinho et al. 2013). Other covalent cross-linkers for chitosan such as diethyl squarate, oxalic acid, or genipin have been investigated to overcome this problem. Ionically cross-linked microparticles or hydrogels are more biocompatible and well tolerated. Ionically cross-linked chitosan hydrogels or microparticles exhibit a greater swelling sensitivity to pH changes compared to covalently cross-linked ones. This fact broadens their potential application, since dissolution can be regulated by pH conditions (Berger et al. 2004).

Chitosan with its positive charges reacts with polyanionic compounds forming polyelectrolytic complexes that can easily incorporate active substances. Tripolyphosphate, citrate, sulfate, and phosphate are used to prepare this kind of complexes. They are normally well tolerated and biocompatible with the human

organism, showing advantages in terms of applications for food and pharmaceutical industry (Berger et al. 2004; Gupta and Jabrail 2006).

Biotechnological application of chitosan: Chitin and chitosan have been found to be useful as a matrix for immobilization of various enzymes for the processing of such products as wine and sugar, the synthesis of organic compounds (Ravikumar 2000), and the construction of sophisticated biosensors for in situ measurements of environmental pollutants and metabolite control in artificial organs (Krajewska 2004).

Chitosan as drug delivery matrix: Chitosan is considered to be the drug carrier for the twenty-first century. For effective drug delivery, it is being used in the form of microspheres, microparticles, nanoparticles, granules, gels, or films. Chitosan microspheres are useful for the controlled release of antibodies, antihypertensive agents, anticancer agents, protein and peptide drugs, vaccines, and nutraceutical compounds (Dash et al. 2011).

Chitosan as wound healing agent: Due to bacteriostatic and fungistatic properties of chitosan, it is used as a wound healing agent in skin ointments. Chitosan implanted in animal tissues encourages wound healing and hemostatic activities. Biocompatible wound dressings derived from chitin are available in the form of hydrogels, xerogels, powders, composites, and films (Gavhane et al. 2013).

19 Chitosan in Water Treatment

Water gets polluted due to metal ions, inorganic anions, phenolic compounds, dyes, and radioactive isotopes. Many of these water pollutants are toxic and can enter the human food chain. The toxic heavy metal ions are discharged into the environment through different industrial activities. The high adsorption potential of chitosan is attributed to (1) high hydrophilicity due to a large number of hydroxyl groups of glucose units, (2) the presence of a large number of functional groups, (3) the high chemical reactivity of these groups, and (4) flexible structure of the polymer chain.

Chitosan and its derivatives are being successfully used in water treatment to remove lead, copper, and cadmium from drinking water, due to complex formation between the amino group and heavy metal ions (Bhatnagar and Sillanpää 2009).

Radionuclides are an important category of metals in terms of environmental impact and interest from nuclear industry. Chitosan is an excellent biosorbent to adsorb radionuclide from aqueous solution in an acid environment (Wang and Chen 2014).

Dyes are usually present in the effluents of textile, leather, paper, and dye manufacturing industries. These effluents are not only toxic to the aquatic biota but also disturb the natural equilibrium by reducing photosynthetic activity of water in streams. Some dyes are reported to cause allergy, dermatitis, skin irritation, and cancer in humans. The removal of dyes from effluents before they are released into natural water bodies is important. Chitosan-based biosorbents have an extremely high affinity for many classes of dyes (Crini and Badot 2008).

Phenol and substituted phenols cause unpleasant taste and odor in drinking water and can exert negative effects on different biological processes. The ubiquitous nature of phenols, their toxicity even in trace amounts, and the stricter environmental regulations make it necessary to develop processes for the removal of phenols from wastewaters. Chitin and chitosan derivatives can remove phenol and substituted phenols from water (Bhatnagar and Sillanpää 2009). The pH primarily affected the degree of ionization of phenol and the surface properties of chitin. The functional groups of chitosan are protonated at low pH values and resulted in a stronger attraction for negatively charged ions in the adsorption medium. Phenol being weakly acidic is partially ionized in solution. These ions are negatively charged and are attracted due to electrostatic forces by the protonated amino groups of chitosan. As the pH increases, the overall surface charge of chitosan becomes negative and adsorption decreases. The equilibrium uptake of phenol is also affected by temperature due to the enlargement of pore size or creation of some new active sites on the adsorbent surface due to bond rupture. In comparison with activated charcoal, chitosan is more efficient in the removal of polychlorinated biphenyls from contaminated water (Venugopal 2011).

Inorganic anions are also an important class of aquatic pollutants, and various inorganic anions are found in potentially harmful concentrations in drinking water sources. The removal of these pollutants from drinking water supplies is an emerging issue. In recent years, chitin and chitosan derivatives have been successfully utilized for some anion removal from water (Bhatnagar and Sillanpää 2009).

Chitosan is currently employed in domestic sewage treatment systems in conjunction with other settling aids such as alum or bentonite clay to promote coagulation and settling of colloidal and other suspended solids. The polyelectrolyte is added at the rate of 1–2 ppm but can also be employed alone without alum when the concentration is raised to around 10 ppm. Being positively charged, it is very effective at agglomerating the negatively charged sludge particles (Venugopal 2011). Chitosan is also employed as a coagulant in the treatment of wastewater from food industries. The production of surimi generates a large amount of wash water that contains sizeable amounts of proteins, showing high turbidity. Chitosan treatment of surimi wash water results in the recovery of soluble proteins. The protein recovery is further increased by adding a complex of chitosan and alginate. It is also used as coagulant to treat wastewater from milk processing plants. Recovered proteins have application in food and feed industry (Wibowo et al. 2005).

20 Chitosan Application in Agriculture

Chitin and chitosan also have potential in agriculture with regard to controlling plant diseases. They are active against soil fungi, viruses, bacteria, and other pests. Addition of chitin and chitosan alters the environmental conditions in the rhizosphere and phyllosphere to shift the microbial balance in favor of beneficial

organisms and to the detriment of plant pathogens. Fragments from chitin and chitosan are known to have eliciting activities for a variety of defense responses in host plants, including the accumulation of phytoalexins, pathogen-related (PR) proteins, proteinase inhibitors, lignin synthesis, and callose formation (El Hadrami 2010).

21 Regulations and Commercial Applications

Chitosan is used as a food quality enhancer in a number of countries. Chitosan preparations in tablet, capsule, and powder form are being used in healthcare industry. In the European market, chitosan is sold in the form of dietary capsules to assist weight loss, and in some countries, such as Japan, it is added to various foods (e.g., noodles, potato crisps, biscuits). Chitosan-fortified fruit juices and chocolates are marketed in the USA. The role of chitosan as fiber is challenged by popular fiber products such as oats, soy, and bran. In spite of some limitations, chitosan promises to offer innovative applications in diverse areas of food processing and other fields (Venugopal 2011).

In the USA, the 1994 Dietary Supplement Health and Education Act permits use of chitosan as a food supplement without premarket approval as long as no health claims are made. The use of chitin and chitosan as ingredients in foods or pharmaceutical products, however, requires standardization of identity, purity, and stability. Manufacturers should consider filing petitions with agencies such as Food Chemical Codex, US Pharmacopoeia, European Pharmacopoeia, and Japan Pharmacopoeia. These organizations establish methods to identify specific products and standards of purity for pharmaceutical and drug use. Such standards will be necessary for future expansion of the use of chitin and chitosan (Heinze et al. 2005). Chitin and chitosan have been approved for pesticide and seed treatments, as fertilizer, and as animal feed additives. The US Environmental Protection Agency has approved the use of commercially available chitosan for wastewater treatment up to a maximum level of 10 mg/L.

Chitin and chitosan products fall within the lowest level of concern for toxicological testing. Being naturally present in living organisms, chitin and its deacetylated derivative chitosan are considered safe. The available literature on chitin and chitosan suggests a low order of toxicity, based on chemical structure and animal studies. Like several high-molecular-weight food polymers of natural origin such as cellulose and carrageenan, chitin and chitosan are not expected to be digested or absorbed from the human gastrointestinal tract. To date, chitosan appears to be clinically well tolerated. The safety of chitooligomers prepared by the enzymatic depolymerization of chitosan has been reported in a short-term mice feeding study. No mutagenicity has been reported, as judged by the Ames test, mouse bone marrow cell micronucleus test, and mouse sperm abnormality test. A 30-day feeding studies did not show any abnormal symptoms and clinical signs or deaths in rats. No significant differences are reported in body weight, food

consumption, food availability, hematology values, clinical chemistry values, or organ/body weight ratios. No abnormality of any organ was found during histopathological examination (Qin et al. 2006).

22 Conclusion and Future Research Needs

Chitosan is a versatile biopolymer that has a variety of commercial applications. However, individual research reports have used chitosans from various sources with varying physicochemical properties. Hence, the question arises as to how to globally produce chitosans with consistent properties. Each batch of chitosan produced from the same manufacturer may differ in its quality. For proper quality control in the chitosan production, there is a critical need to establish less expensive and reliable analytical methods, especially for the evaluation of molecular weight and degree of deacetylation. Functional properties of chitosan vary with molecular weight and degree of deacetylation. With proper modification of chitosan, its functional properties and biological activities can be further enhanced, and more applications are being developed.

Chitosan with different structures shows different biological activities and not all the biological activities are found in one kind of chitosan. Each special type of bioactive chitosan should be developed for its potential application. Moreover, many studies carried out on chitosan and chitooligosaccharide bioactivity have not provided detailed molecular mechanisms. Hence, it is difficult to explain exactly how these molecules exert their activities. Therefore, future research should be directed toward understanding their molecular-level details, which may provide insights into the unknown biochemical functions of chitosan and chitooligosaccharide as well as help accelerate their future applications. The traditional chitosan production process is costly, thus limiting wider applications of chitosan. Simplification of chitosan production, for example, by elimination of deproteinization and/or demineralization or by reduction of reaction time required for deproteinization and demineralization, would considerably reduce production cost due to reduction in chemical usage, process time, and voluminous wastewater discharge. The typical astringent/bitter taste of chitosan limits its use as a food additive or preservative. Incorporation of L-arginine and adenosine monophosphate, both considered as GRAS, can be used to mask or minimize this effect and should be further investigated. Inherent antibacterial/antifungal properties and film forming ability of chitosan make it ideal for use as biodegradable antimicrobial packaging material. One major drawback of chitosan film is its high sensitivity to humidity, and thus, it may not be appropriate for use when it is in direct contact with moist foods. More research is needed to develop antimicrobial chitosan films that are less sensitive to humidity. Numerous researches conducted on food applications of chitosans have been done at a small or laboratory scale. Further research on quality and shelf life of foods, containing or coated with chitosan, should be conducted on scale-up with large volumes typical of commercial conditions.

This would provide a more realistic and practical information required for actual commercialization of food products containing or coated with chitosans.

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Cyclodextrins

9

André Sá Couto, Paulo Salústio, and Helena Cabral-Marques

Contents

Abstract

Cyclodextrins (CDs) were first isolated in 1891 as degradation products of starch from a medium of *Bacillus amylobacter*. They are cyclic water-soluble, nonreducing, macrocycle carbohydrate polymers constructed from α-(1-4)-linked D-glucopyranose units (naturally occurring α, β, and γ formed by 6, 7, and 8 glucose units), in a ring formation and present a toroidal, hollow, truncated cone shape. Their most important property is the ability to establish specific interactions – molecular encapsulation – with various types of molecules through the formation of non-covalently bonded entities, either in the solid phase or in aqueous solution, taking up a whole molecule, or some part of it, into their cavities. This process in part mimics the "lock and key" mechanism of enzyme catalysis. Complexation may cause changes in physicochemical properties of the guest molecule (e.g., solubility, stability, kinetics and bioavailability, toxicity). Their negligible cytotoxic effects promoted them to the GRAS list and led them to be widely used in many industrial products and technologies.

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1 Introduction

1.1 Historical Background

Since the beginning of times, the most important discoveries happen as casual observation due to researchers' curiosity (Table 1). Cyclodextrin (CD) discovery also followed this pattern as they were first isolated in 1891 by Villiers when, in addition to reducing dextrins, beautiful crystals were observed in alcohol waste as degradation products of starch from a medium of *Bacillus amylobacter*. These substances were called "cellulosine" due to its similarity to cellulose (Villiers 1891). In the following years (early twentieth century), another prominent scientist, Schardinger, made important discoveries for CDs' science. In 1903, this author was responsible for the isolation of two crystalline products showing lack of reducing properties: dextrins A and B. In 1904, Schardinger isolated a new organism capable of producing acetone and ethyl alcohol from sugar- and starch-containing plant material (Eastburn and Tao 1994). In 1911, he described that the strain *Bacillus macerans* produces large amounts of crystalline dextrins (25–30 %) from starch. Thus, Schardinger is considered the founder of CD chemistry due to his work to characterize Villiers' "cellulosines" from 1903 to 1911, determining that they were cyclic oligosaccharides (Schardinger 1903a, b, 1904, 1911; Szejtli 1998). It is for this reason that CDs are also termed Schardinger dextrins, especially in the older literature. Since then several fractionation schemes for the production of CDs were also developed (Stella and Rajewski 1997; Matsuda and Arima 1999; Mabuchi and Ngoa 2001). The γ-dextrin was only isolated in 1935.

Pure CDs (i.e., cyclic water-soluble, nonreducing, macrocycle polymers) were prepared in the early part of the last century by Freudenberg and co-workers (Freudenberg and Meyer-Delius 1938; Freudenberg et al. 1947), who reported that CDs are constructed from α-(1-4)-linked D-glucopyranose units, in a ring formation (Saenger 1980; Uekama 1981; Szejtli 1982). In 1948, using X-ray crystallography the γ-CD structure was clarified. Immediately after, the most important property of CDs was recognized: their ability to form inclusion complexes. The general title for this class of complexes, "inclusion compounds" ("einschlussverbindung"), was first used by Schlenk in 1949. Besides the general terms ("occlusion" and "adducts"), other terms that have been used to describe these complexes are "molecular compounds," "cryptates" (Frank 1975; Szejtli 1982), and "clathrates" (Powell 1948). The term "clathrate," derived from the Latin "clathratus," meaning "enclosed or protected by cross bars of a grating," has been used to describe the cage-like structure of the hydroquinone inclusion compounds.

Table 1 Chronological order of cyclodextrin research (Cramer 1954; Bender and Komiyama 1978; Cramer 1987; Frömming and Szejtli 1994; Robyt 1998; Szejtli 1998, 2004; Loftsson and Duchêne 2007; Kurkov and Loftsson 2012)

Historical period	Investigator	Milestones
1808	E-L. Malus	Development of plane polarized light and observation of optical rotation by carbohydrates
1870	Bayer and Fittig	Formula: HO–CH_2–CH(OH)–CH(OH)–CH(OH)–CH(OH)–CHO for glucose
1888–1891	Fisher	Structure determination of several carbohydrates (glucose, fructose, manose, arabinose)
1891	A. Villiers	Discovery of α- and β-CD, pioneering study on composition and chemical properties
1903–1911	F. Schardinger	Isolation of bacteria responsible for CD synthesis; first attempts to distinguish between different CDs
1920–1930	N. Harworth	Proof of hexagonal form for carbohydrate rings
1924		CD first methylation
1930s		Maltose units bound via α-1,4-glycosidic linkages are found to be building blocks for CD molecule; first isolation of pure CDs
1935	K. Freudenberg and Jacobi	γ-CD is discovered
1936		CD cyclic structure is disclosed
1940s	F. Cramer	Idea of inclusion complex formation is suggested
1948	K. Freudenberg	γ-CD structure is clarified
	W. Borchert	Structures of α-, β-, and γ-CDs are determined by X-ray diffraction
1950s	D. French	Discovery of CDs with larger rings
	F. Cramer	Study of inclusion complexation properties of CDs
1953	K. Freudenberg	First patent on CDs
	F. Cramer	
	H. Plieninger	
1954	F. Cramer	Publication of the book "*Einschlussverbindungen*" ("inclusion compounds")
1957	D. French	First fundamental review on CDs containing first misinformation on toxicity of β-CD
1965	T. Higuchi	Development of a mathematical model describing inclusion complexation mechanism
	K. Connors	
1975	M. Furue	First publication on CD polymers
1976	Ono Pharmaceutical Co. Ltd.	Release of the first medicine, Prostarmon E, from CD
1980s		Beginning of industrial application of CDs in food and cosmetics
1981	J. Szejtli	The First International Cyclodextrin Symposium is organized; the first cyclodextrin book is published

(*continued*)

Table 1 (continued)

Historical period	Investigator	Milestones
1983–1985	U. Brauns	HP-β-CD is patented in Europe and the USA
	B. Muller	
	J. Pitha	
1983	K. Miyajima	First suggestion of self-association of parent CDs
1988	Chiesi Farmaceutic (Italy)	Commercialization of Piroxicam/β-CD tablets (Brexin®)
1991	V. Stella	SBE-β-CD is patented
	R. Rajewski	
1994	Oftalder (Portugal)	Commercialization of the first eye drops: chloranphenicol/RM-β-CD (Clorocil®)
1990s	A. Harada	Intensive research activity on CD catenanes and rotaxanes
	M. Kamachi	
2000s	M. Bonini	Intensive research activity on CD aggregation
	A. Coleman	
	G. Gonzalez-Gaitiano	
	T. Loftsson	
	L. Szente	
	A. Wu	

Although Chemical Abstracts and several other reference sources use the term "clathrates" as a general descriptor for inclusion compounds, this term more appropriately describes only cage-like polymolecular inclusion compounds (Cabral-Marques 1994a). In 1961 the existence of larger natural CDs, namely, δ- (delta), ε- (epsilon), ζ- (zeta), and η- (nu) CD, was proved (9–12 glucose residues) (Hirose and Yamamoto 2001). CD chemistry was discussed in detail by Bender and Komiyama (Bender and Komiyama 1978).

1.2 Occurrence/Sources

Although discovered more than 100 years ago as previously referred, the cyclic glucooligosaccharides termed CDs (based on dextrose, which is an old name for glucose) remained laboratory curiosities until the 1970s when they began to be used commercially (Szejtli 1998). Its production results from degrading the starch by the enzyme cyclodextrin glucanotransferase (CGTases), involving excision and reconnection of single turns from the helical α-(1→4)-glucan (amylose) chain (Fig. 1) to provide cyclic α-(1→4)-linked glucooligosaccharides with six (α-CD), seven (β-CD), and eight glucose units (γ-CD) (Rendleman 1999; Lichtenthaler 2010). This intramolecular transglycosylation is called cyclization reaction (Szejtli 1998; Cheirsilp et al. 2010).

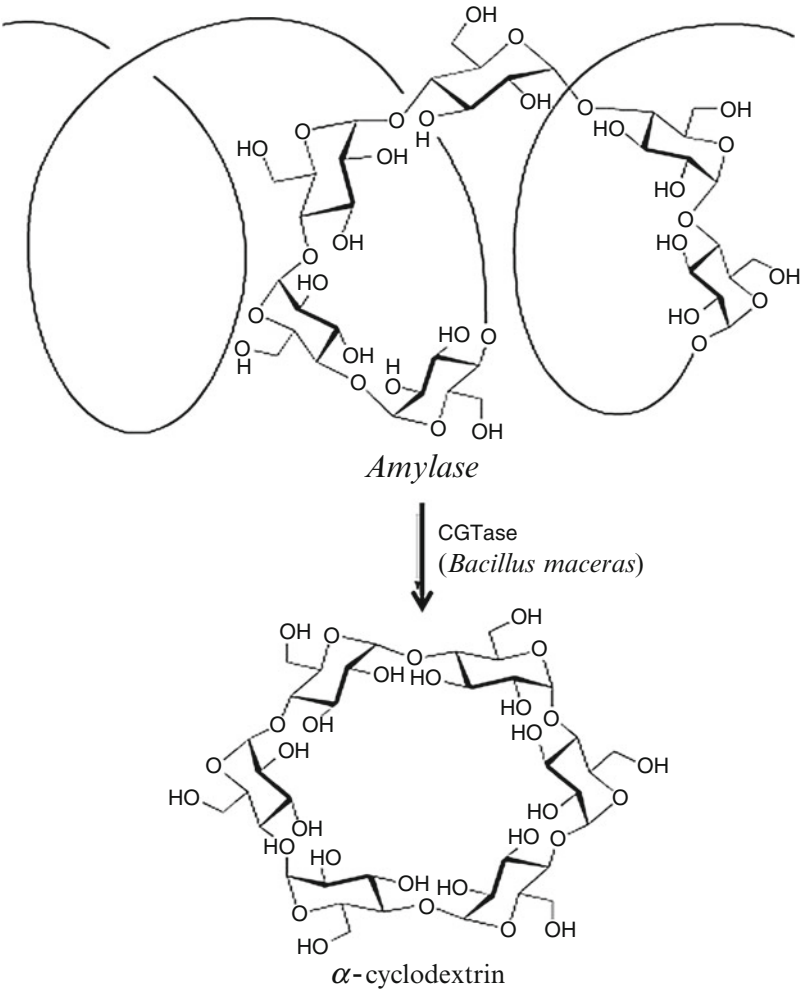

Fig. 1 Representation of a left-handed, single-stranded helix of VH-amylose (*top*) and of α-cyclodextrin (*bottom*), which de facto represents a single turn of the amylose helix excised and reconnected by *Bacillus macerans*-derived enzymes (CGTases). The close analogy allows VH-amylose to be considered as a tubular analogue of *α*-cyclodextrin (Lichtenthaler 2010)

The starch sources used to produce CDs are varied and include according to different authors the following substrates:

- Liquefied starch, maltodextrin, or long-chain maltooligosaccharides (Rendleman 1999)
- Maltodextrin and commercial soluble starch, corn, cassava, sweet potato, and waxy corn starches (Alves-Prado et al. 2008)
- Amaranth starch (Urban et al. 2012)

- Soluble and cocoyam starch (Mora et al. 2012)
- Starch such as potato, tapioca, and corn (Shahrazi et al. 2013)

CGTase is specifically active on both structures of amylose and amylopectin (Shahrazi et al. 2013). Several microorganisms and methods have been used to produce or use CGTase as described in the following:

- *Bacillus amylobacte*r in 1881 (Szejtli 1998).
- *Bacillus macerans* (Rendleman 1999).
- *Bacillus circulans* DF 9R (Rosso et al. 2002).
- *Bacillus clausii* strain E16 (Alves-Prado et al. 2008).
- *Bacillus megaterium* (Zhekova and Stanchev 2011; Sivakumar and Shakilabanu 2013).
- Alkaliphilic *Bacillus licheniformis* (Thombre and Kanekar 2013).
- CGTase was anchored on the surface of *Saccharomyces cerevisiae* and was used as an immobilized enzyme (Wang et al. 2006).
- *Escherichia coli* pAD26 cells immobilized on cotton (Kriaa et al. 2012).
- *Amphibacillus* sp. NPST-10 (Ibrahim et al. 2012).
- *Paenibacillus macerans* CCM 2012 (Urban et al. 2012).
- *Amphibacillus* sp. NRC-WN (Al-Sharawi et al. 2013).

2 Structure and Properties

2.1 Parent Cyclodextrins

The most common of these naturally occurring, ring-shaped molecules are the α- (alpha), β- (beta), and γ- (gamma) CDs formed by six, seven, and eight glucose units (Fig. 2) and cavities' diameters smaller than 0.6, 0.8, and 1.0 nm, respectively (Frank 1975; Szejtli 1982). Because of their sugar backbone, these CDs are also known as cycloamyloses: hexa- (C6A), hepta- (C7A), and octa-amylose (C8A) (Szejtli 1982) or cyclohexa-, cycloheptа-, and cyclooctaglucan and α-, β-, and γ-amylosan (French 1957).

CDs have no well-defined melting point and start to decompose at temperatures above 270 °C, and at 300 °C a sharp endothermic process is detected, which indicates that the melting is accompanied by decomposition (Szejtli 1982). Generally their solubility in water is greatly increased at higher temperatures (except for the dimethyl-β-CD which decreases). The most important characteristics of the natural CDs are summarized in Table 2 (Saenger 1980; Szejtli1998; Pitha et al. 1983; Duchêne et al. 1986; Bas and Rysanek 1987; Uekama and Otagiri 1987; Duchêne and Wouessidjewe 1990a; Salústio et al. 2011).

It is noteworthy that there are differences in the reported central cavity and outer periphery diameters of the CDs. Molecule CDs present a hydrophilic exterior, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix, described as a "micro heterogeneous environment" (Szejtli 1989).

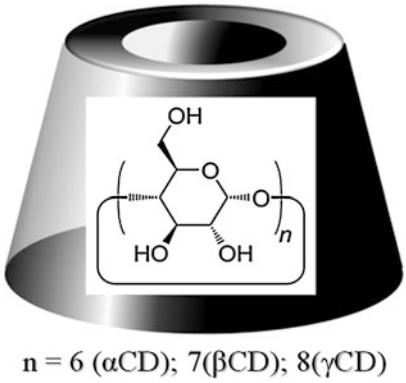

Fig. 2 Schematic cyclodextrins structure (Szejtli 1998; Salústio et al. 2011)

Table 2 Characteristics of α-, *β*-, and *γ*-CDs (Saenger 1980; Szejtli 1998; Pitha et al. 1983; Duchêne et al. 1986; Bas and Rysanek 1987; Uekama and Otagiri 1987; Duchêne and Wouessidjewe 1990; Salústio et al. 2011)

	α	*β*	*γ*
n° of glucose units	6	7	8
mol wt.	972	1,135	1,297
Solubility in water (g/100 ml), 25 °C	14.5	1.85	23.2
$[R]_D$25 °C	150.0 ± 0.5	162.5 ± 0.5	177.4 ± 0.5
Cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3
Height of torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Diameter of outer periphery (Å)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Approx. cavity volume ($Å^3$)	174	262	427
Approx. cavity volume in 1 mol CD (ml)	104	157	256
Approx. cavity volume in 1 g CD (ml)	0.10	0.14	0.20
Crystalline forms (from water)	Hexagonal plates	Monoclinic parallelograms	Quadratic prisms
Crystal water, wt %	10.2	13.2–14.5	8.13–17.7
Diffusion constant at 40 °C	3.443	3.224	3.000
Hydrolysis by *A. oryzae* α-amylase	Negligible	Slow	Rapid
V_{max} value, min^{-1}	5.8	166	2,300
Relative permittivity at pH 5.3, 25 °C (on incorporating the toluidinyl group of 6-*p*-toluidynilnaphthalene 2-sulphonate)	47.5	52.0	70.0
(On incorporating the naphthalene group)	[a]	29.5	39.5
p*K* (by potentiometry) at 25 °C	12.332	12.202	12.081
Partial molar volumes in solution (ml/mol)	611.4	703.8	801.2
Adiabatic compressibility in aqueous solutions ml (mol^{-1} bar^{-1}) × 10^4	7.2	0.4	−5.0

[a]Naphthalene group is too bulky for the α-CD cavity

Nuclear magnetic resonance (NMR) and X-ray diffraction indicate the C-1 chair conformation for the glucose molecule (Rao and Foster 1963). CDs are shown to have a toroidal, hollow, truncated cone structure, where the secondary OH groups on the C-2 and C-3 atoms are located on the wider side of the torus, while the primary OH groups on the C-6 are positioned on the opposite side of the torus (the narrower) and are directed "away" from the cavity except if H-bonded to include guest molecules (Saenger 1982; Szejtli 1988). The CH groups comprising H-1, H-2, and H-4 are located on the exterior of the molecule, while the polar sugar OH groups are oriented to the cone exterior, and as a consequence the external faces of CDs are hydrophilic leading to their aqueous solubility. The interior of the torus offers an environment of much lower polarity than is present in water, so it can be considered as a "hydrophobic cavity," which is lined by two rings of CH groups (H-3 and H-5), and by a ring of glucosidic oxygen-bridge atoms "ether oxygens" (O-4 and O-5) (Saenger 1982; Jones et al. 1984; Uekama and Otagiri 1987), H-6 forms the narrower rim of the truncated cone. The result of this amphipathic property is that CDs can form soluble, reversible inclusion complexes with water-insoluble compounds resulting in compound solubilization.

In all CD crystal structures, H-bonding between adjacent glucoses is found, with O-3....H-O-2 distances in the usually accepted range, 0.28–0.31 nm. H-bonding between these secondary OH groups appears to be preferred because their pKa, around 12.2 (Chin et al. 1968), is relatively low, suggesting enhanced polarization and therefore good H-bond donor and acceptor functions. This ring of H-bonds is, in general, fully established in β- and γ-CDs, but in α-CD, the curvature of the ring produces, on average, longer O-3....H-O-2′ separations, and H-bonding is weaker and can break down, as shown for the "empty" hexahydrate where four O-3....H-O-2′ hydrogen bonds are formed but two are broken. Spectroscopic data also indicate that β-CD is rigid in aqueous solution, whereas α-CD is flexible and undergoes conformational change if inclusion of a guest molecule occurs, as proposed in the "induced-fit" concept for proteins (Saenger 1982; Szejtli 1988). These interactions prevent hydration by water molecules (Szejtli 1985; Uekama and Otagiri 1987) resulting in poor aqueous solubility. In order to improve CDs' aqueous solubility, their structure has been modified via alkylation and hydroxyalkylation (Uekama and Otagiri 1987; Yoshida et al. 1988; Jicsinszky 2014).

The OH groups on C-2, C-3, and C-6 are available as points of structural modification without danger of eliminating the "central void" (MacNicol et al. 1978). The OH groups on C-6 are the most reactive, whereas the OH at C-3 are much less reactive than those at C-2.

2.2 Large Cyclodextrins

CDs with fewer than six glucopyranose residues do not exist, probably for steric reasons (Duchêne and Wouessidjewe 1990b). The existence of CDs comprising more than 8 glycosyl units was referenced in 1948 by Freudenberg and Cramer (Freudenberg and Cramer 1948). A decade later these findings were subsequently

substantiated by French and co-workers, who reported the isolation and partial characterization of large CDs with 9, 10, 11, and 12 glycosyl units in the macrocycle. However, some doubts remained concerning these CDs since they were not able to experimentally distinguish the large CDs from branched CDs (Pulley and French 1961; French et al. 1965; Szejtli 1988). During last decades, the existence of the large CDs has been fully proven. CDs containing up to 31 glycosyl units have been purified and characterized (Koizumi et al. 1999), and the existence of even larger CDs with degrees of polymerization up to several hundreds of glycosyl units has been reported (Kitamura 2000; Larsen 2002). Indeed, large CDs are not commercially exploited due to their extremely small isolation/purification yields and lack of their complexing properties (French 1957; Pulley and French 1961; French et al. 1965; Szejtli 1982).

Large CDs (Table 3) have often been designated "cycloamylose" (abbreviated CD*n*, where *n* designates the number of glucose molecules in the macrocycle) (Kitamura 2000; Larsen 2002).

2.3 Cyclodextrins Derivatives

In order to improve the physicochemical properties (i.e., better solubility, stability, and inclusion formation abilities/release behaviors of the substrate molecules) of the natural CDs, many derivatives were synthesized by various molecular manipulations (Frömming 1987; Frömming et al. 1987).

These derivatives (Table 4) usually are produced by alkylations, aminations, esterifications, or etherifications of their primary and secondary OH groups, leading also to the formation of polymeric and amphiphilic CDs (long alkyl or fluoroalkyl chains at primary and/or secondary sides of the CDs) (Bender and Komiyama 1978; Szejtli 1982; Szente et al. 1999; Sollogoub 2013). Croft and Bartsch (1983) published an interesting review of chemical methods for modifying CDs.

3 Complexation

3.1 Complexes Formation

CDs are able to form inclusion complexes with a wide variety of hydrophobic guest molecules. Thus, guest molecules can be totally or partially entrapped into CDs' cavities and in several molar ratios. The complexation process (Fig. 3) involves the insertion of the less polar part of the guest molecule into the host cavity, while the more polar and often charged group of the guest is exposed to the bulk solvent. During insertion phenomenon the water molecules are removed from the inside of CD cavity due to their thermodynamical instability. The inclusion complex formation is achieved by weak interactions such as van der Waals, hydrogen bonding, and hydrophobic effect, among others (Szejtli 1982; Cabral-Marques 1994a; Rekharsky and Inoue 1998; Sun et al. 2006). This process does not occur by means of ionic,

Table 3 Nomenclature and some properties of large cyclodextrins (Larsen 2002)

Glycosyl units	Semisystematic name	Generic name[a]	Abbreviation	Molecular weight	Aqueous[d,e] solubility (g/100 mL)	Surface[d,e] tension (mN/m^{-1})	Specific[e] rotation $[\alpha]_D^{25}$	Half-life[f] of ring opening (h)	Radius[g] of gyration (Å)
6	Cyclomaltohexaose	α-cyclodextrin	α-CD	972.9[b]	14.5	72	147.8	33	6.0
7	Cyclomaltoheptaose	β-cyclodextrin	β-CD	1135.0[b]	1.85	73	161.1	29	6.7
8	Cyclomaltooctaose	γ-cyclodextrin	γ-CD	1297.2[b]	23.2	73	175.9	15	7.3
9	Cyclomaltononaose	δ-cyclodextrin	CD_9	1459.3[b]	8.19	73	187.5	4.2	–
10	Cyclomaltodecaose	ϵ-cylodextrin	CD_{10}	1621.4[b]	2.82	72	204.9	3.2	–
11	Cyclomaltoundecaose	ζ-cyclodextrin	CD_{11}	1783.6[b]	>150	72	200.8	3.4	–
12	Cyclomaltododecaose	η-cyclodextrin	CD_{12}	1945.7[b]	>150	72	197.3	3.7	–
13	Cyclomaltotridecaose	θ-cyclodextrin	CD_{13}	2107.9[b]	>150	72	198.1	3.7	–
14	Cyclomaltotetradecaose	ι-cyclodextrin	CD_{14}	2270.0[b]	2.30	73	199.7 ± 1.0	3.6	–
15	Cyclomaltopentadecaose	κ-cyclodextrin	CD_{15}	2432.2[b]	>120	73	203.9 ± 0.4	2.9	–
16	Cyclomaltohexadecaose	λ-cyclodextrin	CD_{16}	2594.3[b]	>120	73	204.2 ± 0.7	2.5	–
17	Cyclomaltoheptadecaose	μ-cyclodextrin	CD_{17}	2756.4[b]	>120	72	201.0 ± 0.6	2.5	–
18	Cyclomaltooctadecaose	ν-cyclodextrin	CD_{18}	2918.6[b]	–	–	–	–	–

19	Cyclomaltononadecaose	ξ-cyclodextrin	CD_{19}	3080.7[b]	–	–	–	–	–
20	Cyclomaltoeicosaose	*o*-cyclodextrin	CD_{20}	3242.9[b]	–	–	–	–	–
21	Cyclomaltoheneicosaose	π-cyclodextrin	CD_{21}	3405.0[b]	–	–	–	–	11.5
22	Cyclomaltodoicosaose	–	CD_{22}	3567.2[c]	–	–	–	–	–
23	Cyclomaltotriicosaose	–	CD_{23}	3729.3[c]	–	–	–	–	–
24	Cyclomaltotetraicosaose	–	CD_{24}	3891.4[c]	–	–	–	–	–
25	Cyclomaltopentaicosaose	–	CD_{25}	4053.6[c]	–	–	–	–	–
26	Cyclomaltohexaicosaose	–	CD_{26}	4215.7[c]	–	–	–	–	19.6
27	Cyclomaltoheptaicosaose	–	CD_{27}	4377.9[c]	–	–	–	–	–
28	Cyclomaltooctaicosaose	–	CD_{28}	4540.0[c]	–	–	–	–	–
29	Cyclomaltononaicosaose	–	CD_{29}	4702.2[c]	–	–	–	–	–
30	Cyclomaltotriacontaose	–	CD_{30}	4864.3[c]	–	–	–	–	–
31	Cyclomaltohentriacontaose	–	CD_{31}	5026.5[c]	–	–	–	–	–
n	–	–	CD*n*	$n \cdot 162.14$	–	–	–	–	–

[a]Fujiwara et al. 1990; Endo et al. 1995, 1997a, b, 1998; Miyazawa et al. 1995
[b]Calculated from the molecular formula and confirmed by mass spectrometry (Fujiwara et al. 1990; Endo et al. 1995, 1997a, b, 1998; Miyazawa et al. 1995; Koizumi et al. 1999)
[c]Calculated from the molecular formula and confirmed by mass spectrometry (Koizumi et al. 1999)
[d]Observed at 25 °C
[e]Endo et al. 1995; Ueda et al. 2000
[f]In 1 M HCl at 50 °C
[g]Determined by small angle X-ray scattering at 25 °C (Kitamura 2000)

Table 4 Cyclodextrins derivatives and their release behavior (Salústio et al. 2011)

Hydrophilic derivatives		
Methylated β-cyclodextrin		
Methyl-β-cyclodextrin	Me-β-CD	
Randomly methylated-β-cyclodextrin	RM-β-CD (RAMEB)	
Dimethyl-β-cyclodextrin	DM-β-CD (DIMEB)	
Randomly dimethylated-β-cyclodextrin	RDM-β-CD	
Trimethyl-β-cyclodextrin	TM-β-CD	*Immediate release*
Acetylated dimethyl-β-cyclodextrin	DMA-β-CD	
Hydroxyalkylated β-cyclodextrin		*Enhanced dissolution and absorption of poorly water-soluble drugs*
2-Hydroxyethyl-β-cyclodextrin	2-HE-β-CD	
2-Hydroxypropyl-β-cyclodextrin	2-HP-β-CD	
3-Hydroxypropyl-β-cyclodextrin	3-HP-β-CD	
Hydroxybutenyl-β-cyclodextrin	HBen-β-CD	
2,3-Dihydroxypropyl-β-cyclodextrin	2,3-DHP-β-CD	
Branched β-cyclodextrin		
Glucosyl-β-cyclodextrin	G_1-β-CD	
Maltosyl-β-cyclodextrin	G_2-β-CD	
Glucuronylglucosyl-β-cyclodextrin	GUG-β-CD	
Hydrophobic derivatives		
Alkylated β-cyclodextrin		
2,6-di-*O*-ethyl-β-cyclodextrin	DE-β-CD	
2,3,6-tri-*O*-ethyl-β-cyclodextrin	TE-β-CD	
Acylatedβ-cyclodextrin		*Prolonged release*
2,3,6-tri-*O*-$acyl_{(C2\text{-}C18)}$-β-cyclodextrin	TA-β-CD	
2,3,6-tri-*O*-butanoyl-β-cyclodextrin	TB-β-CD	*Sustained release of water-soluble drugs*
2,3,6-tri-*O*-valeryl-β-cyclodextrin	TV-β-CD	
2,3,6-tri-*O*-octyl-β-cyclodextrin	TO-β-CD	
Ionizable derivatives		
Anionic β-cyclodextrin		
O-carboxymethyl-*O*-ethyl-β-cyclodextrin	CME-β-CD	*Delayed release* (pH-dependent release)
β-cyclodextrin sulfate	β-CD sulfate	
Sulfobutyl ether group-β-cyclodextrin (d.s.4)	SBE_4-β-CD	*Immediate release*
Sulfobutyl ether group-β-cyclodextrin (d.s.7)	SBE_7-β-CD	
Cationic β-cyclodextrin		
Trimethyl-ammonium-β-cyclodextrin	TMA-β-CD	*Prolonged release*
Drug-CD conjugate		*Delayed release* (site-specific release)

d.s. degree of substitution

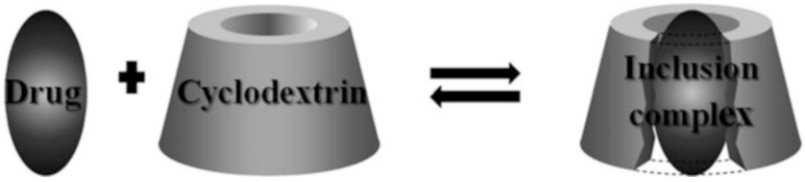

Fig. 3 Interaction of a drug molecule with a CD to form an inclusion complex (Salústio et al. 2011)

covalent, or coordinate covalent bonds (Frank 1975; Cramer 1982; Charoenchaitrakool et al. 2002). The specific association of two molecules results not by a single weak interaction but through the simultaneous cooperation of several weak interactions. Molecular mechanics calculations have also been made to investigate the solute-solvent interactions between CD and water and the molecular interactions in inclusion complexes of CDs. The essential criterion is simply that the enclosed molecule or guest must be of a suitable size and shape (i.e., the best possible filling of space) to fit into a cavity within a solid structure formed by host molecules (Powell 1948, 1954). The main binding contribution between CD and its partners is the geometrical fitting, so complexation occurs in a stereo-specific manner (Cramer 1982). The spatial requirements for the formation of a CD inclusion compound in part mimic the enzyme catalysis "lock-key" mechanism which occurs if the substrate/guest molecule is orientated properly with respect to the active centers of the host (Cramer and Hettler 1967). It is also possible to use the CD to block some reactive sites on the guest and expose others.

CD complex formation, from a pharmaceutical point of view, is equivalent to molecular encapsulation, as the drug molecules are isolated from each other and are dispersed on a molecular level in an oligosaccharide matrix, i.e., a process similar to drug encapsulation in a hydrophilic matrix (e.g., polyvinylpyrrolidine or cellulose) (Pitha et al. 1983; Szejtli 1985).

Inclusion complexes can be obtained by several techniques, such as physical mixing (Menezes et al. 2012), coprecipitation (Sapkal et al. 2007;Nieddu et al. 2014), complexation in a slurry (Fages et al. 2007; Menezes et al. 2012, 2014), complexation in paste (Gil et al. 2004; Menezes et al. 2012, 2014), extrusion (Yano and Kleinebudde 2010), dry mixing (Higashi et al. 2009), solution/suspension with solvents removal by drying methods (spray-drying, freeze-drying) (Salústio et al. 2009), sealed-heating method (Nieddu et al. 2014), and supercritical fluids (Junco et al. 2002). In order to enhance the complex efficiency of the CDs, different methods can be used such as ionization of the drug, salt formation, formation of metal complexes, addition of organic cosolvents to the aqueous complexation media, and the use of supercritical fluids, since all promote an intrinsic solubility enhancement of the drug-favoring complexation (Junco et al. 2002; Loftsson and Duchêne 2007). There are also references to the inclusion of essential oils (EO) into β-CD through a sol-gel process, which consists in the introduction of the suspension containing the EO and β-CD into a colloidal silica, under continuous stirring, at room temperature. This procedure was used to

encapsulate mint and lavender oils, which according to the authors, allowed the EOs to be much more protected against the humidity, temperature, and solar light, among other factors (Răileanu et al. 2013).

3.2 Phase Solubility Studies

Phase solubility studies are a traditional approach to determine not only the stability (also called equilibrium) constant value but also to give insight into the stoichiometry of the equilibrium. Experimentally, an excess of a poorly water-soluble guest molecule (e.g., drug, D, representing the substrate) is introduced into several vials to which a constant volume of an aqueous vehicle containing successively larger concentrations of the CD (representing the ligand) is added. Afterwards the vials are shaken at constant temperature until equilibrium is established. The supernatant is spared and the total concentration of the drug (D_t) is determined by a adequate quantification method (e.g., UV-VIS, HPLC, fluorescence). The phase solubility profile is then constructed by assessing the effect of the CD on the apparent solubility of the drug (D) as shown in Fig. 4 (Higuchi and Connors 1965; Brewster and Loftsson 2007; Cabral-Marques 2010). Several behaviors can be identified between two major types: A and B.

Thus, the following profiles may be described as:

- A-type – formation of soluble inclusion complexes
  - A_L-type – linear increase of drug solubility versus CD concentration
  - A_P-type – positively deviating from the linear profile (i.e., the solubilization is proportionally more effective at higher concentrations)
  - A_N-type – negatively deviating from the linear profile (i.e., the CD is proportionally less effective at higher concentrations)
- B-type – formation of inclusion complexes poorly soluble
  - B_S-type – complexes of limited solubility
  - B_I-type –formation of insoluble complexes

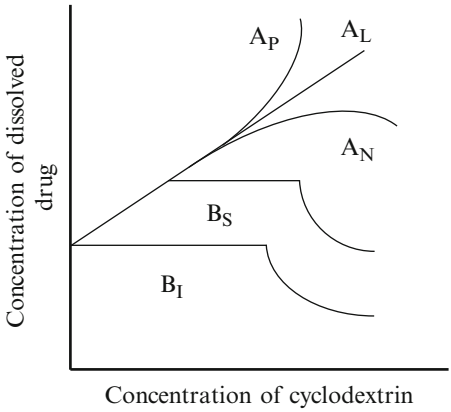

Fig. 4 Profile types in the phase solubility diagrams (Higuchi and Connors 1965)

A_L profiles indicate a linear increase in solubility in function of CD concentration, and water-soluble complexes are being formed with solubilities higher than that of the uncomplexed substrate. A_L-type relationships are first order with respect to the CD and may be of first or higher order with respect to the drug (i.e., *D•CD*, D_2•*CD*, D_3•*CD*, etc.). If the slope of the A_L isotherm is higher than the unity, higher-order complexes are assumed to be involved in the solubilization. Although a slope of less than one does not exclude the occurrence of higher-order complexes, a one-to-one complex is often assumed in the absence of other information. A_P systems suggest the formation of higher-order complexes with respect to the CD, at higher CD concentrations (i.e., *D•CD*, *D•*CD_2, *D•*CD_3, etc.). The stoichiometry of the formed complexes has historically been implied by the extent of curvature of the phase solubility profile. Thus, when an isotherm best fits to a quadratic function, the formation of a one-to-two (*D•*CD_2) complex is suggested, one best fits to a cubic function suggests a one-to-three complex (*D•*CD_3), and so forth. A_N profiles have several explanations including bulk changes imparted to the solvent by the CD at various concentrations (i.e., the CD is acting as a chaotrope or kosmotrope or is altering the bulk properties of the media by changing its viscosity, surface tension, or conductivity) and/or CD self-association at high concentrations. Equilibrium constants can be derived from the linear portion of each phase solubility profile, given by Eq. 2. To this point, intrinsic drug solubility is given as D_o and the formed complex is represented by *D•CD*.

The complexation process is most frequently a 1:1 host/guest ratio; however, 2:1, 1:2, or even higher-order associations may exist at the equilibrium, almost always simultaneously. Equilibrium established between dissociated and associated species can be expressed by the stability (association or equilibrium) constant and designed as K_s or K_a or $K_{1:1}$ (Szejtli 1998). These constants may be determined by several methods, e.g., phase solubility studies (Waleczek et al. 2003; Daletos et al. 2008), NMR, UV-VIS, HPLC (Cabral-Marques 1994a, b), TLC (Lederer and Leipzig-Pagani 1996), and static headspace method (Saito et al. 1999). The CD association with the drug (D) or guest molecules and the dissociation of the formed inclusion complexes are governed by a thermodynamic equilibrium showed by the following equations (Szejtli 1998):

$$\begin{matrix} mD + nCD \overset{K_{m:n}}{\Leftrightarrow} D_mCD_n \\ [a-mx][b-nx]\ [x] \end{matrix} \tag{1}$$

and

$$K_d = \frac{[x]}{[a-mx]^m[b-nx]^n} \tag{2}$$

Then, a dissociation constant can be defined as

$$K_d = \frac{[a-mx]^m[b-nx]^n}{[x]} = \frac{1}{K_{m:n}} \tag{3}$$

Intrinsic drug solubility is given as D_0 and a formed complex is represented by $D{\bullet}CD$.

Since

$$[D] = D_0 \tag{4}$$

$$D_t = D_0 + m[D_m CD_n] \tag{5}$$

$$CD_t = CD + n[D_m CD_n] \tag{6}$$

then the values for $[D_m{\bullet}CD_n]$, $[D]$ and $[CD]$ can be derived as

$$[D_m CD_n] = \frac{D_t - D_0}{m} \tag{7}$$

$$[CD] = CD_t - n[D_m CD_n] \tag{8}$$

where D_0 is the equilibrium solubility of the drug in the absence of CD, D_t is the total concentration of the drug (i.e., the sum of the complexed and uncomplexed forms), and CD_t is the total concentration of CD. For equilibria that are first order with respect to the CD ($n = 1$), the following equation can be obtained:

$$D_t = \frac{mKD_0^m CD_t}{1 + KD_0^m} + D_0 \tag{9}$$

A plot of D_t versus CD_t for the formation of $D_m{\bullet}CD$ should, therefore, give a straight line with the y intercept representing D_0 and the slope defined as

$$\text{slope} = \frac{mKD_0^m}{KD_0^m} \tag{10}$$

Therefore, if m is known, the K can be calculated meaning that for 1:1 complexation, i.e., $m = 1$, the following graphical approach can be applied:

$$K_{1:1} = \frac{\text{slope}}{D_0(1 - \text{slope})} \tag{11}$$

For 1:1 drug $\bullet CD$ complexes, the complexation efficiency (CE) can be calculated from the slope of the phase solubility diagram according to Eq. 12. When selecting CD or complexation conditions during formulation work, it can frequently be more convenient to compare the CE than $K_{1:1}$ values since CE is less sensitive to errors related to estimation of intrinsic drug solubility (Loftsson et al. 2005a):

$$CE = \frac{[D \bullet CD]}{[CD]} = D_0{}^* K_{1:1} = \frac{\text{slope}}{(1 - \text{slope})} \tag{12}$$

The CE is the product of the apparent solubility of the poorly soluble drug (D_0) and the apparent stability constant of the complex ($K_{1:1}$), assuming formation of 1:1 *D•CD*. The CE can be increased by either increasing the value of D_0 or the value of $K_{1:1}$ or both values simultaneously. This parameter increase can be achieved by the use of drug salts, polymers, and cosolvents. In addition, processing approaches to improve CDs, solubilizing functions should be considered, and formulation concepts such as supersaturation may further help in the optimal use and placement of CD in pharmaceutical dosage forms (Loftsson and Brewster 2012; Ciobanu et al. 2013).

4 Biology/Biotechnological Approaches

4.1 Toxicological Considerations

Due to the importance of CDs and the derivatives, their safety and toxicological profiles have been reviewed by several authors (Cabral-Marques 1994b; Irie and Uekama 1997; Thompson 1997; Del Valle 2003; Stella and He 2008; Arima et al. 2011). As CDs possess MW ranging from almost 1,000 to over 2,000 Da and hydrophilicity ($\log_{Ko/w}$ −8 to −12) with a significant number of H-donors and acceptors, they are not significantly absorbed from the gastrointestinal tract (GI) in their intact form. Natural CDs and their hydrophilic derivatives present considerable difficulties to permeate lipophilic biological membranes. Even the somewhat lipophilic randomly methylated β-CD does not readily permeate lipophilic membranes, although it interacts more readily with membranes than the hydrophilic CD derivatives (Totterman et al. 1997); for this reason some β-CD derivatives have been considered skin enhancers due to their ability to complex membrane components causing its disruption (Babu and Pandit 2004; Wang et al. 2014). Several CDs, namely, γ-CD, 2-HP-β-CD, SBE-β-CD, sulphated β-CD, and maltosyl β-CD evaluated in a number of safety studies have proven that they appear to be safe even when administered parenterally. However, these same studies proved that the natural α- and β-CDs and the methylated β-CDs are not suitable for parenteral delivery (Del Valle 2003). The α-CD and β-CD, unlike γ-CD, cannot be hydrolyzed by human salivary and pancreatic amylases even if it can be fermented by the intestinal microflora (Irie and Uekama 1997; WHO 2002). One of the target organs where the toxicity profile of several CDs can be assessed is the kidney (Antlsperger and Schmid1996).

4.1.1 α-CD

Oral administration of α-CD is, in general, well tolerated and is not associated with significant adverse effects (Lina and Bär 2004a, b). Only small fractions of α-CD are absorbed intact from the GI tract, and it is mainly excreted unchanged in the urine after IV injection (Table 5) (Brewster and Loftsson 2007). Studies in rats showed that α-CD p.o. was excreted 60 % as CO_2 (no CO_2 exhalation after p.o. to

Table 5 Safety overview of selected cyclodextrins (Brewster and Loftsson 2007)

	The pharmacokinetics in rats			Acute toxicity, LD50 rat (g/kg)		Maximum dosage in marketed products (mg/day)	
Cyclodextrin	$t_{1/2}$ after IV injection (min)	Fraction excreted unchanged in urine	Oral absorption	IV	Oral	IV	Oral
α-CD	25	≈90 %	2–3 %	0.5–0.8	>10	1.3	
β-CD	20	≈90 %	1–2 %	1	19	Not for parenteral usage	170
HP-β-CD	20	≈90 %	≤3 %	10	>2	16,000	8,000
SBE-β-CD				>15	>10	6,000–14,000	–
RM-β-CD	18	>95 %	0.5–12 %	1.5–2.1	>8	Not for parenteral usage	–
G2-β-CD	23				>5	No product	No product
γ-CD	20	90 %	<0.02 %	4	>8	No product	No product
HP-γ-CD					>2		

germ-free rats), 26–33 % as metabolite incorporation, and 7–14 % as metabolites in feces and urine, being mainly excreted in the unchanged form by the renal route after parenteral injection ($t_{1/2} = 25$ min, LD_{50} oral, rat >10,000 mg/kg, LD_{50} IV, rat between 500 and 750 mg/kg).

Some other relevant characteristics were observed after this CD administration, such as, skin irritation after IM injection, binding with some lipids, eye irritation, and slight absorption (2–3 %) after oral administration in rats. α-CD is not metabolized in the upper intestinal tract and its cleavage is only due to the intestinal flora of cecum and colon (Del Valle 2003).

4.1.2 β-CD

Due to its low aqueous solubility and side effects (e.g., nephrotoxicity), β-CD is not able to be administered by parenteral route but it can be used in p.o. because by this route is normally nontoxic. However, it must be carefully used because in high doses it may be harmful and therefore not recommended. After p.o. administration, the nontoxic effect level of β-CD was determined to be 0.7–0.8 g/kg/day in rats and about 2/g/kg/day in dogs (Bellringer et al. 1995).

It was noticed that β-CD binds cholesterol, is absorbed in small scale (1–2 %) in the upper intestinal tract after oral administration, and is less irritating than α-CD after IM injection. β-CD is not metabolized in the upper intestinal tract, but suffers bacterial degradation and fermentation in cecum and colon which may lead to gas production and diarrhea (LD_{50} oral, rat >5,000 mg\kg; LD_{50} IV, rat = 450–790 mg/kg) (Del Valle 2003). As it will be described in the Regulatory State chapter, β-CD is the most commonly used CD in pharmaceutical formulations, and, thus, it is probably the most studied in humans.

4.1.3 γ-CD

The metabolism of γ-CD closely resembles that of starch and linear dextrins (Munro et al. 2004). Only a small portion of the IV-injected γ-CD is absorbed intact from the GI tract, and it is mostly excreted unchanged in the urine. Oral administration of 8 g γ-CD or 8 g maltodextrin to humans did not reveal any differences in GI tolerance of these two oligosaccharides (Koutsou et al. 1999). The γ-CD showed to promote an insignificant irritation after IM injection and was rapidly and completely degraded by intestinal enzymes in the upper intestinal tract to glucose monomers (even using high daily doses, e.g., 10–20 g/kg). Following p.o. administration γ-CD remained intact, i.e., almost no absorption (0.1 %) was observed and it was practically not metabolized after parenteral administration (LD_{50} oral, rat $\gg$8,000 mg/kg; LD_{50} IV, rat $\approx$4,000 mg/kg) (Del Valle 2003).

Comparing the toxicological profile of the three natural CDs, γ-CD seems to be the least toxic. But its complexes normally have limited solubility in aqueous solutions and tend to self-aggregate; therefore, its complexing abilities are limited compared to those of β-CD and some water-soluble β-CD derivatives (Table 5).

4.2 Metabolism and Pharmacokinetics

4.2.1 Cyclodextrins

Regarding physicochemical and biological characteristics, CDs (ring dextrins) are very similar to water-soluble linear dextrins, but owing to their cyclic structure, they are more resistant to hydrolysis (enzymatic and nonenzymatic) than linear ones (Kurkov and Loftsson 2012). Contrary to starch, CDs are generally resistant to β-amylases, so they are not hydrolyzed from the nonreducing ends, but they cannot resist to the slow hydrolysis caused by α-amylases (hydrolysis from within the carbohydrate chain). α-CD and β-CD are not affected by the stomach acids and salivary and pancreatic enzymes during digestion, but γ-CD is digested partly by these amylases in the GI tract. The mechanism involved in the CDs' hydrolysis resistance is due to all bridge oxygens being hidden within the central cavity; this hydrolytic rate will depend on several factors, namely, the ring size and fraction of free CD (Kurkov and Loftsson 2012).

Linear dextrins as well as starch are digested mainly into glucose through a stepwise enzymatic hydrolysis process which starts when in contact with salivary α-amylase, but by p.o. administration this is overcome as in the stomach the enzyme is inactivated (Dona et al. 2010). Some nonenzymatic specific acid hydrolysis of the dextrins can occur in the stomach, but this phenomena can be delayed by the formation of complexes with food lipids (Singh et al. 2010). In the small intestine (neutral pH), where pancreatic fluid containing α-amylase is released, enzymatic hydrolysis occurs. The dextrin substrates that remained intact along the above enzymatic process are finally submitted to bacterial digestion when they reach the lower sections of the intestine.

Thus, the most important pharmacokinetic parameters for these macromolecules are urinary clearance and hepatic uptake (Nishikawa et al. 1996). As it was shown by several authors, both natural CDs and their derivatives are susceptible to bacterial digestion in the GI tract (Antlsperger and Schmid 1996; Irie and Uekama 1997; Zhou et al. 1998; Zuo et al. 2002; Van Ommen et al. 2004; Stella and He 2008).

Natural CD pharmacokinetics are very similar to each other and to the linear dextrins with similar molecular weights (MW). The insignificant amount that is absorbed from the GI tract after p.o. administration is made by passive diffusion and can be explained by the bulky and hydrophilic nature of the CDs. After absorption, CDs are eliminated by the renal route without undergoing significant metabolism. Any remaining CD is eliminated by other pathways (liver metabolism and biliary excretion) similarly to low MW dextrans.

The main elimination process is dependent on the administration route: Following p.o. administration in rats and dogs, HP-β-CD was mainly excreted via the feces, whereas after IV administration, this CD was excreted via the kidneys. In the case of humans, this excretion process mainly occurs via the kidneys (Gould and Scott 2005). The $t_{1/2}$ of the elimination phase (natural CDs) ranged approx. between 1.4 and 2 h, and the volume of distribution (V_D) was approx. 0.2 L kg^{-1}. The pharmacokinetic studies showed that over 90 % of parenterally administered CD

Table 6 The absorption of cyclodextrins and their derivatives following oral administration (Stella and He 2008)

Cyclodextrin	Species	Dose (mg/kg)	Absorption (% dose)	Reference
α-CD	Rat	200	1	Van Ommen et al. 2004
β-CD	Rat	500	0.6	Kubota et al. 1996
γ-CD	Rat	1,000	0.02	De Bie et al. 1998
G-β-CD	Rat	500	0.3	Kubota et al. 1996
HP-β-CD	Rat	40	3	Gerloczy et al. 1990
SBE-β-CD	Rat	60	1.64	CyDex (unpublished)

will be eliminated from the body within approximately 6 h and over 99.9 % within 24 h (Kurkov and Loftsson 2012). The absorption of natural CDs and their derivatives following oral administration in the rat is summarized in Table 6.

After being absorbed CDs are distributed to several organ tissues, such as the kidney, urinary bladder, liver, adrenal gland, and others (Gerloczy et al. 1990; Monbaliu et al. 1990; Antlsperger and Schmid 1996; Kubota et al. 1996; De Bie et al. 1998; Van Ommen et al. 2004). The absorption of β-CD-methylated derivatives in rats were 6.3–9.6 % for DM-β-CD and 0.5–11.5 % for M-β-CD (Mosher and Thompson 2002). The HP-β-CD oral bioavailability (itraconazoleoral solution) in humans was estimated to be less than 1 % (De Repentigny et al. 1998; Stevens 1999). The IV administration of β-CD and HP-β-CD to permanently cannulated rats resulted in a pharmacokinetic behavior of both CDs similar to that of inulin, showing a rapid distribution over extracellular fluids and elimination through glomerular filtration (Frijlink et al. 1990). CDs via IV route disappeared rapidly from systemic circulation, as they are rapidly eliminated by the kidney, almost in intact form. This makes the kidney the organ with the highest level of CDs among all tissues. Thus, renal insufficiency could result in CD accumulation. As an example, it was observed that after IV bolus administration (10 mg kg^{-1}) of M-β-CD, its concentration in the kidneys (mainly in the renal cortex) remained almost at the same level for at least 6 days (Antlsperger and Schmid 1996; Slain et al. 2001; Gould and Scott 2005; Von Mach et al. 2006; Stella and He 2008).

The β-CD steady-state volume of distribution (V_{dss}) and most of its derivatives in all animal species tested correspond well with the respective extracellular fluid volume (Table 7). Thus, it can be concluded that, after IV administration, CDs distribute mainly in the extracellular compartments without involving the deep compartments or storage pools (Kleijn et al. 2011). Methylated β-CD has a larger V_{dss} and longer $t_{½}$ compared to other CD derivatives which may be due to its ability to interact with cellular membranes and its hemolytic activity (Thompson 1997). The total plasma clearance for HP-β-CD and SBE-β-CD in all species tested is similar to the glomerular filtration rate of individual species (Davies and Morris 1993). In these cases 100 % of an IV dose was recovered in the urine within 6–12 h (Davies and Morris 1993). Most CDs disappear from tissues dramatically within the first several hours after administration. For example, the level of SBE-β-CD in most tissues decreased by more than 90 %; even in the kidney, SBE-β-CD was reduced

Table 7 Pharmacokinetic parameters of some cyclodextrins IV administered (Stella and He 2008)

Cyclodextrin	Species	Dose (mg/kg)	$T_{1/2}$ β (min)	V_{dss} (mL/kg)	Plasma clearance (mL/min/ kg)	Reference
β-CD	Rat	50	21.6	201.8	7.53	Kubota et al. 1996
G-β-CD	Rat	50	17.3	308.1	12.5	Kubota et al. 1996
HP-β-CD	Rat	200	23.9	194	7.2	Frijlink et al. 1990
HP-β-CD[a]	Human	43	102	222	1.57	Szathmary et al. 1990
HP-β-CD[b]	Human	114	114	253	1.54	Zhou et al. 1998
SBE-β-CD	Rat	600	18	300	9.8	CyDex (unpublished)
SBE-β-CD	Rabbit	600	30	200	5.2	CyDex (unpublished)
SBE-β-CD	Dog	240	66	430	4.7	CyDex (unpublished)
SBE-β-CD[c]	Human	100	84	200	1.9	CyDex (unpublished)
M-β-CD[d]	Rabbit	200	420	2,500	3.8	(Grosse et al. 1999)

[a]Calculated as 70 kg body weight. HP-β-CD was administered intravenous injection (IV) at 3 g in normal volunteers

[b]The body weight is calculated as 70 kg. HP-β-CD was administered as itraconazole formulated with HP-β-CD to patients with advanced AIDS at 8 g over 1 h IV infusion

[c]IV infusion for 1 h

[d]Methyl-β-CD with total degree of substitution (DS) of 10.4

by at least 60 % from 0.1 to 1 h following IV bolus injection of 600 mg/kg SBE-β-CD in the rat (Stella and He 2008).

4.2.2 Complexes

The partitioning of the drug in its different forms (dissolved free drug molecules, dissolved complex, solid free drug, and solid complex) depends on the solubility of the free drug and the complexed drug in the given medium, the stability constant of the CD-drug complex, the molar ratio of the components, and the volume of the liquid phase. The same complex may provoke different – enhanced or decreased – biological responses depending on the additional amount of CD or even on the volume of water consumed.

It has generally been believed that following oral administration only an insignificant amount of CD is absorbed simultaneously with the drug, i.e., only free drug molecules can enter the circulation and the increasing excess of the remaining CD will push the equilibrium towards complex formation. CD acts primarily as a carrier agent to the site of absorption transporting the more or less hydrophobic guest

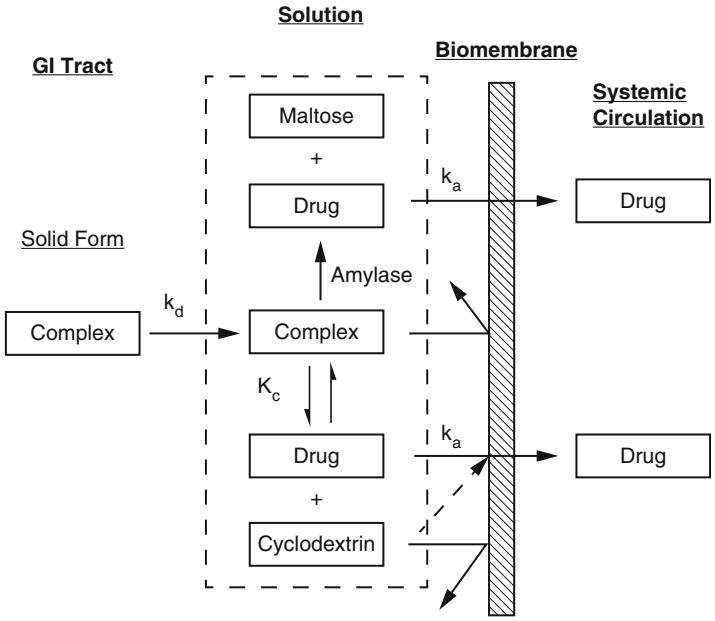

Fig. 5 Mechanism of drug absorption from CD complexes in vivo (where k_d is the rate constant of dissolution, k_a rate constant of absorption, *D* drug in the orally administered dose, *GI* concentration of dissolved drug in the gastrointestinal tract, and *B* concentration of drug in blood) (Cabral-Marques 1994c)

molecule through an aqueous milieu to the lipophilic membrane of cells in the GI tract. There the guest molecule is absorbed, since the membrane has a higher affinity for a lipophilic guest molecule than the CD itself. If the complex is very stable or the CD concentration is high, the equilibrium is greatly shifted towards complexation and absorption is considerably hindered. Some studies have indicated slow absorption of some CDs by passive diffusion across intestinal membranes, which seem to be promoted in the presence of bile salts and Ca^{2+}-chelating agents indicating absorption by the paracellular pathway. It is unclear whether GI tract absorption of drug-CD complexes may constitute a significant level of absorption under certain conditions, but in the case of pulmonary delivery where the barrier membranes are much thinner, this possibility should be considered.

In a model for in vivo drug release, the first step must be the dissolution of the solid adducts (Fig. 5). There then exists an equilibrium between the dissolved complex and the released drug. Only the dissolved, non-complexed drug can be absorbed (Cabral-Marques 1994c).

4.3 Biotechnological/Bioengineering Approaches

CD has been used in several biotechnological/bioengineering studies, some of which are described below:

It is not easy to predict drug absorption after its complexation with CD due to the large range of interacting parameters and their influence on free drug concentration.

Molecular modeling can help by making predictions and providing orientations for the development of new complexes that can be formulated in pharmaceutical dosage forms. Neutral compound complex model demonstrates the mechanism behind a possible variation (increase, decrease or no effect) in the absorption when CD is dosed as an inclusion complex. A model for predicting the CD effect on drug bioavailability, when administered as inclusion complex, was used. This model involved mass transport expressions for drug dissolution and absorption and pseudo-equilibrium *D•CD* complexation. Its predictions were compared with in vitro and in vivo experimental results allowing to predict when CD can be delivered as a preformed complex with drug to enhance bioavailability (Gamsiz et al. 2013).

Different polymers such as plasticized poly (vinyl chloride) (PVC-P), poly (ethylene oxide) (PEO), PEO/poly (propylene oxide) (PPO), and copolymers, e. g., Pluronic F68 (F68), were studied in order to obtain a novel modified biomaterial surface using CDs for increased blood compatibility. The β-CD-PEO and β-CD-F68 associations in certain feeding ratio were synergistic in producing the desired effect (Zhao and Courtney 2006).

Hydrogels have suitable properties for wound-dressing use due to its good biocompatibility and maintenance of a moist environment for better healing. The efficacy of these platforms can be improved by the use of CDs as cross-linking agents. The association hydrogels-CDs may allow the moist environment required for the healing process and the moiety with the ability to protect and modulate the release of bioactive molecules (Pinho et al. 2013). Furthermore, the development of the platforms using CD-containing polyrotaxanes is expected to provide a new paradigm for biomaterials. Thus, the CD-based biodegradable polypseudorotaxane hydrogels could be used as a promising injectable drug delivery system for sustained and controlled drug release. The polyrotaxanes with drug- or ligand-conjugated CDs threaded on a polymer chain with a biodegradable end group could be useful for controlled and multivalent targeted delivery. In the field of gene delivery, cationic polyrotaxanes consisting of multiple OEI-grafted CDs threaded on a block copolymer chain are attractive non-viral gene carries due to the strong DNA-binding ability, low cytotoxicity, and high gene delivery capability (Li et al. 2011).

The bioactive compounds from plants (polyphenolics, alkaloids, and polysaccharides) have well-documented pharmacological properties and have been widely used by the food, cosmetic, and pharmaceutical industries due to its advantages, such as high levels of biocompatibility, low toxicity and good availability. Polyphenolics are currently the major group of interest in view of their anti-inflammatory, antimicrobial, and antioxidant properties, as well as their availability in the human diet (Cowan 1999; Aridogan et al. 2002; Belščak-Cvitanović et al. 2011; Gou et al. 2011). Many studies have been published regarding the encapsulation of natural polyphenolic agents by CDs, for food and drug delivery proposes. CDs have a capacity of improving their aqueous solubility, protect them from elevated temperatures, pH, and light- or moisture-induced degradations

increasing their bioavailability. In the case of flavonoids, the CD derivatives are the best choices to achieve an efficient complexation (Pinho et al. 2014).

Monochlorotriazinyl-β-cyclodextrin (β-CDMCT) has been used as a great tool for surface modification of natural and synthetic fibers (polyester, polyamides, and polyacrylics). These materials after being grafted onto celluloses substrates can be used for fragrance release, odor adsorption (sheets and personal clothing), controlled release (antibacterial, fungicide, or insect repellents), UV protection, and stabilization of active ingredients due to CD cavities. These platforms in β-CD cavities can include an insect repellent and an insecticide. In addition this new tool was designed for the treatment of materials (such as bed nets, curtains, specific clothing, military uniforms, etc.) in order to prevent man-vector contact (Reuscher and Hinsenkorn 1996; Romi et al. 2005).

The structural CD properties make them suitable building blocks to be used in polymeric reactions, since they can be grafted to polymers (natural/synthetic) resulting in different polymer architectures (Zhang and Ma 2013).

Molecular complexation with CDs already cited play an important role in biotechnology and bioengineering area in the domain of selectivity, separation, and solubilization of various biomolecules.

4.4 Applications of CDs

Since CDs are versatile molecules that offer unique features and have negligible cytotoxic effects, they have been widely used in many industrial products, technologies, and analytical methods (Cabral-Marques 1994c; Singh et al. 2002; Del Valle 2003; Cabral-Marques 2008). Some examples of applications are the following.

4.4.1 Pharmaceuticals

CDs have been widely used in pharmaceutical industries, being in the most of the cases as auxiliary substances, improving stability, bioavailability, and organoleptic properties (e.g., palatability, masking odors) of active compounds in multiple pharmaceutical dosage forms (Del Valle 2003; Carrier et al. 2007; Miller et al. 2007; Cabral-Marques 2010). These versatile sugar molecules enabled the production and reintroduction of numerous active substances that, owing to stability, compatibility, or absorption problems, were not in use (Cabral-Marques 1994c). Shortly, the CDs' multifunctional features have been exploited in drug delivery system being potential candidates in many applications because of their ability to alter the physical, chemical, and biological properties of guest molecules through the formation of inclusion complexes (Uekama et al. 1998; Cabral-Marques 2008). All these improvements enabled their application in commercialized pharmaceutical products used in almost every drug delivery systems: α-CD (oral, parenteral), β-CD (dermal, oral, rectal, sublingual), HP-β-CD (ocular, oral, parenteral, rectal), DM-β-CD (dermal, nasal, ocular), SBE-β-CD (parenteral), and HP-γ-CD (ocular) (Cabral-Marques 2008; Tiwari et al. 2010). Among all CDs, β-CD is the most reasonably priced, thus the most used on industrial applications.

Oral delivery: The drug release in this route of administration is controlled either by dissolution, diffusion, osmotic systems, density, or pH (Tiwari et al. 2010).

CDs are particularly useful as they can be used as a way to reduce local drug irritation and increase drug absorption rate (Loftsson et al. 2005b). The hydrophilic CDs provide an enhancement of stability, solubility, dissolution rate, and wettability of poorly water-soluble drugs by means of complex formation (Carrier et al. 2007). CDs are supposed to act only as carriers and help to transport the drug through an aqueous medium to the lipophilic absorption surface in the GI tract. Buccal and sublingual routes have also been an effective way to deliver rapid-dissolving drug-CD complexes. In this case, a rapid increase in the systemic drug concentration takes place along with the avoidance of systemic and hepatic first-pass metabolism (Tiwari et al. 2010).

Pulmonary delivery: Pulmonary administration has been gaining an increased importance for therapeutic usage due to lung's anatomical and physiological characteristics like large surface area available (~100 m^2), high irrigation (5 L/min), very thin absorption membrane (0.1–0.2 μm), and relatively low enzymatic activity (Pilcer and Amighi 2010). This is normally an attractive route for treatment of local diseases (e.g., asthma, DPOC) but is also interesting for systemic delivery. However, this route is usually limited by the characteristics of drugs used, low aqueous solubility, and slow drug dissolution (Loftsson et al. 2005b). CDs are used to solve these drawbacks and to improve the pulmonary delivery of drugs either systemically or locally. Formulations of insulin delivered via pulmonary route (powders and liquids) have low bioavailability (10–15 %), and thus, to get the right dose, a high drug loading is needed in drug delivery system (Ramalhete et al. 2001). Formulations with penetration enhancers (with proven efficacy via other administration routes) such as CDs could help this matter (Cabral-Marques et al. 1991). Current DPIs (Dry Powder Inhalers) for treatment of respiratory diseases generally deliver only 10–20 % of the nominal dose to the site of action. It is possible to engineer particles by spray-drying and using CDs to improve this drawback allowing a more efficient drug delivery to the site of action, with fewer systemic effects than oral therapy (Vozone and Cabral-Marques 2002; Almeida and Cabral-Marques 2004; Drumond et al. 2014).

Nasal delivery: This is another effective route that avoids the extensive GI breakdown and hepatic first-pass metabolism. Due to nasal mucosa's good permeability, this delivery route is a novel approach for systemic delivery of high potency drugs with a low oral bioavailability. As lipophilic CDs can interact with biological barriers (without affecting their barrier function), they have the ability to enhance the drug delivery – a property which makes CDs ideal penetration enhancers for intranasal administration. Methylated CDs are the most commonly used and widely studied. They have shown in several studies very promising results being efficient absorption enhancers, namely, in the delivery of peptides and hormones (Merkus et al. 1999; Loftsson et al. 2005b; Tiwari et al. 2010).

Ocular delivery: In this type of delivery systems, the most used dosage form is the eye drop due to easy instillation in the eye. However, eye drops are a great formulation challenge due to the high probability of its inability to sustain high

local concentration of drug (Tiwari et al. 2010). By this reason CDs have been used to overcome these problems as they present possible advantages in ophthalmic use, such as increase in solubility and/or stability and reduction or avoidance of side effect of drugs (e.g., discomfort and irritation) (Torres Marques et al. 1996; Loftsson et al. 2012). One of the prerequisites for a new vehicle to be used in ophthalmic preparations is that it is not irritating to the ocular surface, because irritation causes reflex tearing and blinking, which result in a fast washout of the instilled drug (SáCouto et al. 2014). Hydrophilic CDs do not penetrate eye cornea biological barriers but enhance the ocular bioavailability of lipophilic drugs by keeping them in solution (Cabral-Marques 2008).

Rectal delivery: This route is by far the chosen one to deliver drugs to the unconscious patients, children and infants. Despite offering a relatively low area for drug absorption, which leads to an erratic release of drugs, the rectal mucosa represents a potential site for delivery of drugs with limitations by the oral route (e.g., organoleptic problems, pH degradation, high first-pass metabolism) (Tiwari et al. 2010). Beraldo et al. (2002) has shown that CDs are quite useful to solve the erratic release problems of this route.

Dermal delivery: This is the most sophisticated and more reliable form to administrate the drug through skin, either for local or systemic action (Tiwari et al. 2010). CDs have a significant safety margin in dermal application and can be used to optimize the transdermal delivery of drugs. CDs improve drugs' solubility and/or stability in topical preparations, consequently enhancing the transdermal absorption of drugs, to sustain the drug release from the vehicle and to avoid undesirable side effects associated with dermally applied drugs (Waleczek et al. 2003; Santos et al. 2004; Loftsson et al. 2005b). CDs may interact with some components of the skin and the lipids such as cholesterol and triglycerides and consequently induce a temporary change in the skin barrier function and an enhancement in the drug absorption. It was shown that DM-β-CD has higher skin permeation effect than HP-β-CD (Cabral-Marques 2008).

4.4.2 Bioconversion and Fermentation

The efficiency of bioconversion and fermentation processes is often restricted by the inhibitory or toxic influence from either the substrate or the conversion products on the biocatalyst. Another problem often found on most organic substrates is their solubility in water which complicates the amount of substrate accessible to the biocatalyst. In order to overcome process limitations such as low solubility of lipophilic substrates, CDs can be added to the bioconversion or fermentation media. In addition CDs may provide a reduction of toxicity by complexation with toxins and biocompatibility improvement (Bar 1989; Cabral-Marques 2010).

4.4.3 Environment Protection

CDs are important to environmental science in terms of remediation by complexation of organic contaminants/pollutants and heavy metals from soil, water, and atmosphere. After treating wastewaters containing environmentally unacceptable aromatic compounds (phenol, p-chlorophenol and benzene) with β-CD, the initial

levels of these aromatic hydrocarbons were significantly reduced. The same CD benefic actions may also be used in the treatment of gaseous effluent from chemical industries (Cabral-Marques 1994b; Del Valle 2003).

4.4.4 Agrochemical Industry

CDs may be used to improve a variety of properties of bactericides, insecticides, herbicides, and fungicides. Complex formation with β-CD has been reported, including examples of increased or decreased activity of pesticides (Cabral-Marques 1994b).

4.4.5 Catalysis

The CDs ability to mimetize enzymes, i.e., molecular recognition phenomenon, led them to be used in catalysis. Thus, CDs have to be modified through substituting various functional compounds or by attaching reactive groups. In addition CDs possess the ability of enantiomeric recognition. Resulting from the chelating effect of the CD catalysts, rates of reaction may be enhanced by almost 1,000-fold when compared to free solution (Del Valle 2003).

4.4.6 Analytical

The CDs are very useful in separation process due to its molecular recognition ability to discriminate positional isomers, functional groups, and homologues/enantiomeric compounds. Depending on the substrate to be separated, the choice of the CDs is essential since shape, size, and selectivity will influence separations. CDs have been used in HPLC and GC (being CDs in the stationary phase columns), in NMR (chiral shift agents), in circular dichroism (selective agents altering spectra), and also in electrochemical chemistry (to mask contaminating compounds) (Del Valle 2003). These substances serve as an ideal selector by molecular recognition and further enhance the complex forming ability and selectivity in various type of separations (Eastburn and Tao 1994; Han 1997; Loung and Nguyen 1997; Szejtli 1998; Del Valle 2003; Cabral-Marques 2010).

4.4.7 Food and Flavors

In food industry the CDs are used as multifunctional food ingredients. They can be used as carriers for molecular encapsulation forming inclusion complexes with a variety of molecules including fats, aromatic agents, flavors, and colors. The inclusion complexes improve both physical and chemical stability of sensitive ingredients leading to better conservation and, consequently, to extended food shelf life. CDs also can act as a controlled release agent of desired food constituents and can provide a promising alternative to the conventional encapsulation technologies by removing and/or masking undesirable components (e.g., removal of cholesterol from butter, eggs, milk; decreasing of juice's bitterness). In addition, CDs can protect the food ingredient throughout many rigorous food-processing methods of freezing, thawing, and microwaving (Prasad et al. 1999; Del Valle 2003; Cabral-Marques 2010).

4.4.8 Cosmetics, Toiletry, and Personal Care

Cosmetic preparations are another area where CDs have been largely used to protect molecules against physical and chemical instabilities (e.g., oxidation, decomposition, hydrolysis). By complex formation included guest molecules may increase their stability, suppress volatility (in the case of perfumes), reduce/prevent skin irritation, and improve room fresheners and detergent's action. Active ingredients are released from inclusion compounds by controlled release. In summary, it is possible to say that major benefits of CDs in the cosmetics, toiletry, and personal care products are stabilization, odor control, process improvement upon conversion of liquids to solids, absorption increase/decrease of various compounds into skin, flavor protection, enhanced water solubility, and thermal stability of oils. Moreover, CDs are used in toothpaste, lipsticks, skin creams, liquid and solid fabric softeners, paper towels, tissues, shampoos, and deodorants (Del Valle 2003; Cabral-Marques 2010).

4.4.9 Packaging and Textile Industry

Textile industry is another area in which CDs are increasingly attracting attention (Cabral-Marques 2010). Fabrics can be imbued with novel properties by means of these substances. In order to permanently transfer the versatile properties of CDs to textiles, Wacker-Chemie (the world's largest producer of γ-CDs) covalently attached reactive CD derivative with monochlorotriazinyl (MCT) substituents to the fiber. This substituted CD provided excellent textile finishing to cottons, blended materials, and woolens. Using hydrophobic tosyl derivative of β-CDs, threefold increase in the binding of fluorescent dye to the polyester fiber was attained.

CDs also play a major role in the packaging industry. Inclusion complex containing oily antimicrobial and volatile agents are coated on a water-absorbing sheet with a natural resin binder, which is used for wrapping fresh products. It was found that food-packaging bag manufactured using CD with ethylene-tetracyclo-3-dodecane copolymer and hinokitiol showed no odor and good antifungal properties after 1 week of storage at room temperature, which proved useful for food-packaging materials (Hedges 1998; Ishibashi et al. 1999; Hirose and Yamamoto 2001).

5 Current Regulatory State

Despite of being characterized as pharmacologically inactive compounds, pharmaceutical excipients are treated as active compounds in the regulatory perspective, especially when used for the first time in humans (Demerlis et al. 2009; Koo 2011; Osterberg et al. 2011). Their acceptance is often supported by previous usage in commercialized pharmaceutical products or in the food industry. This is also valid if the excipient is already being used at the same levels and administration routes as in marketed products. In these cases a full safety evaluation may not be needed (Kurkov and Loftsson 2012).

Table 8 Food approval status of Cyclodextrins (Cabral-Marques 2010)

	α-CD	β-CD	γ-CD
WHO/FAO[a]	ADI = not specified (June 2001 and June 2004)	ADI = 5/mg/kg/day (Jan. 1995)	ADI = not specified (1999 and 2000)
USA	GRAS[b] (Jan. 2004)	GRAS[c] (Oct. 2001)	GRAS[b] (Sept. 2000)
Canada	Filed for novel food status (July 2006)		
EU	Novel food approved (2008)	Carrier for food additives (<g/1 kg)	Novel food filed (Jan. 2010)
Japan	Natural product	Natural product	Natural product
Mexico	Follow FDA approvals with an import license	Follow FDA approvals with an import license	Follow FDA approvals with an import license
Mercosur States[d]	Food approved		
FSANZ[e]	Novel food (Jan. 2004)		Novel food (2003)
Korea	Approved for dietary supplement	Approved for dietary supplement	Approved for dietary supplement
Philippines	Food approved		Food approved
Thailand			Approved for dietary supplement
Taiwan	Approved for dietary supplement		

[a]*WHO*/*FAO* World Health Organization/Food and Agriculture Organization of the United Nations
[b]GRAS in a wide range of intended use in food
[c]GRAS as a flavor protectant
[d]Mercosur States – Argentina, Brazil, Paraguay, Uruguay, and Venezuela
[e]FSANZ – Food Standards Australia New Zealand

The regulatory status of CDs has evolved over the last years even with the skepticism within the regulatory agencies concerning their usage. Consensus seems to be building among regulators that CDs are excipients and not part of the drug substance although divergent opinions exist (Brewster and Loftsson 2007). Products containing CDs undergo rigorous evaluation, and their strengths and weaknesses are understood and agencies will become more comfortable with them and lower the barriers to their use (Davis and Brewster 2004; Loftsson et al. 2004; Stella and He 2008). Table 8 shows an overview of the food approval status of CDs by the different worldwide regulatory agencies.

Monograph for natural β-CD is listed in some pharmacopoeia sources such as the US Pharmacopoeia/National Formulary (USP/NF), European Pharmacopoeia (Ph. Eur.), and Japanese Pharmaceutical Codex (JPC). α-CD is also listed in the Ph. Eur., USP/NF, and JPC; γ-CD is referenced in the JPC and soon will be included in the Ph. Eur. and USP/NF. A monograph for HP-β-CD is available in the Ph. Eur., and a draft has been circulated for the USP/NF; SBE-β-CD can be found in the USP/NF. Other derivatives are not yet compendial but efforts are being made for their inclusion. α-CD, β-CD, and γ-CD were also introduced into the generally regarded as safe (GRAS) list of the FDA for use as a food additive in 2004, 2001,

and 2000, respectively; HP-β-CD and γ-CD are cited in the FDA's list of Inactive Pharmaceutical Ingredients. SBE-β-CD is also available in various dosage forms and listed in the FDA's compilation of Inactive Pharmaceutical Ingredients (Cabral-Marques 2010; Kurkov and Loftsson 2012).

Currently different marketed pharmaceutical products (Table 9) as well as numerous food products (Cabral-Marques 2010) can be found using different CDs: α-CD, β-CD, HP-β-CD, RM-β-CD, SBE-β-CD, γ-CD, and HP-γ-CD.

6 Conclusion/Prospects

Since the begin of nineteenth century CDs have been showing their potentialities, and up to today they have gained a great importance and different applications in distinct areas from pharmaceutical and food to chemical and environmental industries. So a prosperous future can be envisaged to CDs being one of the most adaptable and relevant excipient. All this was only possible due to their remarkable characteristics which make them a versatile compound with the ability to form inclusion complexes with guest molecules possessing a hydrophobic moiety (Tiwari et al. 2010).

Due to science evolution, the discovery of new molecules with solubility, bioavailability, and permeability limitations or other undesirable properties (stability, taste and odor, irritation potential, etc.) raises the need for CDs' usage as they can be useful tools for scientists.

CDs can be useful drug carriers in delivery systems as they are bio-adaptable particularly in parenteral applications (especially HP-β-, SBE-β-, and maltosyl-β-CDs), having the ability to control the rate and time of drug release. Peracylated CDs may serve as novel hydrophobic carriers to control the release of water-soluble drugs including peptides and proteins in various routes of administration. Amphiphilic or ionizable CDs can modify the rate or time of drug release and bind to the surface membrane of cells, which may be used for the enhancement of drug absorption across biological barriers. A combination of molecular encapsulation with other pharmaceutical excipients is effective and valuable in the improvement of carrier properties of CDs. Conjugates of a drug with CDs can be a versatile drug site-specific carrier for colon therapy; they form a new class of colon-targeting prodrugs (Uekama et al. 1998; Uekama 2004; Li and Loh 2008).

There are more than 100 different CD derivatives commercially available as fine chemicals, mainly for use in chromatography, in diagnostics, and as intermediate for further synthesis. The ideal derivative does not exist yet. It is expected that more derivatives will be developed. For organ or receptor targeting, extremely stable, bio-adaptable, and specific affinity-showing CD complexes will be needed (Cabral-Marques 2008; Kurkov and Loftsson 2012).

Despite of all the unique architecture and the chelating properties that CDs present, the usage of this excipient is not by itself a reason to achieve a successful goal without previous studies. In fact a great knowledge of the characteristics of all the compounds intended to be used (CDs, guest molecule, additives to the product)

Table 9 Commercialized pharmaceutical products containing cyclodextrins (Adapted from Szejtli 2005; Hincal et al. 2011; Kurkov and Loftsson 2012)

Cyclodextrin	Drug	Therapeutic usage	Formulation	Trade name
α-CD	Alprostadil	Treatment of erectil dysfunction	Intracavernous solution	Caverject, rigidur, dual, edex
	Alprostadil (PGE1)	Chronic arterial occlusive disease, etc	Intra-arterial infusion	Prostodin
	Alprostadil (PGE1)	IA or IV vasodilator	IV solution	Prostarmon
	Alprostadil (PGE1)	IA or IV vasodilator	IV solution	Prostavasin
	Cefotiam hexetil HCl	Antibiotic	Oral tablet	Pansporin T
	OP-1206	Buerger's disease	Tablet	Opalmon
β-CD	Benexate HCl	Anti-ulcerant	Oral capsule	Ulgut, Lonmiel
	Betahistidine	Antivertigo drug	Tablet	Betahist
	Cetirizine	Antibacterial agent	Chewing tablets	Cetrizin
	Cephalosporin	Antibiotic	Tablet	Meiact
	Chlordiazepoxide	Tranquilizer	Tablet	Transillium
	Dexamethasone	Anti-inflammatory steroid	Ointment, tablets	Glymesason, glyteer
	Dextromethorphan	Antitussive (cough suppressant)	Xyrup	Rynathisol
	Diphenhydramine, chlorotheophylline	Travel sickness	Chewing tablets	Stada travel pill
	Flunarizine	Calcium channel blocker	Tablet	Fluner
	Iodine	Throat disinfectant	Gargle	Mena-Gargle
	Meloxicam	Nonsteroidal anti-inflammatory drug	Tablet, suppository	Mobitil
	Nicotine	Nicotine replacement product	Sublingual tablets	Nicorette, nicogum
	Nimesulide	Nonsteroidal anti-inflammatory drug	Tablets	Nimedex, mesulid fast
	Nitroglycerin	Coronary dilator	Sublingual tablets	Nitropen
	Omeprazole	Proton-pump Inhibitor	Capsule	Omebeta
	PGE2	Induction of labor	Sublingual tablets	Prostarmon E
	Piroxicam	Nonsteroidal anti-inflammatory drug	Tablets	Brexin, brexidol
	Piroxicam		Suppository	Cycladol

(*continued*)

Table 9 (continued)

Cyclodextrin	Drug	Therapeutic usage	Formulation	Trade name
	Piroxicam	Analgesic for pediatric use	Liquid	
	Rofecoxib	Nonsteroidal anti-inflammatory drug	Tablet	Rofizgel
	Tiaprofenic acid	Analgesic	Oral tablet	Surgamyl
	Thiomersal	Antiseptic/ antifungal	Eye drop	Vitaseptol
HP-β-CD	Alfaxalone	Veterinary anesthetic		
	Cisapride	Gastroprokinetic agent	Suppository	Propulsid
	Hydrocortisone	Mouthwash (aphtha, gingivitis, etc.)	Liquid	Dexacort
	Indomethacin	Nonsteroidal anti-inflammatory drug	Eye drop solution	Indocid
	Itraconazole	Antifungal agent	Oral and IV solutions	Sporanox
	Mitomycin	Anticancer agent	IV infusion	Mitozytrex MitoExtra
	Telavancin	Bactericidal lipoglycopeptide	IV infusion	Vibativ
RM-β-CD	17β-Oestradiol	Hormone	Nasal spray	Aerodiol
	Chloramphenicol	Antibiotic	Eye drop solution	Clorocil
SBE-β-CD	Aripiprazole	Antipsychotic drug	IM solution	Abilify
	Amiodarone	Antiarrhythmic agent	IV	Nexterone
	Insulin	Diabetes	Nasal spray	
	Maropitant	Antiemetic drug (motion sickness in dogs)	Parenteral solution	Cerenia
	Voriconazole	Antifungal agent	IV solution	Vfend
	Ziprasidone mesylate	Antipsychotic drug	IM solution	Geodon, Zeldox
γ-CD	Minoxidil	Stimulate hair growth	Solution	Alopexy
	Sugammadex (as sodium salt)	Antidote (modified γ-CD)	IV solution	Bridion
HP-γ-CD	Diclofenac sodium salt	Nonsteroidal anti-inflammatory drug	Eye drop solution	Voltaren Ophtha
	Tc-99 Teoboroxime	Diagnostic aid, cardiac imaging	IV solution	CardioTec
SL-CD	Modified porcine (circovirus inactivated)	PCV2 vaccine	Suspension for injection	Suvaxyn PCV

and prior work are extremely necessary to try to predict and find out any possible interaction between them, just because these interactions can adversely affect the performance of these constituents, which can lead to incompatibilities, formulation problems, lack of stability, etc., and consequently have a great impact in the final commercialized product. In conclusion, the ideal scenario would be to choose the CD that has the most appropriate characteristics to include the desired guest molecule, predict all the different variables that can influence the complex formation process, and in the end be able to prepare a product with the desirable properties that can be an innovation or an improvement and at last but not the least be economically viable.

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Cellulosic Biomaterials

10

Holger Wondraczek and Thomas Heinze

Contents

Abstract

Cellulose, the most abundant polymer on earth, holds a big potential for different applications in the biomedical field. This book chapter summarizes recent advances of cellulose research with respect to the new biomaterials paradigm. In this context, it is intended to give the reader an overview on the huge variety of cellulosic structures that are provided by nature or can be man-made. It highlights important examples of historical and recent cellulosic biomaterials and touches certain aspects of ongoing developments, which may form the basis for future applications.

Keywords

Structure • Biosynthesis • Bacterial cellulose • Nanomaterials • Tissue engineering • Wound treatment • Drug delivery • Cellulose derivatives

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1 Introduction and Scope

Although cellulose and many of its derivatives possess unique properties including hydrophilicity, polyfunctionality, inherent biocompatibility, and last but not least, the ability to form supramolecular structures, they were typically not considered as classical biomaterials. According to the Consensus Conference on Definitions in Biomaterials Science, in 1986, which determined a biomaterial to be "a non-viable material used in a medical device, intended to interact with biological systems" (Williams 1987), only a very limited number of cellulosics were understood as biomaterials. However, in the context of the continuous development in materials science and health technology, the biomaterials paradigm has evolved to:

> A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine. (Williams 2009)

As a consequence of this changed understanding of what biomaterials are, cellulose and its derivatives are constantly studied for their applicability, for example, in tissue engineering, drug delivery, and diagnostics.

This chapter is not intended to exhaustively cover the published literature on cellulosic biomaterials. Instead, important concepts regarding the use of cellulose and cellulose derivatives in the field will be presented with the aim to enable the reader to further explore these concepts towards new applications in medical technology. For this purpose fundamental information on the structure, processing, and derivatization of cellulose will be provided as well as selected examples of well-known and quite recent developments of advanced cellulosic (bio)materials.

2 Sources of Cellulose

Cellulose is distributed throughout nature in plants, animals, algae, and fungi (Fig. 1). However, the majority of cellulose sources are plant fibers. Cellulose contributes approximately 40 % to the carbon fraction in plants, serving as structuring element within the complex architecture of the plant cell walls. Cellulose may occur in pure form in plants but it is usually accompanied by hemicelluloses, lignins, and comparably small amounts of extractives. Wood contains about 40–50 wt% cellulose. Comparable amounts can be found in bagasse (35–45 wt%), bamboo (40–55 wt%), and straw (40–50 wt%) and even higher in flax (70–80 wt%), hemp (75–80 wt%), jute (60–65 wt%), kapok (70–75 wt%), and ramie (70–75 wt%), whereas cotton represents a pure cellulose source containing more than 90 wt% (Hon 1996). It is an impressive amount of cellulose that is produced in one year, not only by wood fibers from trees with 1,750,000 kt world production but also in 1-year plants including bamboo (10,000 kt), cotton linters (18,450 kt), jute (2,300 kt), flax (830 kt), sisal (378 kt), hemp (214 kt), and ramie (100 kt), for instance (Eichhorn et al. 2001). In addition, several fungi and green algae produce cellulose

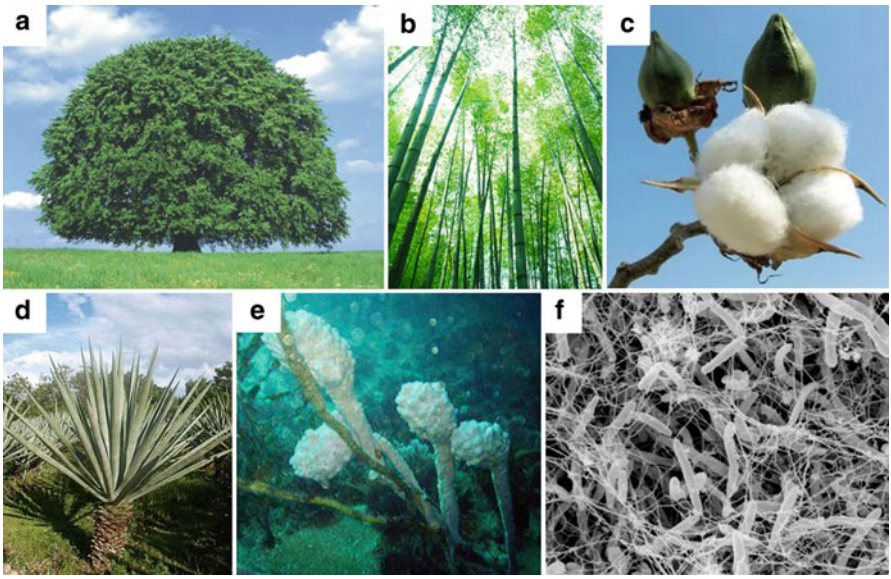

Fig. 1 Selection of important cellulose sources: (**a**) hardwood (beech tree), (**b**) bamboo, (**c**) cotton, (**d**) sisal, (**e**) tunicine, and (**f**) *Gluconacetobacter xylinus* (Reproduced from Schubert et al. (2011) with permission)

(e.g., *Valonia ventricosa*, *Chaetomorpha melagonicum*, *Glaucocystis*) and some animals such as ascidians, which are marine animals that contain cellulose in the outer membrane. Moreover, bacteria of the genera *Gluconacetobacter*, *Agrobacterium*, *Pseudomonas*, *Rhizobium*, and *Sarcina* can synthesize bacterial cellulose from glucose and various other C sources (Vandamme et al. 1998; Jonas and Farah 1998). Bacterial cellulose, which is produced directly in the form of a fibrous network, contains no lignin, pectin, hemicelluloses, and other biogenic products, is highly crystalline, and possesses high DP values.

3 Structure of Cellulose

3.1 Molecular Structure

The molecular entity of cellulose is a chain of D-anhydroglucopyranose units (AGUs) connected by glycosidic β-(1 → 4) linkages (Fig. 2). The six-membered AGU rings are in the energetically favored chair conformation with C4 and C1 out of the reference plane containing the four other ring atoms ($^4C_1$ conformation) (Rao et al. 1957). The AGUs are decorated with two secondary hydroxyl groups in positions 2 and 3 and one primary hydroxyl group in position 6, while the oxygen atom at position 4 (and 1, respectively) is part of the interunit linkage. Resulting from the β-(1 → 4) linkages, cellulose chains have a twofold helical conformation,

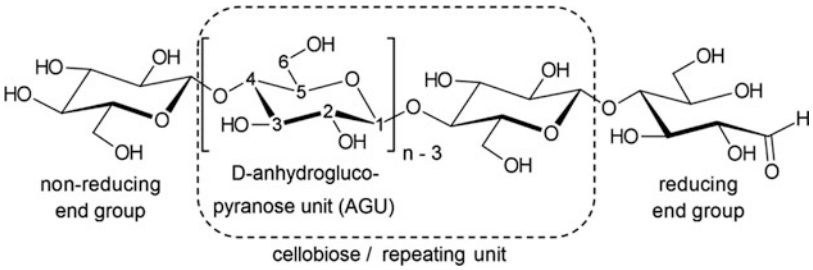

Fig. 2 Schematic drawing of the molecular structure of cellulose including conventional numbering of the C atoms of D-anhydroglucopyranose units; n = degree of polymerization (DP)

meaning that the main planes of every second AGU is turned by 180° (French et al. 2003). Thus, the structural repeating unit of cellulose molecules is cellobiose with a linear repeat length of 1.3 nm (Krässig 1993). The terminal groups of the polymeric chains are chemically different. The anomeric carbon atom of one end group is part of a glycosidic linkage, and this end group carries an additional secondary hydroxyl group in position 4. The other end group is a D-glucose unit, which is present in the cyclic hemiacetal form in equilibrium with the open-chain aldehyde form. Since the aldehyde group possesses reducing properties, the latter group is termed as reducing end group, while the other one is termed as nonreducing end group. The number of AGUs plus two for the end groups is defined as the degree of polymerization (DP) of cellulose.

Overall, cellulose has the most clearly defined molecular structure among all polysaccharides (and many synthetic polymers) in terms of chemical purity and structural identity, which is independent of the raw material cellulose is isolated from. Some minor deviations from the ideal molecular structure occur due to processing and purification steps required for isolation of cellulose from its natural sources as well as due to environmental stress and aging. For example, small amounts (4–40 mmol/kg) of oxidized functionalities like carbonyl and carboxyl groups are formed during the isolation of cellulose from wood (pulping) or cotton.

3.2 Hydrogen Bonds and Crystallinity of Cellulose

There are numerous possibilities for the formation of hydrogen bonds between the three hydroxyl groups of the AGU and the oxygens of both the ring and the glycosidic linkage. They are considered to have a strong influence on the properties of cellulose and give rise to various three-dimensional structures beyond the molecular entity. For example, the intramolecular hydrogen bonds between the OH groups of C3 and the adjacent ring oxygen of the AGUs as well as those between the hydroxyl oxygens at C6 and the adjacent OH groups at position C2 in combination with the β-glycosidic linkage are responsible for the relatively high stiffness and rigidity of cellulose molecules, on the one hand (Fig. 3) (Krässig 1993;

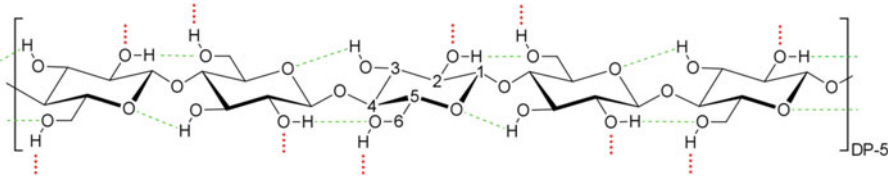

Fig. 3 Schematic representation of intra- (*green dashed*) and intermolecular (*red dotted*) hydrogen bonds of cellulose (Adapted from Tashiro and Kobayashi 1991)

Tashiro and Kobayashi 1991). On the other hand, the intermolecular hydrogen bonds, in particular between the OH groups of C6 and the oxygen of C2 of adjacent AGUs of other cellulose chains, are the main bonds stabilizing the cellulose network.

The intramolecular hydrogen bonds and the resulting chain stiffness as well as the intermolecular secondary valences cause the organization of cellulose in structures sufficiently regular to exhibit a crystalline X-ray diffraction pattern. Thus, X-ray diffraction has been extensively employed to determine the structure of cellulose including the index of crystallinity $I_C = [I_{Crystalline}/(I_{Crystalline} + I_{Amorphous})]$, for the calculation of the crystallite length and width, and for probing the inter- and intramolecular hydrogen bonds in native cellulose. As a result, cellulose polymorphism as a prominent structural feature was revealed. It was shown that native cellulose exits in two crystalline allomorphs, namely, cellulose I_α and cellulose I_β. Cellulose I_α is characterized by a one-chain triclinic unit cell with all the glycosyl linkages and the hydroxymethyl groups identically oriented and the chains organized in sheets packed in a "parallel-up" fashion. The structure of I_β differs from that of I_α in having two unique sheets containing conformationally distinct chains with different hydrogen bonding in a monoclinic unit cell (Fig. 4) (Nishiyama et al. 2003).

Besides cellulose I, several other polymorphs can be obtained from native cellulose by different treatments (Fig. 5). The most prominent and, from a technical point of view, most important one is cellulose II. It is obtained when cellulose I is either dissolved and precipitated (regeneration) or treated with a swelling agent, e.g., aqueous sodium hydroxide of >18 wt %, and washed with water (mercerization). The transformation of cellulose I to cellulose II is irreversible since cellulose II is thermodynamically more stable. The crystal structure of cellulose II is characterized by a two-chain monoclinic unit cell with antiparallel orientation of the chains.

The crystalline modification of cellulose III is obtained by treating native cellulose with liquid ammonia (below −30 °C) or an organic amine such as ethylene diamine, followed by washing with alcohol. Small differences in lattice dimensions exist between the two submodifications cellulose III_I and III_{II}. As the fourth modification reported so far, cellulose IV is formed upon treatment of cellulose III in a suitable liquid at high temperature and under tension.

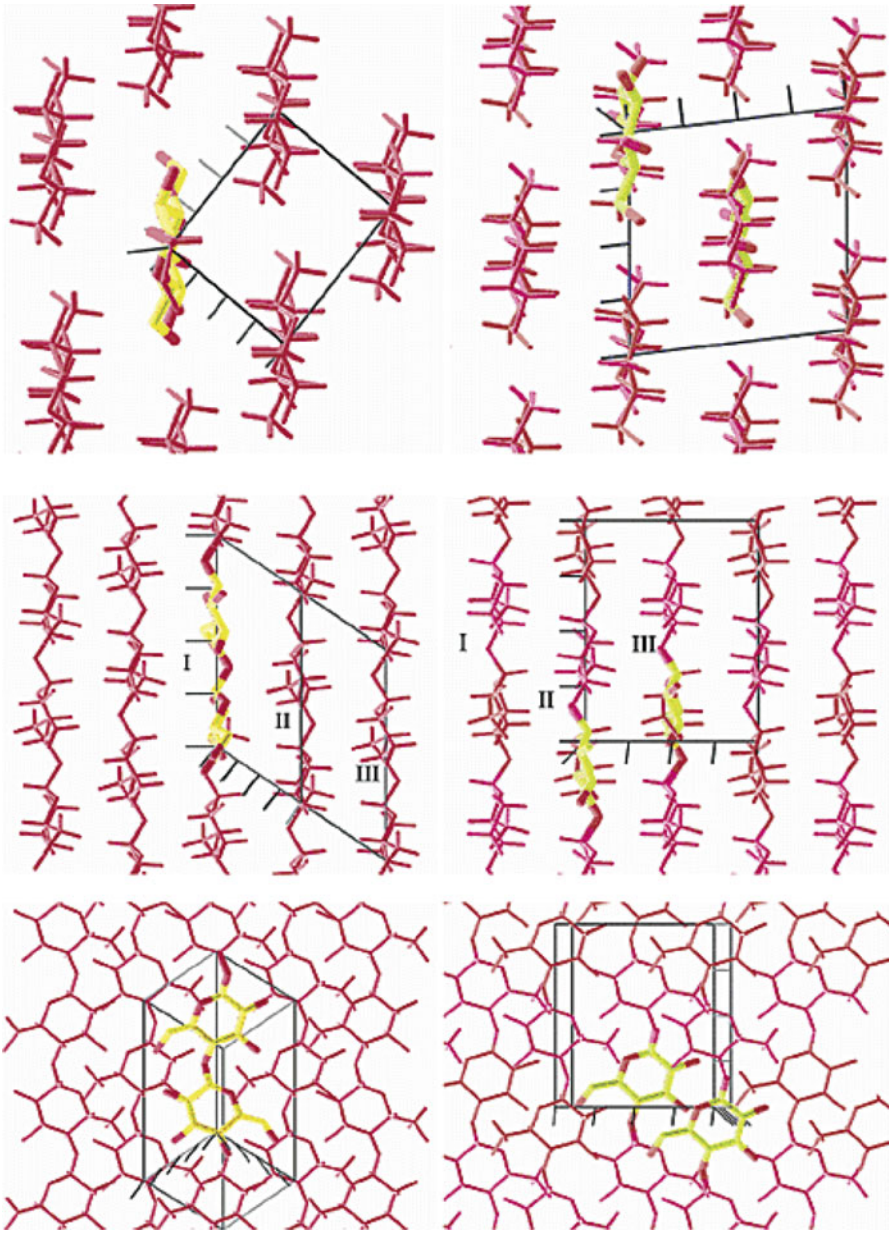

Fig. 4 Projections of the crystal structures of cellulose I_α (*left*) and I_β (*right*) down the chain axes (*top*), perpendicular to the chain axis and in the plane of the hydrogen-bonded sheets (*middle*), and perpendicular to the hydrogen-bonded sheets (*bottom*). The cellulose chains are represented by *red* skeletal models. The asymmetric unit of each structure is also represented in *thicker lines* with carbons in *yellow*. The unit cell of each structure is shown in *black* (Reproduced from Nishiyama et al. (2003) with permission)

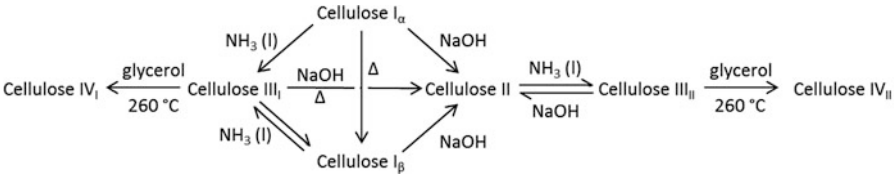

Fig. 5 Transformation of cellulose into its various allomorphs

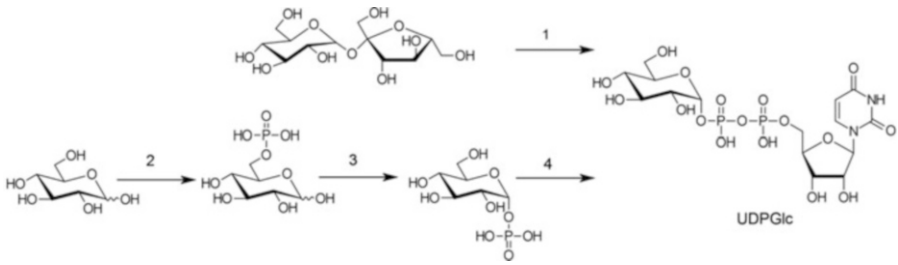

Fig. 6 Different pathways of the intracellular formation of uridine 5′-diphosphoglucose (*UDPGlc*) from sucrose (*top*) and glucose (*bottom*); *1* sucrose synthase, *2* glucokinase, *3* phosphoglucomutase, *4* UDPGlc pyrophosphorylase

3.3 Biosynthesis of Cellulose and Morphological Structure of Native Cellulose

3.3.1 Glucose Polymerization

The glucose polymerization toward glycans is in general accomplished by β-glycosyltransferases that catalyze the transfer of sugar residues from activated donor sugars to acceptor molecules. In the case of the cellulose synthase family, the only substrate is uridine 5′-diphosphoglucose (UDPGlc), which can be formed along two different pathways (Fig. 6) (Coleman et al. 2006). The first is the cleavage of sucrose by sucrose synthase, liberating fructose and UDPGlc. The second one relies on phosphorylation of D-glucose in position 6 by the enzyme glucokinase and the active isomerization of D-glucose-6-phosphate to α-D-glucose-1-phosphate by the enzyme phosphoglucomutase. Finally, α-D-glucose-1-phosphate is converted to alpha-linked UDPGlc.

The actual polymerization of UDPGlc toward cellulose is accomplished by the catalytic subunits, i.e., cellulose synthase (CESA), of a complex oligomeric assembly of enzymes. The precise structure and the way of working of this enzyme complex (frequently termed as terminal enzyme complex, TEC) as well as of its subunits are, almost 40 years after their discovery, not fully understood and thus still subject of intensive research efforts (Mutwil et al. 2008; Joshi and Mansfield 2007). According to the actual knowledge, the CESA proteins are tightly anchored in the cytoplasmic membrane, both in plants and in prokaryotes. It is presumed that the globular fragments of the CESAs are located at the cytoplasmic face. These

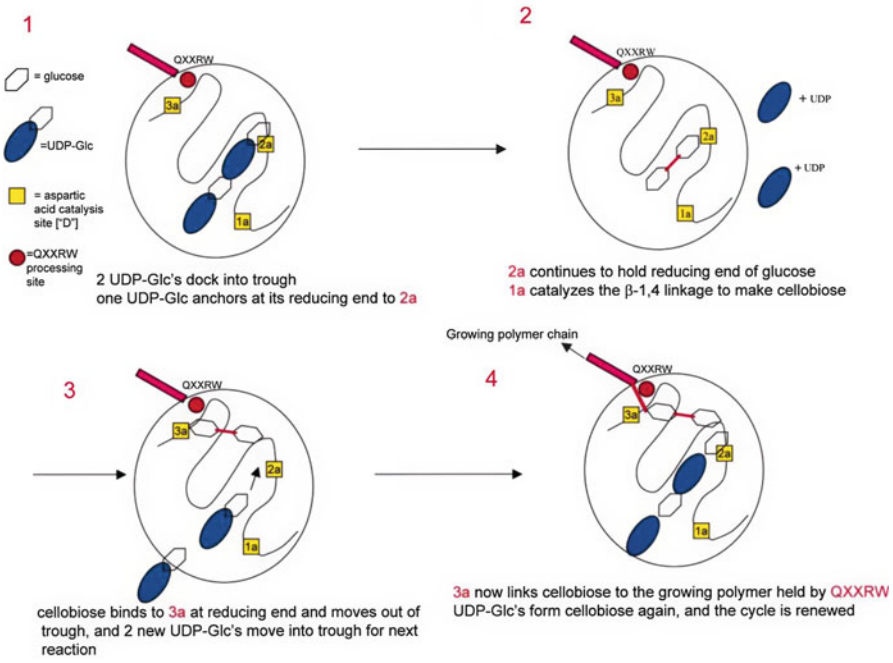

Fig. 7 Generalized model for the polymerization reactions leading to the formation of β-1,4-glucan chains in cellulose biosynthesis (Reproduced from Brown and Saxena (2000) with permission)

globular fragments contain three catalytic sites with aspartic acid residues as crucial elements, which transfer the glucopyranose units from UDPGlc to the nonreducing end of the growing glucan chains in consecutive manner. The generalized model of Brown and Saxena (2000) suggests that one catalytic site (2a) dock two UDPGlc molecules in the binding pocket (Fig. 7(1)). The second catalytic site (1a) catalyzes the formation of the β-1,4-linkage between the two glucose units under the release of two UDP molecules and thus accomplishes the synthesis of the cellobiose unit as the polymeric repeating unit (Fig. 7(2)). The third catalytic site (3a), which is in the vicinity of a Gln-X-X-Arg-Trp (QXXRW) motif, drags the reducing end of the cellobiose unit out of area 1a/2a, where subsequently two new UDPGlc molecules are bound and the next cellobiose unit is formed (Fig. 7(3)). In the next step, the QXXRW motif forces the reducing end of the cellobiose unit to the site of extrusion. Simultaneously, 3a drags the next cellobiose unit toward QXXRW and links it to the nonreducing end of the growing chain, while the 1a/2a region binds two more UDPGlc to repeat the cycle (Fig. 7(4)). Interestingly, this mechanism would require no primer for the initiation of cellulose biosynthesis, and the two UDPGlcs simultaneously docking to the binding pocket to first form cellobiose, which is then transferred to the growing chain, give an elegant explanation for the twofold helical conformation of cellulose (French et al. 2003). Moreover, this

model, based on structural data of different CESAs derived from the encoding genome, is in good agreement with experimental data, for example, from microdiffraction studies, which prove both that the reducing ends of nascent cellulose chains are directed away from the cells and the "parallel-up" packing in native cellulose I crystals (Koyama et al. 1997).

3.3.2 Morphological Organization of Cellulose

Besides the underlying molecular structure, the morphological structure of cellulose is of high importance for the apparent properties of cellulosic materials. In the case of native cellulose, the extrusion of cellulose chains and the associated hydrogen bond-based organization gives rise to a broad variety of properties, which are evolutionarily related to the physiological function of cellulose in the originating organism. In this regard, the most interesting aspect is the great diversity of the structure of the terminal enzyme complexes (TECs), along the cellulose-producing species, which implies a fundamental diversity of the supramolecular arrangement (Fig. 8). As highlighted by Brown (1996), the structure of the TEC is related, for example, to the size and shape of the microfibrils as well as to the crystallization process. Thus, the wish of many researchers to understand the diversity of the TEC complexes is not only scientifically motivated but plays also a role for the applicability of cellulosics, for example, as biomaterials. Moreover, the enormous developments in the field of genetic engineering allow nowadays to tune the properties of cellulosic materials by changing the characteristics of the cellulose source, in particular in the case of bacteria, at the very fundamental level of the structure of TECs.

In the following the role of the organization of the TECs and the resulting morphological structure of native celluloses will be discussed based on the examples of plant and bacterial cellulose.

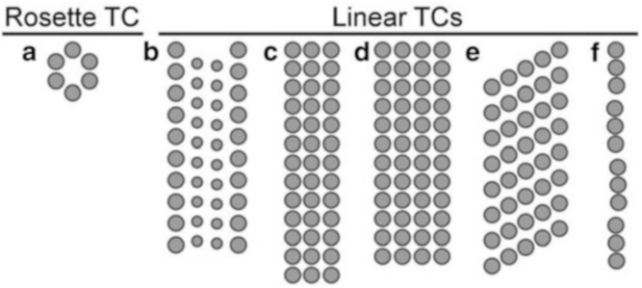

Fig. 8 Schematics of rosette and linear terminal enzyme complexes (*TCs*), for (**a**) plants (6 chains/subunits) and green algae (*Micrasterias*), (**b**) tunicate (*Metandroxarpa uedai*) (unknown number of chains/subunit), (**c**) green algae (*Valonia*) (10–12 chains/subunits), (**d**) red algae (*Erythrocladia*) (4 chains/subunits), (**e**) yellow-green algae (*Vaucheria*) (1 chain/subunit), and (**f**) bacterial (*Acetobacter*) (16 chains/subunits). Each *dark circle* represents a single subunit (Reproduced from Moon et al. (2011) with permission)

3.3.3 Plant Cellulose

For vascular plants, the ultrastructural morphology of the TECs was first described by Mueller and Brown (1980). By freeze-fracturing of rapidly frozen, untreated plant cells of *Zea mays*, they could show that the structures of the cell membranes, which are associated with the ends of individual cellulose microfibrils, have a sixfold symmetrical arrangement of transmembrane particle subunits (Fig. 9a). More recently, it was demonstrated that each subunit of these rosette TECs consists of six individual CESA proteins (Kimura et al. 1999). Interestingly, at least three different types of CESA are required to form a functional rosette complex that is capable to produce cellulose and crystallize it into cellulose I_α in the cell wall matrix (Desprez et al. 2007) (Fig. 9b).

During the coordinated action of enzymatic polymerization of cellulose by the 36 individual CESAs at the TEC, 36 highly extended cellulose chains are generated and simultaneously crystallized (Fig. 10). It has been shown that the first products of the crystallization phase are β-glucan minisheets, which each consists of six cellulose chains originating from the subunits of the TEC and holds together predominantly by van der Waals forces (Cousins and Brown 1995). The minisheets are directed to the center of the rosette complex and crystallize there to the metastable cellulose I, so-called elementary fibril, predominantly on the basis of hydrogen bond interactions (Brown and Saxena 2000). Besides the crystalline parts, the areas containing crystalline defects such as dislocations, chain ends, and twists as well as the crystal surface contribute to the less ordered amorphous cellulose. Altogether, the basic units generated by the TEC have lateral dimensions between

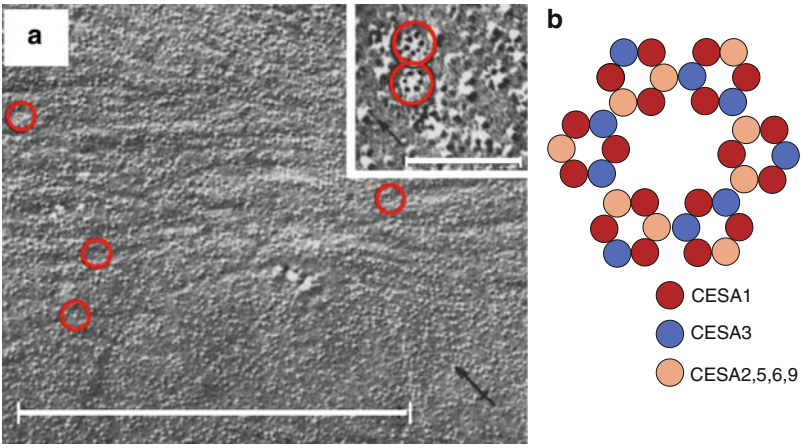

Fig. 9 Structure of terminal enzyme complexes in vascular plants: (**a**) scanning electron microscopy image of freeze-fractured cell membrane of *Zea mays* showing intermembrane particle (*circles*) possessing a symmetrical, rosette-like arrangement of six subunits (*inset*), scale bar is 1 μm; (**b**) schematic view on rosette complex visualizing the six individual CESA proteins of the rosette subunits. CESAs 1 and 3 occupy positions that are exclusively for these CESAs. CESAs 2, 5, 6, and 9 appear to be interchangeable for the other positions (Reproduced from Mueller and Brown (1980) (**a**) and Mutwil et al. (2008) (**b**) with permission)

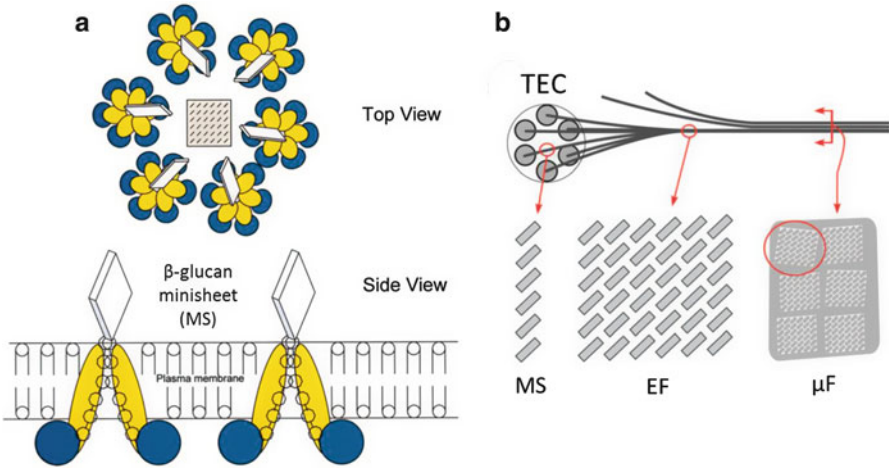

Fig. 10 Schematic model of the formation of supramolecular structures of cellulose by the terminal enzyme complex (*TEC*) in vascular plants: (**a**) spatial organization of the cellulose synthase proteins in the TEC and its impact on the formation of elementary fibrils of cellulose; (**b**) formation of the different structural levels within the TEC. *MS* denotes for the minisheet cross section believed to be formed from the rosette subunits, whereas each *gray box* represents a cellulose chain looking down the chain axis. *EF* denotes the cellulose I crystal lattice of an elementary fibril cross section formed by six minisheets. *μF* denotes the cross section of a microfibril formed by consolidation of multiple elementary fibrils (Reproduced from Brown and Saxena (2000) (**a**) and Moon et al. (2011)(**b**) with permission)

1.5 and 3.5 nm and lengths of about several 100 nm (Moon et al. 2011). Depending on the synthesizing species and the distribution pattern of the TECs on the cell surfaces, the elementary fibrils are assembled to higher structures like micro- and macrofibrils or fibrillar bands, which possess diameters/widths between 10 and 100 nm and lengths in the micrometer range.

In higher plants the fibrils and fibrillar bands represent the construction units of the cell wall. As exemplarily shown for spruce wood, in which the macroscopic fibers originate from single plant cells, the cell wall architecture is characterized by different layers, namely, the primary wall (P), the secondary wall composed of an outer (S1) and inner layer (S2), and the lumen wall (S3) (Fig. 11) (Agarwal 2006). Each of the cellulosic layers (P, S1, S2, and S3) is basically a mesh of fibrils with different density, texture, and thickness. The primary cell wall, which contains besides cellulose larger amounts of hemicelluloses, pectins, and proteins, is less than 0.1–0.2 μm and contributes only 1–4 % to the total weight of wood fiber. The different lamellae of the P-wall are characterized by the orientation of their cellulose microfibrils. In the outer lamella, the microfibrils form an irregular network, whereas in the inner lamella, they have a nearly transverse orientation. The three-layered secondary wall is in total 3–5 μm thick. The S2 layer with a thickness of 2–4 μm and 80–85 wt % of the total cellulose content is the dominant feature, whereas the S1 and S3 layers are 0.2–0.3 and 0.1 μm thick, respectively. In general, the microfibrils of the secondary cell wall are oriented parallel to each

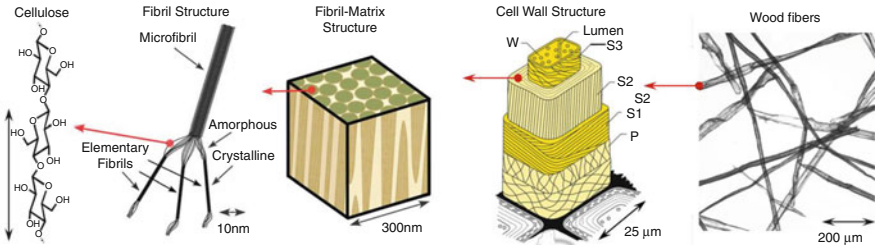

Fig. 11 Different structural levels of cellulose molecules in the plant cell wall (Adapted from Moon et al. 2011 and Agarwal 2006)

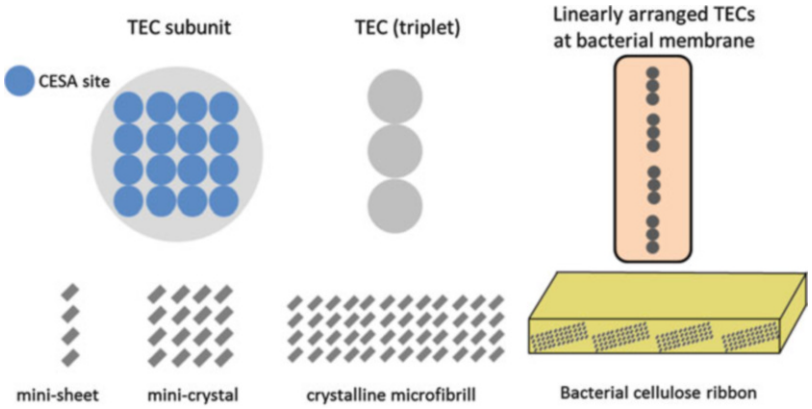

Fig. 12 Schematic representation of the structure of the terminal enzyme complex of *Acetobacter xylinum* and the related structural levels of the bacterial cellulose produced (Adapted from Brown 1996)

other, but the particular texture differs between the layers. In the S1 and S3 layers, the fibrils are tightly wound in helices at nearly transverse orientation with an angle of 60–90° with respect to the cell axis. In contrast, the microfibrils in the S2 layer are arranged in parallel extended helices possessing a comparably small angle of 5–30° to the cell axis.

3.3.4 Bacterial Cellulose

The morphological structure of cellulose formed by bacteria such as particular *Acetobacter* strains (reclassified as the genus *Gluconacetobacter*) differs remarkably, although the principal way of working of the CESA proteins is the same as in plants and consequently the molecular structure is identical. The most important difference is the alternative structure and organization of TECs of cellulose-producing bacteria. In *Gluconacetobacter xylinus*, the TECs are linear assemblies of a minimum of three massive, basket-shaped subunits (Brown 1996). It is presumed that the subunits contain at least 16 catalytic CESA sites in a not completely understood spatial distribution (Fig. 12). It was shown that the subunits

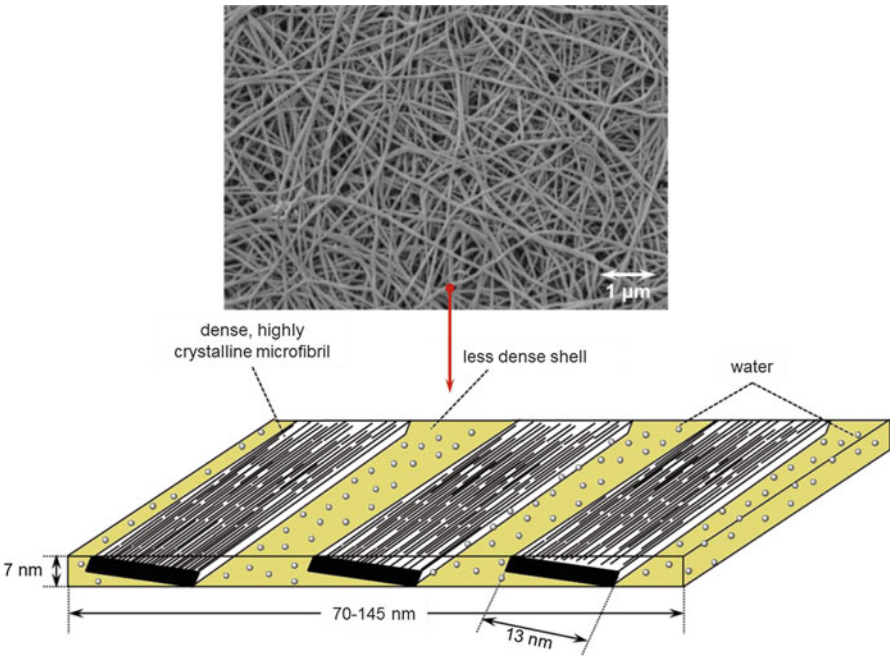

Fig. 13 Scanning electron microscopy image showing the microfibrillar ribbons of bacterial cellulose from *Acetobacter xylinum* (*top*) and model of the ribbons in the wet state as produced by the bacteria (*bottom*) (Adapted from Fink et al. 1997 and Astleya et al. 2001)

produce four-membered minisheets, which are held together predominantly by van der Waals interactions (Brown et al. 1982; Cousins and Brown 1995). These minisheets further assemble to minicrystals, which can be isolated as the smallest crystalline cellulose entity when producing cellulose with a model system consisting of individual subunits of bacterial TECs in vitro (Lin et al. 1985). In vivo, the linear arrangement of the three subunits of the TEC along the longitudinal axis of the bacteria ensures the hydrogen bond-driven consolidation of minicrystals in a highly crystalline microfibril with a rectangular cross section of about 5 × 13 nm.

As a consequence of the presence of 50–80 TECs at the membrane of each single bacterium, the microfibrils are associated into several twisted ribbons, which represent the largest morphological entities of bacterial cellulose. As exemplarily shown in Fig. 13, the ribbon structure is characterized by the dense, highly crystalline cellulose microfibrils surrounded by a shell of noncrystalline polymer chains (Astleya et al. 2001; Fink et al. 1997). The ribbon has a rectangular cross section of about 10 × 50 nm. Although Fig. 13 shows a regular arrangement of microfibrils within the ribbon, it is still debated how many microfibrils are in a single ribbon and how they are packed (Astleya et al. 2001; Fink et al. 1997; Brown et al. 1976). Thus, the arrangement shown represents merely a possibility. Overall, bacterial cellulose (BC) appears as a highly hydrated (>90 % water) network of nanoscale ribbon-like structures that can be obtained as a chemically very pure

cellulosic material by a simple washing process. The very porous structure readily permits the introduction of solutes and solids in the structure and thus makes BC an even more interesting material. Moreover, the morphological structure and thus the properties of BC can be tuned toward desired applications. For this purpose, it is possible to vary the culture conditions or to tailor the bacterial strain in terms of the density and arrangement of the TECs at the cell membrane by means of methods of genetic engineering (Lin et al. 2013).

4 Application of Native Cellulosics as Biomaterials

Historically, native cellulose materials, i.e., cellulosics in which the supramolecular and ultrastructural features formed during the biosynthesis are preserved, were not considered as biomaterials at all. However, during the last two decades, the special properties arising from the nanoscale architecture of native cellulose brought it into focus of biomaterial science and led already to different commercially available products. This section will illustrate these ongoing developments and will also include some aspects, which demonstrate that the full potential of native cellulose regarding biomaterials is far from being completely explored yet.

4.1 Bacterial Cellulose

The network of numerous nanoscale ribbons composed of highly crystalline and almost uniaxially oriented microfibrils (see Sect. 3) makes bacterial cellulose (BC) a resilient and elastic material, possessing a relatively high tensile strength (even in the wet state), an extremely hydrophilic surface, a high water binding capacity, and an excellent biological affinity. Moreover, compared to plant cellulose, BC can be obtained in a very pure form by simple washing (containing no hemicelluloses and lignin) and only a very low extent of carbonyl and carboxyl moieties is present (Schubert et al. 2011). As a consequence, BC possesses also an excellent biocompatibility as demonstrated by an in vivo study of subcutaneous BC implantation in rats (Fig. 14) (Helenius et al. 2006). Another important feature of BC is the in situ moldability, i.e., the shaping during the biosynthesis, which allows the fabrication of complex scaffold structures (Roberts et al. 1986). These properties are drawing more and more attention and BC has been explored as material for wound dressing, tissue repair, and medical implants.

4.1.1 Wound Treatment

BC is considered to recover the biological and structural functions of the skin in wound treatment, and it has been shown that it fulfills a set of requirements to be a modern wound care material (Czaja et al. 2006). It is nontoxic, non-pyrogenic, biocompatible, and able to provide barrier against infection. Due to its osmotic-diffusive properties (mainly water holding capacity and water release rate), BC is able to control fluid loss, to create and maintain a moist environment in the wound, and to absorb exudates during the inflammatory phase of wound healing. Moreover,

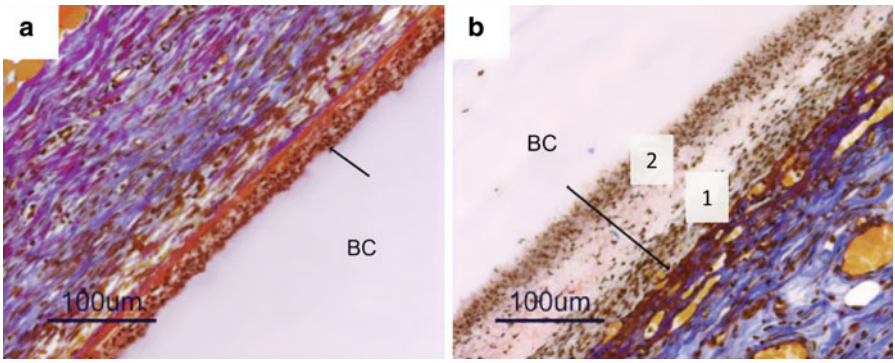

Fig. 14 Microscope images showing the interfaces of a bacterial cellulose sheet 1 week after subcutaneous implantation to rat. Ladewig's trichrome staining shows erythrocytes and muscle tissue in *yellow*/*orange*, collagen in *blue*, and nuclei in *dark blue*/*black*. *Arrows* indicate the approximate surface between the implant and the surrounding tissue: (**a**) compact side and (**b**) porous side; *1* indicates the area with fibroblasts with elongated and round regular nuclei, and *2* shows the invasive front of cells with small irregular nuclei. No foreign body reactions (i.e., fibrosis, encapsulation of the implant or giant cells) are seen. The *BC* implant integrates with the surrounding tissue. Blood vessels, with *yellow* erythrocytes inside, are present around and growing into the implant (Reproduced from Helenius et al. (2006) with permission)

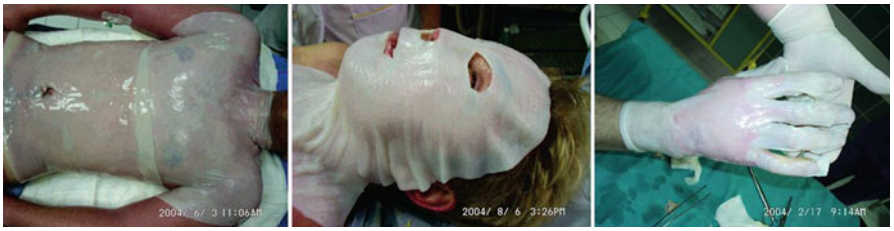

Fig. 15 Never dried bacterial cellulose wound dressing applied to different wounds. The unique physical properties of bacterial cellulose allow an excellent shaping, displaying a high degree of adherence to various body contours and even moving parts (Reproduced from Czaja et al. (2006, 2007) with permission)

the unique network structure enables the introduction of (pharmaceutical) active ingredients and their release into the wound including antimicrobial agents, extracellular matrix materials (collagen, elastin, hyaluronan), or growth factors (Wei et al. 2011; Lin et al. 2011). Last but not least, BC stands out for its conformability, which enables easy and close wound coverage and allows an easy and painless release from the wound (Fig. 15).

The clinical performance of BC wound dressings has been evaluated for several skin injury treatments such as basal cell carcinoma/skin graft, severe body burns, facial peeling, sutures, dermabrasions, skin lesions, chronic ulcers, and both donor and receptor sites in skin grafts (Fontana et al. 1990). In particular, in the treatment of chronic wounds such as venous leg ulcers, bedsores, and diabetic ulcers, which

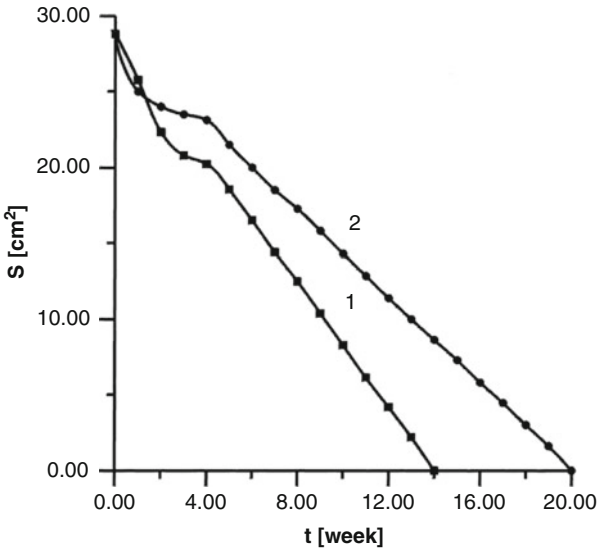

Fig. 16 Results of the comparative therapy of nonhealing venous leg ulcers. Reduction of ulcer areas (*S*) in group *1* treated with bacterial cellulose wound dressing and group *2* treated with Unna's boot (Reproduced from Kucharzewski et al. (2003) with permission)

represent a significant clinical challenge for the difficulty to heal them, BC displays a performance superior to other wound-healing materials. For example, for the therapy of nonhealing venous leg ulcers, it could be shown that a topological treatment with a BC wound dressing compared to a conventional treatment shortened the healing time and decreased the costs of treatment significantly (Fig. 16) (Kucharzewski et al. 2003). Also for the treatment of second- and third-degree burns, which represent the most traumatic and complex skin injuries, can be carried out by means of BC-based wound-healing systems (Czaja et al. 2004). It has been demonstrated that the fully biocompatible BC wound dressings protect burn wounds from excessive fluid loss and absorb wound exudates to the nano-porous structure at the same time. Thus, the entire process of healing could be significantly accelerated (Krystynowicz et al. 2000).

Beyond its use just as a wound-healing treatment, BC can be applied as a scaffold seeded with epithelial cells, thanks to its biocompatibility (see Fig. 14). In this role it could potentially be grafted as a semipermanent artificial skin, inducing epithelial recovery even after the severest epithelial damage. In cases when autografting, often the treatment of choice, is not an option, cell-seeded BC skin substitutes could be an interesting alternative, for example, for indications like large area burns or Stevens-Johnson syndrome.

The commercialization of BC for wound care products was initiated already in the 1980s by Johnson & Johnson who pioneered in investigations of the production of BC on large scale (Ring et al. 1986, 1987). Independently, the Brazilian company BioFill Produtos Biotecnologicos (Curitiba, PR, Brazil) created a new wound-healing

system based on BC (Farah 1990). At present, commercial products are distributed by Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany, under the brand Suprasorb X® and Xylos Corporation, Langhorne, PA,USA, under the brand XCell®.

4.1.2 Implantable Biomaterials

As demonstrated by the pioneering work of Roberts et al. (1986), it is possible to model BC into different shapes during its synthesis without causing significant alterations of its physical properties like mechanical strength. Going beyond the traditionally fabricated flat BC sheets gives rise to its potential application also as implantable biomaterials. In this regard, science follows two approaches for the use of BC. In the first one, BC acts as a scaffold that is surgically integrated into a biological system and is populated subsequently in vivo by cells of the surrounding tissue. The second approach is the use of BC for in vitro tissue engineering, where the cells are seeded onto the three-dimensional scaffold matrix, which enables the cells to develop into a fully functional tissue. Both approaches require the control of the macro- and microstructure of the material. For this purpose, a variety of techniques is used to direct the BC-producing bacteria to produce virtually any shape or form. Furthermore, the micro- and nanoscale structures can be controlled toward the tailoring of properties such as surface chemistry, porosity, and fiber orientation (Petersen and Gatenholm 2011). Ultimately, the ongoing research in each of these areas led to a promising array of techniques being used for structures whose application as biomaterials will be illustrated in the following.

Vascular Tissue Engineering

As shown by Klemm and coworkers, it is possible to shape BC into tubes with an inner diameter down to 1 mm, lengths of several mm, and a wall thickness of about 0.7 mm by means of a special technique involving aspects of a static culture where cellulose is growing on the oxygen-rich surface of the medium (Klemm et al. 2003). The tubes formed by this technique (brand name BASYC®) possess a smooth inner surface, which avoid the formation of blood clots since it resembles normal blood vessels. Moreover, the tensile strength of about 800 mN is compatible with normal blood vessels, and the tensile tests showed as well that the tubes withstand the blood pressure in living organisms. By implanting such a tube to rat as a replacement of a 4–6 mm-long part of the carotid artery, the excellent performance of BC in small-diameter artificial blood vessels applicable in microsurgery was impressively demonstrated (Fig. 17) (Klemm et al. 2001). It could be shown that the bacterial cellulose/carotid artery complex was covered with connective tissue and infused with small vessels in the living animal after 4 weeks after the implantation. Moreover, histological observations revealed that the inner surface of the tube was completely covered with properly oriented endothelial cells, which actively participate in the inhibition of thrombosis and serve as an anticoagulant surface (Heyligers et al. 2005).

Interesting, besides artificial blood vessels, is also the application of the same type of BC tubes for nerve surgery, which was clinically tested as well (Klemm et al. 2001). It was shown that when using the BC tubes as a protective cover for the sciatic nerve of rat, it prevented connective tissues from growing into the nerve gap

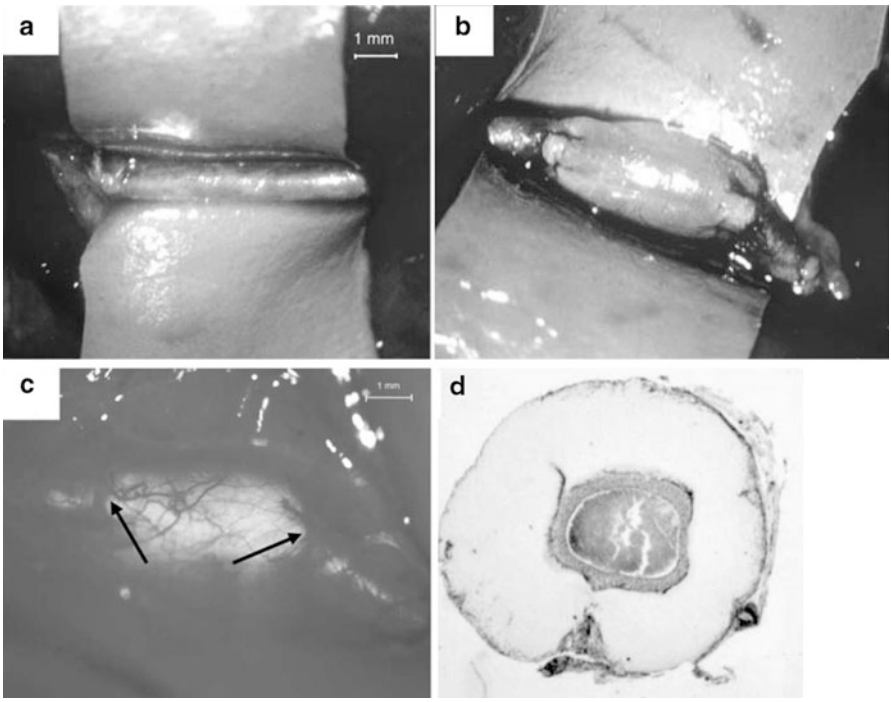

Fig. 17 Usage of a bacterial cellulose tube (BASYC®) as artificial blood vessel for the partial replacement of the carotid artery in rat. (**a**) Preparation of the carotid artery; (**b**) carotid artery with BASYC® interposition immediately after operation; (**c**) carotid artery-BASYC® complex 4 weeks postoperatively (anastomosis areas are indicated with *arrows*); (**d**) histological preparation of carotid artery-BASYC® complex in the *middle* of the region of the interposition 4 weeks after implantation (Reproduced from Klemm et al. (2001) with permission)

and thus facilitated the early regeneration of the nerve. Additionally, it was demonstrated the possibility to augment the BC tubes with a neuroregenerative compound. As a result, an accelerated innervation and improved walking ability was observed in the case of the animals treated with the drug-infused implant.

Avascular Tissues

Recently, BC has also been investigated for the guided regeneration or as scaffold for the engineering of avascular tissues like cartilage and bone. In the guided tissue regeneration, bacterial cellulose is applied as a barrier membrane to enhance the healing process. Like described above for nerve surgery, the implantation of the barrier prevents the formation of scar tissue and can even be used to actively support the regeneration. As, for example, shown in the guided regeneration of maxillofacial, cranial, and periodontal bone defects, the physical barrier prevents the fast ingrowth of fibroblast cells and thus allows the slower process of osteogenesis to occur (Mendes et al. 2009). A good performance for this kind of application was obtained when using Gengiflex® membranes, which are a

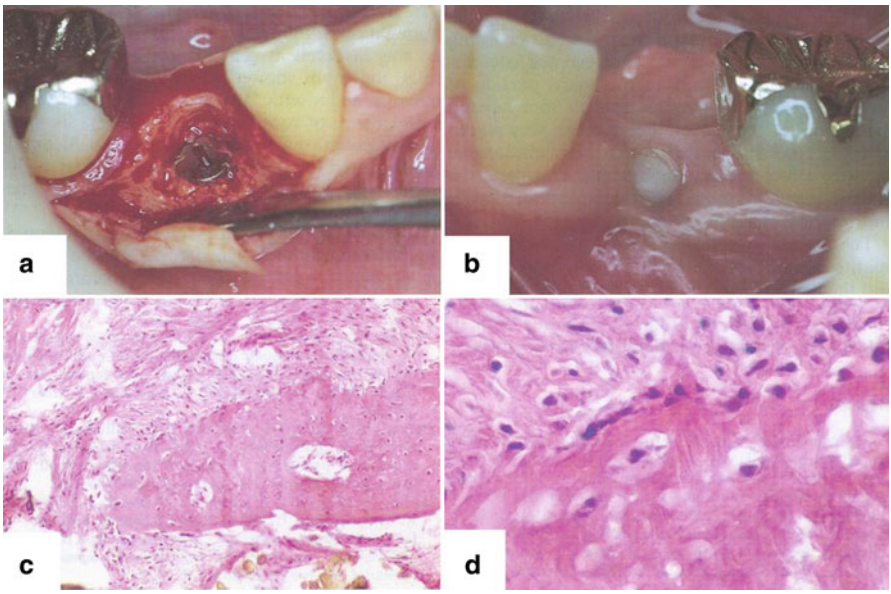

Fig. 18 Formation of bone over a titanium implant placed into an extraction socket by tissue regeneration guided by a bacterial cellulose membrane: (**a**) implant has been inserted, assuring that 4 mm are firmly anchored in healthy bone; (**b**) aspect of recently formed bone over the implant possessing osteocytes in its interior, osteoblast aligned on its surface, and continuity with young connective tissue; (**c**) histological evidence for the formation of bone tissue at the beginning of the restorative phase; (**d**) after complete healing (Reproduced from Novaes and Novaes (1993) with permission)

two-layered assembly of a pure BC (internal) layer and a chemically modified alkali cellulose (external) layer. As demonstrated by Novaes, new bone forms over a $TiAl_6V_4$ implant placed into a circumferential type defect on the buccal aspect (extraction socket) when using BC-based Gengiflex® membranes augmented with hydroxyapatite for guided regeneration (Novaes and Novaes 1993). The complete healing of the bone could be determined clinically and histologically (Fig. 18).

As an alternative to the approach of guided regeneration, BC is also discussed as scaffold for the in vitro engineering of tissue. For example, there is an ongoing research on the usage of BC as a substitute for collagen in biomimetically engineered bone constructs. In this context, the mineralization of the BC scaffolds with inorganic calcium phosphate minerals, such as hydroxyapatite, toward new hybrid materials is intensively studied since these minerals are known to increase osteoblastic differentiation of progenitor cells, increase cell alkaline phosphatase activity, and generally promote bone tissue formation (Stevens et al. 2008; Sibilla et al. 2006). Besides the ratio and morphology (in particular crystal size) of the dopant, also the porosity and nanoscale topography are key elements permitting the migration, adhesion, differentiation, and proliferation of osteogenic cells (Karageorgiou and Kaplan 2005; Dulgar-Tulloch et al. 2009). As, for example, shown by Zaborowska et al. (2010) by means of confocal fluorescence microscopy

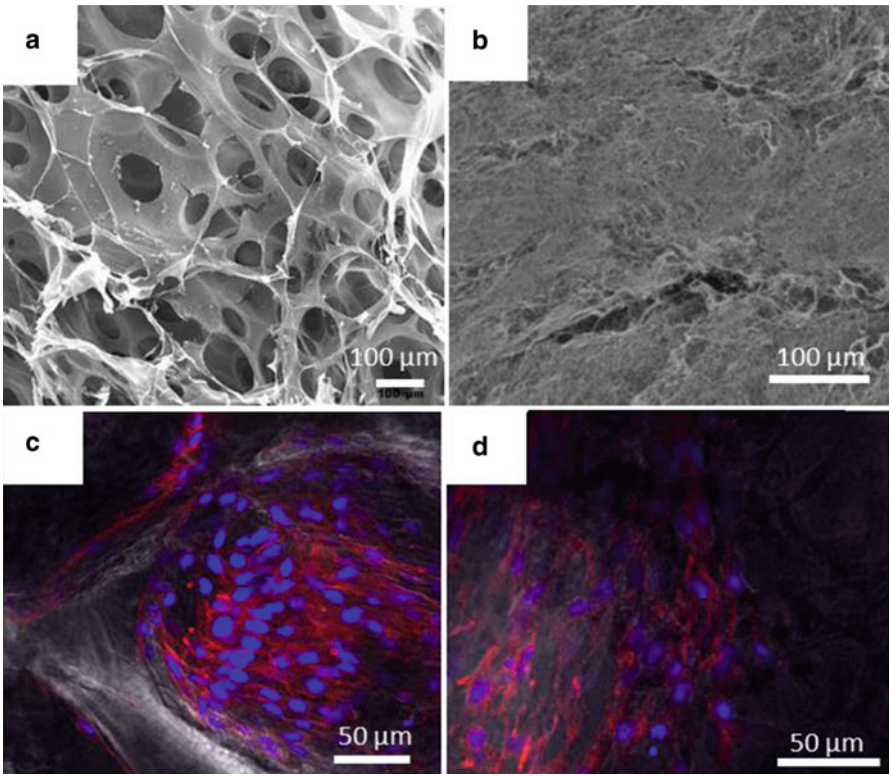

Fig. 19 (**a**) Scanning electron microscopy image showing the morphology of a bacterial cellulose scaffold material possessing highly interconnected pores of about 300–500 µm besides its nanostructure; (**b**) bacterial cellulose scaffold material without microporous morphology; (**c** and **d**) confocal fluorescence microscopy images of MC3T3-E1 osteoprogenitor cells seeded on abovementioned scaffolds. Cell nuclei are stained with DAPI (*blue*) and actin cytoskeleton is stained with rhodamine-phalloidin (*red*) (Reproduced from Zaborowska et al. (2010) with permission)

and histological studies, BC scaffolds, which possess besides their nanostructure highly interconnected pores of about 300–500 µm, are a promising material for bone tissue engineering applications. While the nanometer-sized structures have positive effects on the cell adhesion, the microporosity permits a better penetration of cells, their accumulation in clusters, and an improved osteoplastic differentiation (Fig. 19). Overall, an enhanced bone and capillary formation can be expected by such tailor-made scaffolds.

Another promising field of application is the use of BC as scaffold material for the engineering of cartilage tissue. In this context, native, phosphorylated, and sulfated BCs were evaluated using bovine chondrocytes (Svensson et al. 2005). The results demonstrated native BCs' potential utility as a cartilage scaffold, noting that it showed significantly higher chondrocyte growth than conventional tissue culture plastics and alginate. Although BC showed a chondrocyte proliferation

level of only 50 %, when compared to a collagen type II substrate it provided significant advantages in terms of mechanical properties, i.e., a Young's modulus in the same range as articular cartilage. Bodin et al. (2007) could show that BC has a high potential as meniscus implant for the regeneration of meniscal tissue after partial meniscectomy. The authors demonstrated the five times better mechanical properties of BC compared to the collagen material that have been used in clinical practice, even though natural menisci surpassed BC at higher loads. However, the ongoing research in particular on the preparation of highly ordered BC materials might help to advance even in such challenging applications (Zang et al. 2014).

4.2 Plant Cellulose

Unlike bacterial cellulose, native plant cellulose is not at all considered to be used as a biomaterial nowadays. However, from the author's point of view, micro- and nanostructured materials, which can be prepared from plant cellulose by applying specific so-called top-down approaches (Fig. 20) (Pääkkö et al. 2007), may open new perspectives in biomaterial science by using the remarkable supramolecular and ultrastructural features formed during the biosynthesis of cellulose. Therefore, some aspects of preparation, structure, and properties of such cellulosic nanomaterials will be presented in the following.

4.2.1 Microcrystalline Cellulose (MC)

MC is derived from native cellulose by acid-catalyzed depolymerization using HCl, SO_2, or H_2SO_4 at temperatures well above 100 °C (Dufresne 2008). The acidic hydrolysis occurs in the regions of low lateral order with the consequence that water-insoluble, highly crystalline residues are obtained, forming a stable suspension after vigorous stirring. The DP of the starting cellulose decreases with time and finally reaches a nearly constant value (the level-off DP) of 25–300, which can be controlled by the depolymerization conditions and especially the starting material (Steege and Philipp 1974). The dialysis, commonly applied to remove the acid, and the final spray drying result in re-agglomeration of smaller crystalline domains and, thus, MC may appear stubby to fibrillar (Bondeson et al. 2006).

Commercial MC is a fine, white, and odorless crystalline powder (commercial products are Avicel®, Heweten®, Microcel®, Nilyn®, or NOVA Gel®) and currently used in pharmaceutical (tablet binder), food (rheology control), and paper applications as well as in composite manufacturing (Samir et al. 2005).

An interesting property of MC crystallites is their ability to self-assemble into chiral nematic phases with crystal pitches in the order of the wavelength of visible light, which consequently reflect circularly polarized light of the same handedness as the chiral nematic phase. Above a critical concentration, the MC suspension changes spontaneously into a chiral nematic liquid crystalline phase and forms regularly twisted fibrillar layers, which after drying mimic the structural organization of natural helicoids (de Vries 1951; Revol et al. 1992). This phenomenon is also observed for the cellulose nanocrystallites, namely, cellulose whiskers, which are described below.

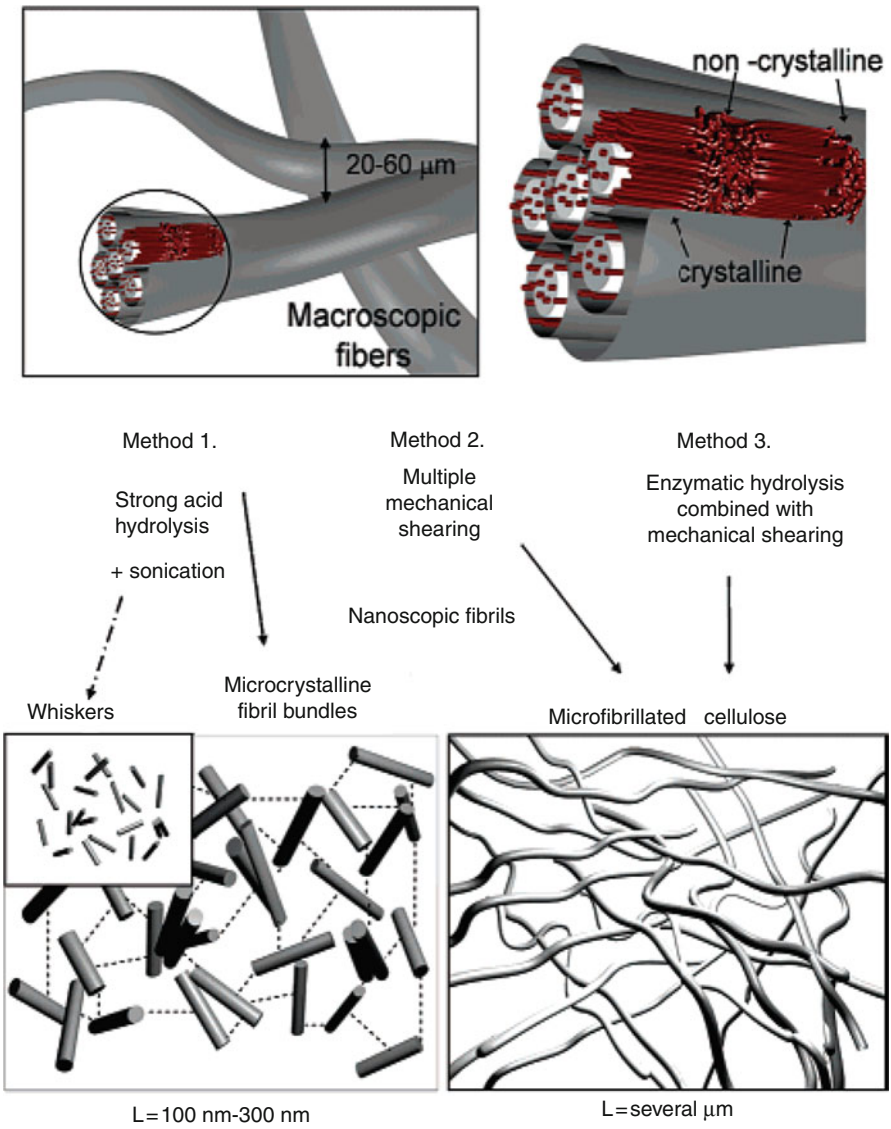

Fig. 20 Mechanical treatment and hydrolysis as top-down approaches for preparing nanoscale cellulosic materials (Reproduced from Pääkköö et al. (2007) with permission)

4.2.2 Cellulose Whiskers

A more intensive hydrolysis than that applied for the preparation of MC results in rigid rodlike cellulose crystallites, which form a stable suspension after treatment with ultrasound (Dufresne 2008). The crystals possess typical dimensions in the order of a few 100 nm in length and of a few nm in diameter (Fig. 21). They resemble a cat's whiskers in terms of straightness and the length to width ratio and

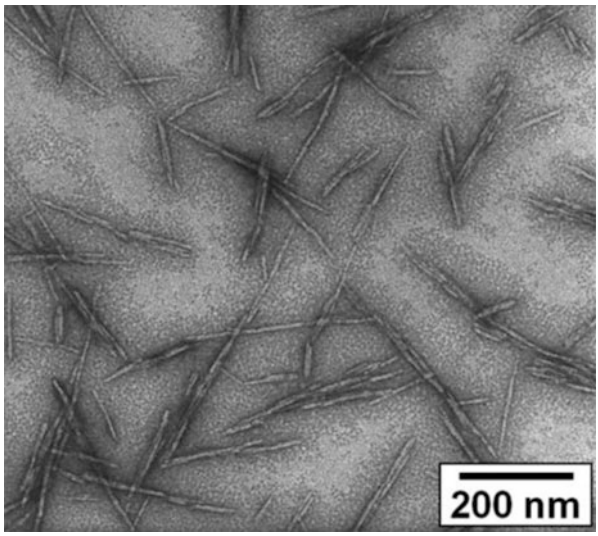

Fig. 21 Transmission electron microscopy image of cellulose nanocrystals obtained from wood pulp showing a whiskers-like structure (Reproduced from Moon et al. (2011) with permission)

are thus frequently called cellulose whiskers. The precise dimensions depend on the origin of the substrate used for preparation (in particular of its amount of amorphous regions), the hydrolysis conditions, and the ionic strength.

The fact that cellulose whiskers show no chain folding and contain only a small number of defects results in mechanical properties, making them very attractive as reinforcing dopant for both thermoplastic and thermoset polymers. The whiskers have a large modulus of elasticity (~150 GPa), strength (~7 GPa), a very low coefficient of thermal expansion (~10^{-7} K^{-1}), and last but not least they possess a comparably low density (Kroon-Batenburg et al. 1986; Nishino et al. 2004). Therefore, latex, poly(β-hydroxyalkanoate), starch, cellulose acetate butyrate, poly(vinyl chloride), poly(vinyl alcohol), polyamide 6, and several other natural and synthetic polymers were blended with cellulose whiskers, resulting in improved mechanical characteristics of the products even when the whiskers content was only a few percent (Favier et al. 1995; Dubief et al. 1999; Dufresne et al. 1999; Angles and Dufresne 2000; Grunert and Winter 2002; Chazeau et al. 2000; de Souza Lima and Borsali 2004; Correa et al. 2014). In this regard, cellulose whiskers might be an attractive material for reinforced polymeric composites. They potentially would allow to enter new fields of application by benefiting from both the proven performance of classical biomaterial plastics and the outstanding properties of the cellulosic material.

4.2.3 Micro- and Nanofibrillated Cellulose (MFC/NFC)

When wood pulp is disintegrated by applying high shear forces, the macroscopic fibers are moderately degraded and opened into their substructural micro- and nanofibrils (Fig. 22a) (Turbak et al. 1982). These individualized fibrils and fibril aggregates are highly entangled and inherently connected, forming mechanically strong networks or gels (Fig. 22b). MFC/NFCs are reminiscent of elementary fibrils

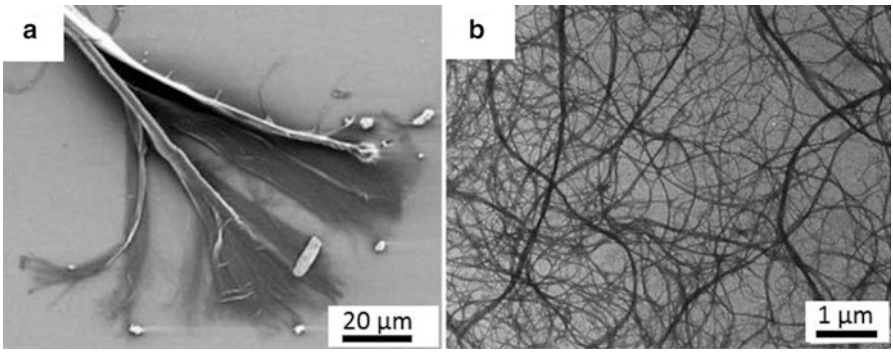

Fig. 22 (**a**) Scanning electron microscopy image visualizing the micro-/nanofibrillation of a pulp fiber by high shear forces; (**b**) transmission electron micrograph showing individualized fibrils and fibril aggregates of micro-/nanofibrillated cellulose gel (Reproduced from Johnson et al. (2009) (**a**) and Dufresne et al. (1997) (**b**) with permission)

in plant cellulose and can be applied in reinforced plastic composites similar to cellulose whiskers (Moon et al. 2011). For example, it is possible to prepare new-generation hemodialysis membranes, which combine active ion exchange with passive ultrafiltration, and the large surface area (Ferraz et al. 2012). Moreover, it is possible to prepare neat MFC/NFC films and membranes that possess properties making them comparable to bacterial cellulose in some aspects (Moon et al. 2011). Thus, there might be a potential of these native plant cellulose in biomedical applications like wound dressing, tissue repair, and medical implants as well.

5 Man-Made Cellulosics as Biomaterials

Excluding the large-scale production of pulp and paper from wood and textiles from cotton, the cellulosics processed by mankind can be classified with respect to the industrial significance into the four classical categories:

- Regenerated cellulose
- Cellulose esters of organic and inorganic acids
- Cellulose ethers
- Oxidized cellulose

While regeneration of cellulose is carried out via the state of dissolution applying either derivatizing or non-derivatizing solvents (Liebert 2010), the synthesis of cellulose derivatives is carried out exclusively under heterogeneous reaction conditions, at least in the conversions used nowadays in the industrial scale. From the authors' point of view, these established fields are mostly of historical or minor importance for the application of cellulose as a biomaterial. Therefore, this section will only include selected aspects regarding classical man-made cellulosics and give some examples of recent advances in polysaccharide science, which not

necessarily lead to actual biomaterials yet but illustrates the potential of cellulose when thinking beyond the ken.

5.1 Conventional Regenerated Cellulosics as Biomaterials

The majority of wood cellulose, which is not used in pulp and paper industry, forms the basis for the so-called regenerated cellulose. In principle, regeneration of cellulose refers to a two-step process for the shaping of cellulose into various forms including fibers, beads, sponges, and films/membranes. In the first step, cellulose needs to be dissolved. Otherwise, a shaping would not be possible since the extensive hydrogen bond network of cellulose causes its degradation before melting would occur. In the second step, the cellulose solution is solidified by precipitation using equipment convenient to obtain the desired shape. During the last century, by far the largest part of the artificial cellulose-based materials was manufactured by the viscose process invented by Cross and coworkers in the early 90s of the nineteenth century (Cross et al. 1893a, b). The most important industrial alternative to the viscose process is the dissolution of cellulose in the non-derivatizing solvent *N*-methylmorpholine-*N*-oxide (NMMO), which allows to produce products with different structural features than those from viscose (e.g., shape, degree of crystallinity, crystallite dimensions, orientation of noncrystalline chain segments) and have less environmental impact (Liebert 2010). Another prominent process used for the shaping of cellulose is based on a discovery made by the Swiss chemist Schweizer in 1857 and later on commercialized as an artificial silk process (Schweizer 1857). Schweizer found that cotton could be dissolved in a solution of copper salts and ammonia (Cuam) and then regenerated in a coagulating bath (Schweizer 1857). In addition to the application of shaping of cellulose, this solvent is very useful for the analysis of cellulose, and it was among the reagents the afterward Nobel Prize-decorated Hermann Staudinger exploited to elucidate the macromolecular structure of cellulose (Nobel laureate 1953).

The viscose, NMMO, and Cuam processes were initially invented for the preparation of fibers predominantly for textile applications. However, by specialized techniques, different geometries including flat and tubular cellulose membranes, sponges, and beads were prepared from cellulose. Membranes produced by the viscose process, namely, cellophane, were used in the early treatments of acute or reversible kidney failure by Kolff et al. (1944). For such treatments, a long cellophane tube was spirally wrapped around a cylinder and rotated in a stationary dialysis fluid bath. This kind of apparatus with its cellulose membrane as an essential part became the first system for the treatment of renal failure in widespread clinical use. Also in new designs including coil, parallel flow, and hollow fiber dialyzers, cellulose membranes were in use in the beginning. Figure 23 shows a typical hollow fiber produced by the Cuam process as it was used in hollow fiber dialyzers. However, since the 1970s, the use of membranes in the treatment of renal failure tends to move away from cellulose toward synthetic membranes, although recent clinical studies could not prove any benefit in terms of reduced mortality or

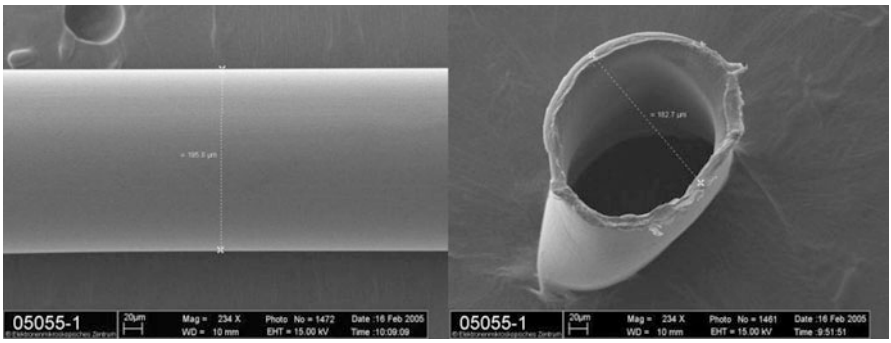

Fig. 23 Hollow fiber used for blood dialysis prepared via the Cuam process (Reproduced from Liebert (2010) with permission)

the reduction in dialysis-related adverse symptoms (Grassmann et al. 2005; Macleod et al. 2005). From the authors' point of view, this trend might change when taking into account the potential arising from the multifunctionality of cellulose in future developments. It might be possible to chemically modify cellulose membranes in a way that it not only acts as an inert phase separator but takes an active role, for example, in reducing oxidative stress or hypersensitivity reactions associated with the dialysis treatment (Yu et al. 2013; Sanchez-Villanueva et al. 2014).

A more recent use of regenerated cellulose is the application of cellulose sponges in tissue engineering approaches similar to BC as described above. For example, the formation of granulation tissue in viscose cellulose sponges with different cellulose contents and sizes was studied after subcutaneous implantation in rats (Martson et al. 1998). The sponges were studied histologically and histomorphometrically 1–16 weeks after implantation. It was shown that the implants with lower cellulose content, i.e., higher porosity and larger pore size, and smaller dimensions were invaded by more cells and filled with connective tissue more rapidly than those with the higher cellulose content and larger dimensions. A similar study by Pulkkinen et al. (2006) demonstrated the suitability of viscose cellulose sponges as a scaffold for cartilage tissue engineering. They showed that the sponges alone or with recombinant human type II collagen cross-linked inside the material were biocompatible and a significant cell proliferation could be obtained. The constructs remained soft during the observation period and were devoid of extracellular matrix composition, typical for normal articular cartilage. However, as a drawback of viscose sponges in tissue engineering approaches, the more pronounced foreign body tissue reaction, compared to BC, must be considered. It may arise from minor impurities or deviations from the idealized cellulose structure caused by the viscose process (Martson et al. 1999). Moreover, viscose is like other neat cellulosic materials as well, hardly degradable in the body of living animals or humans.

The NMMO process described above offers various possibilities for the physical modification of fibers. Thus, a variety of filled fibers possessing functional properties became accessible in recent years. For example, fibers with 33 wt % of a

superabsorbent resin without a pronounced deterioration of its textile properties could be produced, applying NMMO as solvent. Instead of using powdered organic compounds as absorbent materials, embedding into fibers provides the chance for applications of superabsorbent materials in areas which were not considered so far (Kolbe and Meister 2006). Other filled fibers are accessible in a comparable manner in particular for health-promoting applications. For example, fibers possessing antimicrobial and antifungal activities could be prepared by immobilizing about 0.04 g of silver per kg of fibers. As shown by Hipler et al. (2006), the antimicrobial activity remains constant over approx. 60 washing cycles, and the textiles made from these fibers can be applied as an excellent antimicrobial barrier dressing without causing allergic or toxic effects.

5.2 Conventional Cellulose Derivatives as Biomaterials

Chemical modifications of cellulose are generally based on the characteristic reactions of alcohols involving the hydroxyl groups at positions 2, 3, and 6 of the AGUs of the polymer. In this regard, esterification and etherification are of particular importance. Esterification is usually performed with acids in the presence of a dehydrating agent or by the reaction with acyl chlorides or acid anhydrides. The resulting esters can be classified into esters of inorganic acids, such as cellulose nitrate, sulfate, and phosphate, and esters of organic acids, such as cellulose acetate, propionate, acetobutyrate, and acetophthalate. Cellulose etherification is generally performed by Williamson ether synthesis with alkyl halides in the presence of a strong base, with alkylene oxides in weakly basic medium, and by Michael addition of acrylic or related unsaturated compounds (Wertz et al. 2010).

Like neat regenerated cellulose, its esters and ethers had historically been applied in hemodialysis. For example, hollow cellulose acetate-based fibers were introduced by Dow Chemical in the late 1960s of the last century to overcome some of the shortcomings associated with Cuam fibers like problems with leukopenia (Gotch et al. 1969). With similar intent, cellulose etherified with small amounts of tertiary amino groups, namely, N,N-dimethylaminoethyl- or benzyl moieties were used for the production of hemodialysis membranes under the brand names HEMOPHAN® and SMC®, respectively (Schaefer et al. 1987; Hoenich et al. 1997). Although these membranes showed some advantages in terms of biocompatibility, they did not become accepted in widespread clinical use and are of minor importance nowadays (Yu et al. 2013).

Another field where cellulose derivatives find a widespread application is in the controlled drug release from tablets. In such, the cellulose derivatives constitute a hydrophilic excipient system possessing a variety of different release modalities, which are dependent on the actual structure of the derivative as well as on the formulation process. It has been shown by a model system that the synergistic interactions of the heterodisperse cellulose derivatives determine the release profile of the active pharmaceutical ingredient (Staniforth and Baichwal 1993). For example, the cellulose ether hydroxypropylmethyl cellulose (HPMC) is one of the most

widely used hydrophilic matrices for drug delivery systems. It is used in tablets prepared by wet granulation (Lapidus and Lordi 1966), slugging (Huber and Christenson 1968), and direct compression (Conte et al. 1993). In such formulations, the drug release is controlled by the molecular weight of the HPMC, the drug to HPMC ratio, the particle size of both the drug and HPMC, and the solubility of the drug (Kawashima et al. 1998; Pillay and Fassihi 1999). None of the inventors considered the cellulose derivatives in such classical drug delivery applications as biomaterials. However, keeping in mind the new biomaterials paradigm, the reader will notice that they actually were used as such.

5.3 New Aspects of Man-Made Cellulosics for Biomaterials

As stated before, the constant development in cellulose science and technology gives rise to new perspectives for the use of cellulose as biomaterials. From the authors' point of view, two aspects are of major importance in this context. The first one is the development of new processing techniques for cellulose toward hybrid- and nanoscale materials. The second is related to new methods for the chemical modification of cellulose. This includes in particular the great opportunities to design novel and unconventional cellulose derivatives by homogeneous-phase chemistry. The following discussion on these two aspects is not intended to give a comprehensive review. Instead, it should give the reader an impression of what is possible even though some of the examples might not have an obvious link to biomaterial science.

5.3.1 New Cellulosic Nanomaterials

Electro-spinning of Cellulose and Cellulose Derivatives

Electro-spinning is a technique widely used for the preparation of nanofibers, which opens up a way to realize high effective surface area materials (Greiner and Wendorff 2007). The nanofibers may be produced from different polymers and found to be interesting for various application fields, namely, in biomedicine, composites, and filters, as well as in catalytic and textile products (Reneker and Chun 1996; Frenot and Chronakis 2003; Xie et al. 2008). The nanofibers regulate the water vapor and wind permeability and may improve the thermal isolation of textiles. Moreover, they may possess special properties like aerosol filtration, binding of chemical and biological contaminants, or improved surfactant release (Li et al. 2010), for which air cleaning of contaminated environments is a typical example (Scholten et al. 2011).

Cellulose dissolved in DMA/LiCl; NMMNO (Kim et al. 2006); ionic liquids, e.g., 1-butyl-3-methylimidazolium chloride (Viswanathan et al. 2006); or sodium hydroxide/water/urea (Qi et al. 2010) could be transferred to nanofibers of different morphology by electro-spinning (Fig. 24). Also conventional cellulose derivatives like cellulose acetate can be easily processed by electro-spinning. Other more specialized polysaccharide derivatives including water-soluble polyelectrolytes

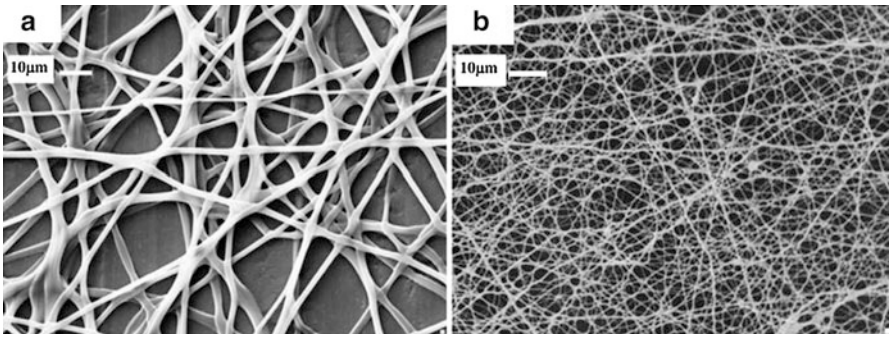

Fig. 24 Scanning electron microscopy images of electro-spun cellulose fibers (**a**) from NMMO and (**b**) from DMA/LiCl (Reproduced from Kim et al. (2006) with permission)

like 6-deoxy-6-trisaminoethyl-amino cellulose can be electro-spun by using blended solutions of polymers like polyvinyl alcohol (Römhild et al. 2013).

In the field of biomaterial science, electro-spun cellulose-based fibers are considered for applications like the immobilization and delivery of bioactive substances as well as for cell culture and tissue engineering approaches (Konwarh et al. 2013). For example, drug-loaded electro-spun cellulose acetate mats were prepared for transdermal drug delivery. It has been shown that it is possible to deliver drugs for systemic effects at a predetermined and precisely controlled rate, which is predominantly controlled by the design of the nanomaterial, i.e., the size and morphology of the fibers (Kumar 2011). In terms of tissue engineering, it has to be stated that the principal possibilities to actively control factors such as interfiber distance and alignment, surface functionalization, gradient organization, and biodegradability make electro-spinning of cellulose or its derivatives the most promising alternative to the tissue engineering approaches on basis of bacterial cellulose. However, up to now the application of electro-spun cellulosics is not quite well elaborated compared to bacterial cellulose. Nevertheless, the interested reader will find a series of references supporting the growing endeavor to bring electro-spun cellulosics to the field of tissue engineering in the recent review of Konwarh et al. (2013).

Nanospheres

Nanoscale particles can be obtained from different cellulose esters including the commercially available cellulose acetates, cellulose acetate propionate, and cellulose acetate butyrate and also from some organo-soluble cellulose ethers. For this purpose, an emulsification-solvent evaporation procedure or the low energy-consuming methods of solvent displacement may be applied (Wondraczek et al. 2013; Hornig and Heinze 2008). Comparing the methods, it could be shown that large amounts of small and very uniform nanoparticles could be obtained by the emulsification-solvent evaporation procedure, whereas the solvent displacement technique yields narrowly distributed particles of somewhat larger particle diameters (Fig. 25).

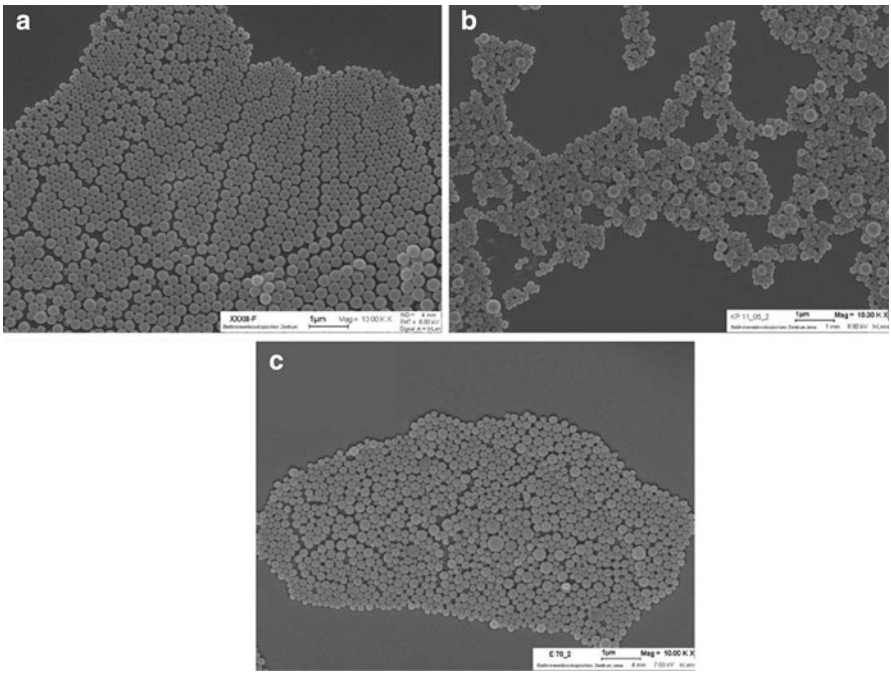

Fig. 25 Field-emission scanning electron microscopy images of the nanoparticles prepared from cellulose acetate: (**a**) by emulsification-solvent evaporation, (**b**) dialysis, and (**c**) dropping technique (Reproduced from Wondraczek et al. (2013) with permission)

It is important to point out that even spherical nanoparticles of polymers containing hydrophilic moieties like 6-deoxy-6-(ω-aminoalkyl)aminocellulose-carbamates could be prepared. Such nanoparticles are of particular interest because they possess primary amino groups that can be modified much easier than OH moieties. Thus, labeling with rhodamine B isothiocyanate is very simple and does not change the size, stability, and shape of the nanoparticles. Incorporation of the nanoparticles in human foreskin fibroblasts BJ-1-htert and breast carcinoma MCF-7 cells could also successfully be carried out without any transfection reagent (Nikolajski et al. 2012).

Although an organo-soluble cellulose derivative must be used for the technique of nanoprecipitation, even pure cellulose nanoparticles could be prepared. Using trimethylsilyl cellulose (TMSC), the formation of nanoparticles by dialysis of the organic solvent against water is accompanied by a complete removal of the TMS functions. The particle size distribution analysis shows that cellulose particles with a size of 80–260 nm are accessible in this simple manner (Kostag et al. 2010). The aqueous suspensions of the pure, spherical cellulose nanoparticles are storable for several months without any demixing. Covalent labeling of the cellulose nanoparticles with fluorescein isothiocyanate (FITC) has no influence on particle size, shape, and stability. The particles can be sterilized and suspended in biological

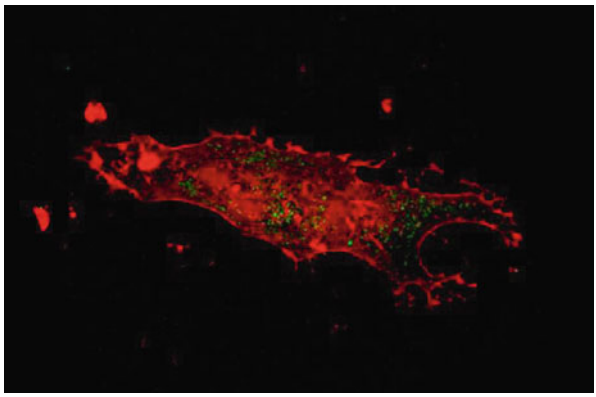

Fig. 26 Confocal micrograph overlay of 21 stacks of human fibroblasts (*red* = cell membrane) incubated with fluorescein isothiocyanate-labeled cellulose nanoparticles (Reproduced from Liebert et al. (2011) with permission)

media without structural changes. As can be seen in Fig. 26, the FITC-labeled cellulose nanoparticles can penetrate into living human fibroblasts by endocytosis without transfection reagents or attachment of a receptor molecule as studied by means of confocal LSM (Liebert et al. 2011).

5.3.2 Amino Celluloses: A Promising Example for Novel Cellulose Derivatives

From the authors' point of view, a large mostly unexplored field of applications for cellulose (also with respect to biomaterials) arises from the tailored chemical modification of cellulose under homogeneous reaction conditions. In the following, one outstanding class of such novel cellulose derivatives, namely, amino celluloses, will be explained as an example in order to stimulate the reader to think beyond the current borders of cellulosic biomaterials.

Conversion of cellulose tosylate with di- or oligoamines yields polymers of the type P–CH_2–NH–(X)–NH_2 (P = cellulose, (X) = alkylene, aryl, or oligoamine, Fig. 27). The structure of the amino celluloses can be designed in terms of different distances of the terminal NH_2 groups from the cellulose backbone (spacer effect) and different basicity and reactivity. Moreover, the di- and oligoamines provide different properties including pK_b value, charge distribution, hydrophilic/lipophilic balance, and redox-chromogenic properties (Tiller et al. 1999, 2000; Berlin et al. 2000, 2003). The chromogenic (electron mediator) properties play an important role for the inherent property of most amino celluloses that they form films on a vast amount of different materials and thus can be applied as a primer, for example, for the immobilization of antibodies for biosensor applications (Jung and Berlin 2005; Becher et al. 2004).

Due to the multifunctionality of cellulose and the stability of tosylates, a modification of the secondary OH groups prior the S_N reaction may be carried out to design the properties of the products additionally. The OH groups at positions 2 and 3 are preferably esterified to adjust the properties including the solubility of the polymer. While the amino celluloses possessing mainly OH groups at the secondary positions are water soluble, the additionally esterified polymer derivatives are soluble in organic solvents like DMAc and may form nanoparticles (see above).

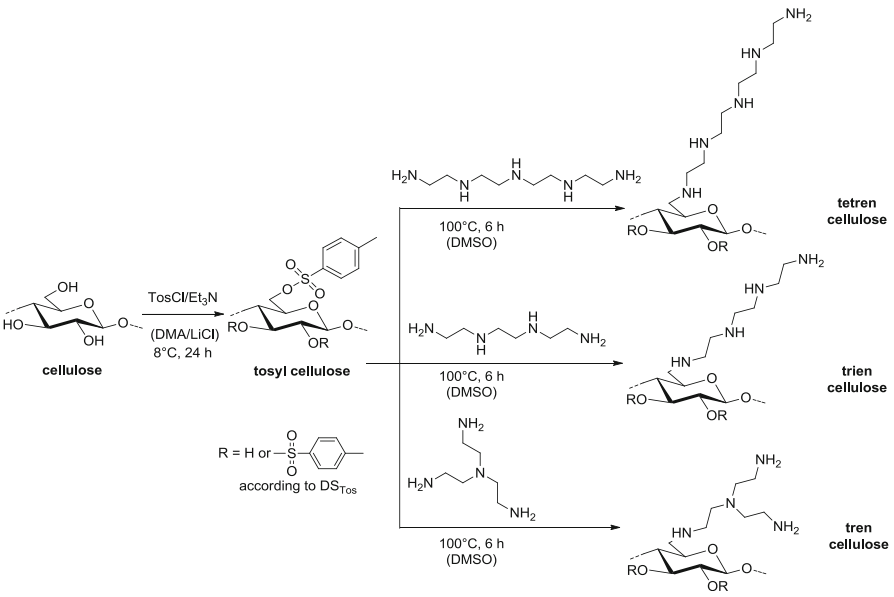

Fig. 27 Reaction path for the synthesis of 6-deoxy-6-amino cellulose ester derivatives by nucleophilic displacement of tosyl cellulose

An extremely interesting property of 6-deoxy-6-amino cellulose is the fact that they form multiple oligomeric species as it was found by using the hydrodynamic technique of analytical ultracentrifugation (Heinze et al. 2011). For every amino cellulose studied, the sedimentation coefficient distributions show between 4 and 5 discrete species with a stepwise increase in sedimentation coefficient across a range of six different solute loading concentrations (from 0.125 to 2.0 mg/ml). For example, the lowest sedimentation coefficient of the 6-deoxy-6-(2-(bis(2-aminoethyl)aminoethyl)-amino) cellulose was detected at 1.8 Svedbergs (S). Additional species sedimenting at peak maxima 2.8, 4.0, 5.1, and 6.5 S were also clearly found. It became obvious that even a fully reversible self-association (tetramerization) within this family of 6-deoxy-6-amino cellulose occurs. Remarkably, these carbohydrate tetramers are then seen to associate further in a regular way into supramolecular complexes (Fig. 28). This behavior was found for the first time for a carbohydrate, while it is well known for polypeptides and proteins like hemoglobin and its sickle cell mutation (Ferrone et al. 1985). The large self-assemblies of cationic structures render them as possibilities for mimicking the properties of histones and for their use as condensing or packing agents in DNA-based therapies (Teif and Bohinc 2011). Most importantly, however, our traditional perceptions as to what is "protein-like" and what is "carbohydrate-like" behavior may need to be reconsidered (Nikolajski et al. 2014).

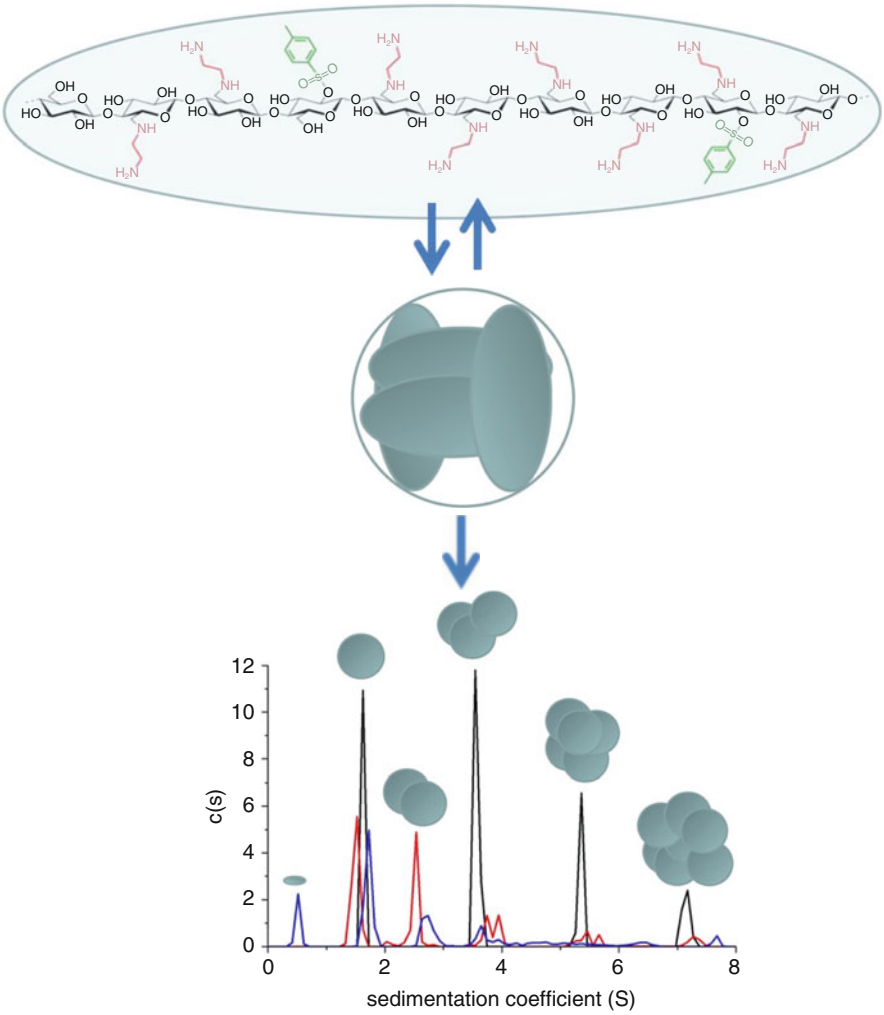

Fig. 28 Reversible tetramerization and further higher-order association of the polysaccharide 6-deoxy-6-(ω-aminoethyl)aminocellulose (AEA cellulose). *Top*: monomer unit of degree of polymerization ~10, degree of substitution at C-6 $DS_{Amine} = 0.83$, and degree of substitution at C-2 of tosyl residues $DSTosyl = 0.2$, yielding a M ~ 3,250 g/mol and s ~ 0.5 S. *Middle*: assembly into tetramers with M ~ 13,000 g/mol and s ~ 1.7 S. *Lower*: sedimentation coefficient distribution for AEA cellulose at different concentrations, 2.0 mg/ml (*black*), 1.0 mg/ml (*red*), 0.75 mg/ml (*blue*), 0.25 mg/ml (*green*), and 0.125 mg/ml (*pink*). Based on the s ~ M2/3 scaling relationship, the super-monomers associate into super-trimers, super-hexamers, and super-9-mers with evidence also for some super-dimers, although the latter were not evident at the highest loading concentration. The proportion of the super-monomers drops relative to the higher-order species showing partial reversibility even with the higher-order association (Reproduced from Nikolajski et al. (2014) with permission)

6 Conclusions

Cellulose is the most important renewable resource and a unique polymer regarding structure and properties. Based on its remarkable properties, it may serve as starting material for various biomaterials. Keeping in mind the new biomaterials paradigm, in particular the research and development in the field of micro- and nanostructured cellulosics (both native and man-made) hold a big potential for applications beneficial to human life. As shown in this book chapter, the versatile characteristics of the different cellulose-based architectures can be used to address specific functions in applications as different as drug delivery and tissue engineering. Consequently, it can be expected that the application fields of micro- and nanostructured cellulose will further be broadened, for example, by applying chemical and physical surface treatments. Moreover, it can be expected that new materials will blur the boundaries between classical biomaterials, drugs, and functional daily life materials. Last but not least, the ongoing research in somewhat exotic fields as, for example, the homogeneous functionalization of cellulose will in the future for sure open new areas of application also in biomaterial research.

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Starch Metabolism in Green Plants

11

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Contents

Abstract

Starch is a substantial component of the human diet providing about 50 % of daily energy uptake, mostly through unrefined cereals. Starch and sucrose are the primary products of photosynthesis. Starch represents the main plant storage carbohydrate that provides energy during heterotrophic growth. Its synthesis and degradation have been studied deeply, reaching a good level of knowledge of the metabolism as a whole. Not only the enzymes involved but also the intracellular localization of the reactions, its regulation, its dependence on light–dark cycle, its evolution from ancestral bacteria, and its correlation with parameters of agronomic interest have been studied. In this work we have attempted a comprehensive review of the starch metabolism in *Arabidopsis thaliana* and other species of agronomic interest and the modular structures present in starch-related enzymes from *Arabidopsis thaliana*.

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1 Introduction

Polyglucans are the most important and widespread carbohydrate storage compounds found in nature, being cellulose, glycogen, and starch the most abundant forms. These two last polysaccharides are comprised of glucose chains linked by an α-1,4 bond and branched at α-1,6. Glycogen is a homogeneous water-soluble polymer with relatively uniformly distributed branches (Roach 2002) and is found in organisms such as archaea, bacteria, and certain eukaryotes (Busi et al. 2012). Starch is the main form in which plants and algae accumulate carbohydrate storage, because cellulose, the most abundant carbohydrate in plants, complies with structural functions. Starch is present in the cytosol of Glaucophyta and Rhodophyceae (red algae) (Dauvillee et al. 2009); however, it is confined to the plastid stroma (chloroplasts in green tissues and amyloplasts in reserve organs) in green algae and higher plants. Starch is essential for the carbon economy of the different organs and tissues of the plants and it can be found in large amounts in fruits, seeds, leaves, roots, and tubers (Smith and Zeeman 2006).

Starch molecules are polymers of glucose in two configurations: amylose, which is mostly unbranched, and amylopectin, which is branched. Amylose is predominantly linear and made up of α-1,4-linked glucosyl moieties, while amylopectin is branched and contains both α-1,4 and α-1,6 linkages. These branches pack together into ordered arrays to form semicrystalline starch granules (Ball et al. 1996; Grennan 2006). Starches from different plant sources vary in size, composition, and structure of amylopectin. These factors influence the physical properties and end-uses for the different natural starches (Santelia and Zeeman 2011) (Table 1).

The structural features of the starch also determine the ability to be digested. This polysaccharide can be hydrolyzed by enzymes of the human gut and its digestion occurs in the small intestine, except for a portion named resistant starch (RS) which is degraded in the large intestine (Busi et al. 2012). RS is defined as the set of starch and products of starch degradation (oligosaccharides and other sugars)

Table 1 Amylose and amylopectin properties

Property/trait	Amylose	Amylopectin
Granule weight	15–35 %	65–85 %
Molecular mass	10^4–10^5	10^7–10^8
Average chain length	3–1,000	3–50
Ramifications	<1 %	4–6 %
Degree of polymerization[a]	10^2–10^3	10^3–10^4
Iodine affinity (g/100 mg)	19–20.5	0–1.2
λ_{max}[b]	630–660 nm	530–570 nm

[a]Molecule average size, expressed as glucose units
[b]With Lugol (I_2/KI)

that are not absorbed in the small intestine but are fermented in the colon producing short-chain fatty acids such as butyrate, and it promotes the normal function of the colonocytes. RS functions as dietary fibers, including prebiotic effect on the colon microflora, improving lipid metabolism. There are at least four mechanisms by which RS is obtained: RS1, a physically inaccessible starch, usually encapsulated on indigestible tissues (encapsulated or embedded within a lipidic or proteic matrix); RS2, starch granules highly resistant to degradation, which has two subtypes, RS2a, with lower amounts of amylose (0–30 %), which typically loses its strength after cooking, and RS2b, starches with high amylose content which retains its granular structure during processing; RS3, retrograde starch which requires cooking to be released from the granules, and the starch retrograde capacity is affected by the intrinsic biosynthetic process; and finally, RS4, chemically modified starches. Although the last mechanism is the most used to produce resistant starch, there are no reports of changes in plant that can mimic those obtained by chemical methods (Topping et al. 2003; Morell et al. 2004; Busi et al. 2012).

2 Generalities About Location, Timing, and Regulation of the Synthesis and Degradation of Starch

Starch synthesis is restricted to the Archaeplastida, which is thought to be originated via an endosymbiotic event between ancestors of cyanobacteria and a heterotrophic host (Cavalier-Smith 2009), resulting in organelles called plastids that are able to perform oxygenic photosynthesis. Recently, phylogenetic studies show that the plastidial starch pathway is highly complex and made up of genes belonging from cyanobacteria and other eukaryotes (Patron and Keeling 2005; Deschamps et al. 2008a, b) and is in sharp contrast to the lower-complexity pathway of cytosolic starch synthesis found in the Rhodophyceae and Glaucophyta (Deschamps et al. 2008a, b; Busi et al. 2012).

Phylogenetic analysis of the enzymes of the starch biosynthetic pathway strongly suggests that the pathway was originally cytosolic in the common ancestor of the Archaeplastida and then redirected to plastids via three discrete steps, leaving some enzymes involved in the metabolism of malto-oligosaccharides (MOS) and amylopectin degradation in the cytoplasm (Busi et al. 2012). The three evolutionary steps involved are (i) plastidial synthesis of unbranched MOS; (ii) glycogen synthesis (including priming steps and branching activities); and (iii) plastidial starch synthesis, resulting in the eventual loss of cytosolic starch synthesis. Interestingly, the relocation of the starch synthesis pathway to plastids coincides with the evolution of light-harvesting complexes (Deschamps et al. 2008a, b; Tetlow 2011; Busi et al. 2012).

Plants live and grow in an environment where the light period (day) is followed by dark (night). The sunlight is the only source of energy for photosynthetic CO_2 assimilation and also constitutes a source of energy for other metabolic processes that occur into the cell. During the night, plants use the storage metabolites that accumulated during the day (Smith and Stitt 2007). When the plants grow, the

accumulation of starch produced during the day (in a near-linear manner) is offset by the degradation overnight, so that most of the reserve polysaccharide is depleted before the start of the new light period. This pattern is conserved under different conditions of irradiances, day lengths, nutrients, water amount, and CO_2 levels (Gibon et al. 2009; Tschoep et al. 2009; Hummel et al. 2010; Graf and Smith 2011; Stitt and Zeeman 2012), indicating that it represents a system feature maintained and adapted to large fluctuations in environmental conditions. This balance requires adequate changes in the rates of synthesis and degradation of starch (Smith and Stitt 2007; Stitt and Zeeman 2012).

Furthermore, it has been found indirect evidence for a role of the biological clock in regulating starch breakdown. Transcripts of genes involved in starch breakdown show large and coordinated diurnal changes, which are at least driven in part by the clock (Harmer et al. 2000; Smith et al. 2004; Lu et al. 2005; Usadel et al. 2008; Stitt and Zeeman 2012; Streb and Zeeman 2012).

Usadel et al. (2008) showed the response of genes involved in starch degradation based on data from Smith et al. (2005) and Zeeman et al. (2007). Most of them (PHS1, ISA3, DPE1, PHS2, DPE2, GWD, PWD, and SEX4 among others) show a marked circadian cycle showing a minimum at the end of the dark period and a peak near the end of the light period (Harmer et al. 2000; Lu et al. 2005), which is retained in a light/dark cycle. However, this increase is weakened or abolished early in an extended night when the clock is antagonized by darkness or falling carbon. This provides a potential mechanism to decrease the rate of starch breakdown when carbon stocks are decreasing in the light period, although the impact depends on the levels of proteins involved (Usadel et al. 2008).

Finally, we should note that the redox-related signals are crucial for the regulation of starch synthesis. ADP-glucose pyrophosphorylase (ADPGlc PPase), the key regulatory enzyme of starch synthesis in the plastid, is subject to posttranslational redox regulation. It was reported that the isoforms from pea leaf chloroplasts and potato tuber are activated by thioredoxin (Ballicora et al. 2000; Geigenberger et al. 2005). In addition, various enzymes involved in starch degradation have been found to be redox regulated, which may imply a coordinated regulation of starch synthesis and degradation by redox signals (Kotting et al. 2010; Geigenberger 2011).

One example of the redox regulation of the starch breakdown is the case of the glucan, water dikinase (GWD). This enzyme catalyzes the phosphorylation of the starch granules, an essential step for the initiation of starch degradation in plant leaves and tubers (Lorberth et al. 1998; Yu et al. 2001a; Ritte et al. 2002, 2004). It was shown that a major fraction of GWD is attached to the surface of the starch granule when isolated from dark-adapted plants, whereas the protein is predominantly found in a soluble form upon isolation from illuminated plants (Ritte et al. 2000). Mikkelsen et al. (2005) showed that GWD is subject to redox activation mediated by thioredoxin f and m, which is followed by the reversible reduction of a specific intramolecular disulfide bond. It was demonstrated that the redox state of GWD affects binding of the enzyme to the starch granules in a selective and reversible manner, which would promote granule association of

GWD in the dark (Mikkelsen et al. 2005). More studies are needed to clarify the in vivo relevance of this mechanism to initiate granule degradation at the beginning of the dark period. Similar to GWD, other enzymes involved in starch degradation such as β-amylase or pullulanase have been found to be activated upon reduction (Spradlin and Thoma 1970; Dauvillee et al. 2001; Schindler et al. 2001; Wu et al. 2002) or to interact with thioredoxins (Balmer et al. 2003), but whether this affects granule binding or multi-protein complex formation was not studied yet. It was suggested that the degradation of the crystalline starch granule may involve a coordinated association of several enzymes to the surface of the granule that is triggered by a decrease in the reduction state of thioredoxins. This mechanism may link starch degradation to light and the carbon status of the leaf (Geigenberger et al. 2005).

Figure 1 presents an outline of starch synthesis and degradation, indicating the main reactions and protein involved.

3 Starch Synthesis: Enzymes Involved

Starch synthesis initiates in chloroplasts when the intermediate of the Calvin cycle fructose-6-phosphate (Fru6P) is converted into glucose-6-phosphate (Glc6P) by the action of phosphoglucose isomerase (PGI). After that, a phosphoglucomutase (PGM1) converts Glc6P into glucose-1-phosphate (Glc1P) (Caspar et al. 1985; Streb et al. 2012).

On the other hand, in non-photosynthetic plastids that accumulate starch, this polysaccharide is produced from sucrose synthesized in the leaves (Comparot-Moss and Denyer 2009). Then, sucrose is catabolized to Glc6P in the cytosol, which could be imported into the plastids via specific hexose-P translocators (Neuhaus and Emes 2000). On the other hand, part of the ATP is also generated in cytosol after sucrose breakdown, transported into plastids via an adenylate transporter and used as energy source for starch synthesis. Inside the plastid, Glc6P is converted to Glc1P by a plastidial phosphoglucomutase. It was also reported that in specific situations, different substrates such as Glc1P may be imported into the chloroplast by the same hexose-P transporter to maintain starch biosynthesis (Tetlow et al. 1996; Fettke et al. 2010).

Three functional phosphoglucomutase proteins are present in Arabidopsis (Egli et al. 2010), one plastidial (PGM1) and two cytosolic (PGM2 and PGM3). Arabidopsis mutants with reduced PGI or PGM1 activities have significantly lower levels of leaf starch (Caspar et al. 1985; Lin et al. 1988a, b; Kofler et al. 2000); however, these plants still contain small amounts of starch in their chloroplasts, showing unaltered ADPGlc levels, suggesting the presence of another pathway that could synthesize the sugar-nucleotide precursor (Vitha et al. 2000; Munoz et al. 2005; Streb et al. 2009). One possibility to explain this situation is that as mentioned above, the Glc1P is translocated to the plastid from the cytosol. However, another possible explanation is that the ADPGlc molecule could be synthesized by alternative enzymatic activities in the mutants.

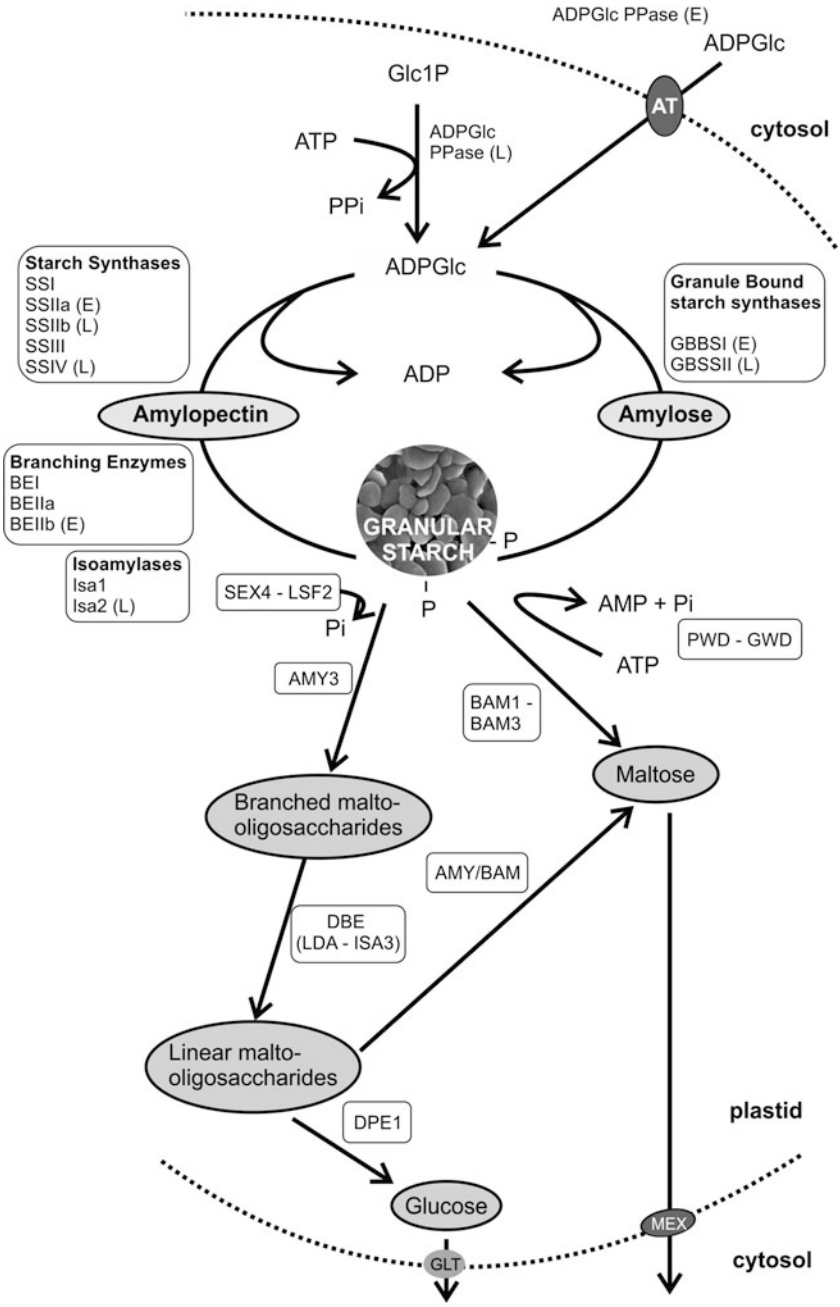

Fig. 1 Synthesis and degradation reactions of starch in plants. The scheme indicates the involvement of different isoforms in leaf (*L*) or endosperm (*E*). When not specified, the enzymes are dual localized in both compartments. All proteins concerned are listed in Tables 2, 3, 4, and 5 (AT, adenylate transporter; GLT, glucose transporter; MEX, maltose transporter)

Arabidopsis PGM2 and PGM3 single mutants present wild-type-like phenotypes; however, the loss of both cytosolic phosphoglucomutases significantly reduces the gametophyte function, probably due to a flaw on the distribution of sugars in the different metabolic pathways (Egli et al. 2010). In addition, the repression of cytosolic PGM in potato causes a significant delay in growth, a decrease in tuber number, an inhibition of sucrose synthesis in leaves, and a decrease level of sucrose and starch in tubers (Fernie et al. 2002).

The ADPGlc pyrophosphorylase (ADPGlc PPase) catalyzes the synthesis of ADPGlc from Glc1P and ATP, and this reaction constitutes the rate-limiting step for starch biosynthesis. This enzyme usually contain two catalytic (small) and two regulatory (large) subunits. Homotetrameric potato tuber ADPGlc PPase is the only PPase from plants which the 3D structure have been solved (Jin et al. 2005). The analysis of the structure show that there are two interacting domains: the N-terminal domain (catalytic) which is similar to a dinucleotide-binding Rossmann fold, whereas the C-terminal domain participates in cooperative allosteric regulation and oligomerization.

ADPGlc PPase from photosynthetic cells is tightly regulated by allosteric modulation, comprising of 3-phosphoglycerate (3PGA) activation and orthophosphate (Pi) inhibition. There is a strong evidence that the regulation by these metabolites is relevant in vivo, being the 3PGA/Pi ratio a main signal determining the starch synthesis within chloroplasts (Preiss et al. 1991; Ball et al. 1996; Preiss and Sivak 1998; Gomez-Casati et al. 1999; Gomez Casati et al. 2000; Ballicora et al. 2004). However, 3PGA can inhibit the enzymatic activity at high concentrations (Hwang et al. 2005). In contrast, whereas some ADPGlc PPases from non-photosynthetic tissues such as maize and rice endosperm and cassava roots are regulated by 3PGA/Pi, the enzymes from pea embryos, barley and wheat endosperm, and bean cotyledons are insensitive to regulation (Gomez-Casati and Iglesias 2002). In addition, as mentioned above, the small subunits of ADPGlc PPase from vascular plants are subjected to redox regulation (Ballicora et al. 2000).

All the plants analyzed contained at least three genes encoding ADPGlc PPase subunits (Deschamps et al. 2008a, b) including at least one large (L) and one small (S) subunit each. Arabidopsis mutants lacking the S or L subunit exhibit a starch-deficient phenotype (Lin et al. 1988a, b). Besides, the first maize mutant plants with altered amounts of either ADPGlc PPase S or L subunits were described almost 50 years ago. These mutant plants also showed starch-deficient phenotype, confirming a relevant function of ADPGlc PPase in starch synthesis (Tsai and Nelson 1966; Dickinson and Preiss 1969). On the other hand, transgenic plants with increased ADPGlc PPase activity triggered elevated yields in several starch-producing crops, including rice, maize, wheat, and potato (Stark et al. 1992; Greene and Hannah 1998; Huang et al. 2014).

Plant ADPGlc PPases differ in spatial and temporal expression. Cereal endosperm ADPGlc PPases are mainly located in the cytosol with only a minor amount in amyloplasts; meanwhile, they are rigorously plastidial located in other species and tissues. The differential expression of subunits in different organs results in variable degrees of sensitivity of AGPase to allosteric effectors (Wang et al. 2014).

Both cytosolic and plastidial AGPases would contribute to the production of endosperm starch (Comparot-Moss and Denyer 2009; Geigenberger 2011). Such compartmentalization requires distinctive modes of regulation and metabolic control of starch biosynthesis compared with other plants, including transport of ADPGlc and hexose-P from the cytosol into the amyloplast (Fig. 1) (Denyer et al. 1996; Thorbjornsen et al. 1996; Tetlow et al. 2003; Patron et al. 2004). Recently, it has been identified that the HvBT1 transporter in barley transports ADPGlc, the main precursor for starch biosynthesis during grain filling, from the cytosol into the amyloplasts of endospermic cells (Soliman et al. 2014). Its localization in the plastid envelop and specific expression in the endospermic barley cells indicate its significance in determining starch yield in this cereal and confirm the distinct modes of regulation between leaf and endosperm starch synthesis (Soliman et al. 2014).

Several starch synthases are thought to catalyze amylopectin synthesis, while granule-bound starch synthases (GBSS) are responsible for the biosynthesis of amylose. These enzymes are tightly bound to starch granules; for this reason they are called "granule bound" (Smith et al. 1997). *Arabidopsis thaliana* presents only one GBSS (Deschamps et al. 2008a, b), while cereals present two types of GBSS called GBSSI and GBSSII (Jeon et al. 2010). Recently, the 3D structure of the catalytic domain of rice (*Oryza sativa* japonica) GBSSI has been solved (Momma and Fujimoto 2012). It has been found that the structure was similar to those reported for the bacterial and archaeal glycogen synthases belonging to the glycosyltransferase family 5 (GT5). According to the transcripts found on the different organs, it has been shown in wheat that GBSSII is expressed predominantly in pericarp, leaf, and culm tissues, suggesting that almost all transient starch is produced by this granule-bound starch synthase (Vrinten and Nakamura 2000), whereas GBSSI (encoded by the Waxy loci (Wx) of cereals) is solely responsible or the biosynthesis of amylose in storage tissues (Smith et al. 1997). In rice it has been shown that a mutation at the Wx locus generates starch but lacking the amylose fraction, whereas a 15–30 % of amylose content is present in wild-type rice endosperm (Sano 1984). Similar results were observed in wheat (Nakamura et al. 1995). However, there is increasing evidence that GBSS is not only involved in amylose synthesis, but it is also involved in amylopectin synthesis. Studies carried out by Yoo et al. have demonstrated that there is a decreased amount of extra-long unit chains of amylopectin in Wx mutants of wheat (Yoo and Jane 2002).

SSI is one of four starch synthases present in the plastid stroma of those organisms that synthesize starch (Ball and Morell 2003). The activity of this isoform, as is the case of the other ones, depends on the organism and the tissue. For example, in developing rice endosperm, the activity of this enzyme represents the 62–66 % of the total starch synthase activity in the soluble fraction, followed by SSIII with a 26 % of the total SS activity, consistent with results obtained also in maize endosperm (Fujita et al. 2006).

SSI from barley is the only plant-soluble starch synthase of which crystallographic structures have been solved (Cuesta-Seijo et al. 2013). Unlike other SSs which present more than one isoforms in cereal species, excepting some cases like

Arabidopsis thaliana, there are no known isoforms of SSI (Deschamps et al. 2008a, b; Jeon et al. 2010). As seen on deficient SSI organisms, it was suggested that this enzyme is the major determinant for the synthesis of amylopectin, mainly involved in the biosynthesis of small outer chains (Delvalle et al. 2005).

Studies on SSI-deficient rice surprisingly shows normal seed morphology, seed weight, amount of endosperm starch, and morphology and crystallinity of endosperm starch granule. On the other hand, the structure of amylopectin is affected by this mutation. SSI prefers short glucan chains as substrate, and they propose that when the chain becomes long enough, this isoenzyme becomes inactive and actually it is entrapped inside the starch granule during the glucan deposition by others SSs (Commuri and Keeling 2001). When SSI becomes deficient, there was a reported increase of chains with degree of polymerization (DP) 6–7 and 16–19, while those of DP 8–12 decreased, indicating that SSI generates chains ranging from DP 8–12 (Fujita et al. 2006). Similar studies have been done on *Arabidopsis thaliana* and the results were in agreement with what was observed in rice. In Arabidopsis, the SSI deficiency leads to a dramatic reduction in the amount of chains with a DP of 8–12, while the ones of DP 17–20 increased (Delvalle et al. 2005). From these results it was suggested that SSI functions from early to late stages of endosperm development. In addition, the complete absence of SSI, despite being a major starch synthase isoform in endosperm, had no effect on the morphology of the starch granules, indicating that the other SS isoenzymes could partially complement SSI deficiency, leading to the modification of the length of the chains (Fujita et al. 2006). In summary, this study strongly suggested that amylopectin chains are synthesized by the coordinated actions of different SS isoforms such as SSI, SSIIa, and SSIIIa.

The second soluble starch synthase from plants, SSII, plays an essential role synthesizing the mid-length amylopectin chains. In general, SSII deficiency in different species results in increased amounts of short glucan chains (DP6 to DP11) within amylopectin, decreased abundance of DP12 to DP25 chains, and in most instances an elevated amylose to amylopectin ratio (Zhang et al. 2008). SSII deficiency in Arabidopsis showed a decreased of DP12 to DP18 chains of amylopectin, while DP6 to DP10 were significantly increased. The starch granules from *Arabidopsis* SSII mutant exhibit distorted morphology and the amount of amylose produced in leaves was increased, presumably by an increased activity of GBSS (responsible of amylose synthesis) in these mutants (Zhang et al. 2008).

In dicots only one SSII is present, in contrast to monocots that contains more than one isoform. SSII in rice presents three isoforms named osSSIIa, osSSIIb, and osSSIIc. The reason for the requirement of multiple isoforms is unknown at the present (Nakamura et al. 2005). The expression analysis of rice SSII isoforms showed that osSSIIa is expressed in endosperm, leaves, and roots, while osSSIIb is primarily expressed in leaves and osSSIIc mainly in endosperm (Jiang et al. 2004). Even though the three SSII have similar structures and the same function, it has been demonstrated that actually they are not redundant. For example, osSSIIa deficiency in endosperm leads to the increase of short chains of

amylopectin, suggesting that this isoform (and not osSSIIc) is responsible for the differences on the rice starch structure and properties. The higher content of amylopectin short chains could explain the different quality of this starch such as an easier disintegration in alkali and lower gelatinization temperature (Umemoto et al. 2002).

In maize and wheat, there are also three SSII present. In these cases, the deletion of SSIIa leads to an increase in amylose content, a decrease of longer chains of amylopectin (DP13 to DP25), and an increase of shorter chains (DP6 to DP11). Thus, it could be postulated that SSIIa has a major role, while SSIIc has a minor function in starch synthesis in endosperm (Yan et al. 2009a).

Taking in account the intron and exon arrangements of the SSII genes of both mono- and dicots, it seems that the development of the SSII genes predates the separation of these two classes (Jiang et al. 2004) and the SS gene duplication in starchy seeds species indicates that this duplication benefits storage starch synthesis in seeds (Yan et al. 2009a).

SSIII has been proposed as the responsible for the synthesis of longer amylopectin "spacers" chains extending between clusters (Maddelein et al. 1994; Wang et al. 2014) and has been proposed to perform a regulatory function in starch biosynthesis (Zhang et al. 2005; Valdez et al. 2008; Wayllace et al. 2010). However, this isoform also performs other functions that overlap with SSII to generate single-cluster chains.

Arabidopsis, wheat, and potato have only one SSIII gene (Wang et al. 2014); on the other hand, two genes are responsible for the expression of SSIII in the endosperm and leaves of rice and maize (Hirose and Terao 2004; Dian et al. 2005). The analysis of Arabidopsis *ssiii* mutants has shown that SSIII deficiency produces a starch-excess phenotype and an increase in total SS activity (Zhang et al. 2005). Loss of SSIII activity in potato tubers leads to a significant reduction of total SS activity and alterations in starch structure and physical properties (Edwards et al. 1999). Starches from SSIII-deficient maize and rice mutants show a decrease in long-branch (DP $>$ 30) proportion in addition to altered granule morphology (Gao et al. 1998). In addition, it was also documented that loss of SSIII in the maize endosperm causes decreased activity of SBEIIa and an increase in other SS activities (Boyer and Preiss 1981).

SSIII from higher plants contains two regions: an N-terminal non-catalytic SSIII-specific domain and a C-terminal domain, the catalytic domain (CD) common to all SS isoforms (Dian et al. 2005; Busi et al. 2008). It has been demonstrated that the N-terminal region of SSIII contains three starch-binding domains (SBD) that show starch-binding capacity (Palopoli et al. 2006; Busi et al. 2008; Valdez et al. 2008). These N-terminal SBDs have also a regulatory role since they modulate the catalytic properties of SSIII (Valdez et al. 2008; Wayllace et al. 2010).

Current experimental evidences provided by Gamez-Arjona et al. (2014) show that Arabidopsis SSIII exhibits a localization pattern close to the surface of the starch granule and suggest that this specific localization is mediated by its three N-terminal starch-binding domain (Gamez-Arjona et al. 2014).

Besides, we previously demonstrated the presence of protein–protein interactions between the SBDs and the catalytic domain of SSIII from *A. thaliana*. The starch-binding capacity of the SBDs and the interactions between the N- and C-terminal domains are critical in the modulation of the catalytic activity of the protein (Wayllace et al. 2010). Furthermore, it was described that the N-terminal region of maize endosperm SSIII and the central SSIII region each bind to different components of the starch biosynthetic pathway (Hennen-Bierwagen et al. 2008). Specifically, in vivo protein–protein interaction studies demonstrate that the amino terminal portion of SSIII can bind to a branching enzyme (BEIIa), whereas the SSIII central domain binds to SSI (Hennen-Bierwagen et al. 2008). Suggested potential functions for the starch synthesizing machinery complexes include substrate channeling and/or effects on the crystallization of the linear chains of amylopectin to form crystalline lamellae (Hennen-Bierwagen et al. 2008, 2009).

Meanwhile, Arabidopsis possesses just one SSIV isoform; two isoforms of SSIV exist in rice, potato and wheat, which are expressed differentially in endosperm and leaves (Dian et al. 2005; Deschamps et al. 2008a, b; Leterrier et al. 2008). Arabidopsis SSIV mutant presents just 1–2 starch granules per chloroplast and the core of these granules has a different structure from normal granules (Roldan et al. 2007). These observations indicate that SSIV is involved in starch granule initiation in Arabidopsis, granule number control, and production of short branches, although the mechanisms involved remain unclear (Roldan et al. 2007; Szydlowski et al. 2009). On the other hand, no SSIV-deficient mutant has yet been characterized in cereal plants at the present (Roldan et al. 2007; Jeon et al. 2010).

Recently, Gamez-Arjona et al. (2014) described that SSIV displays a different localization from other starch synthases. This enzyme is located in specific areas of the thylakoid membrane and interacts with fibrillin 1a (FBN1a) and 1b (FBN1b), which are mainly located in plastoglobules. The localization pattern of SSIV is mediated through its N-terminal region, which contains two long coiled-coil motifs (Gamez-Arjona et al. 2014). The localization of this isoform in specific areas of the thylakoid membrane suggests that starch granules are originated at specific regions of the chloroplast.

A summary of the enzymes involved in starch synthesis in Arabidopsis as well as in other species are shown in Tables 2 and 3.

4 Starch Degradation Pathway

Leaf starch breakdown occurs at night via the coordinated actions of a suite of enzymes constituting a network of reactions rather than a linear pathway. Many of the enzymes involved are represented by numerous isoforms. The chloroplastic starch degradation pathway is summarized and illustrated in Fig. 1 with the different *A. thaliana* isoforms implicated listed in Table 4 and the isoforms from other species in Table 5.

As it is generally known, starch is a water-insoluble polymer whose surface is inaccessible to most enzymes; thus, the starch granule surface reversible

Table 2 Arabidopsis proteins involved in starch synthesis and coding loci

Name	Locus	Localization/mutant phenotype	References
Glc6P < = > Glc1P			
PGM1	At5g51820	Plastidial, mutant near-starchless	Caspar et al. (1985), Streb et al. (2009)
PGM2	At1g70730	Cytosolic; functionally redundant with PGM3	Egli et al. (2010)
PGM3	At1g23190	Cytosolic; functionally redundant with PGM2	Egli et al. (2010)
Glc1P + ATP < = > ADPGlc + PPi			
ADG1	At5g48300	Plastidial/major catalytic subunit, (m) low starch phenotype	Lin et al. (1988a)
APS2	At1g05610	Plastidial/likely nonfunctional	Crevillen et al. (2003)
ADG2	At5g19220	Plastidial, major regulatory subunit in leaf/ catalytic, (m) low starch phenotype	Lin et al. (1988b)
APL	At1g27680	Plastidial, minor regulatory subunit in leaf/ catalytic	Ventriglia et al. (2008)
APL3	At4g39210	Plastidial, regulatory subunit in root,	Ventriglia et al. (2008)
APL4	At2g21590	Plastidial, regulatory subunit in root,	Ventriglia et al. (2008)
(1,4-α-D-Glc) (n) + ADPGlc = > (1,4-α-D-Glc)(n + 1) + ADP			
GBSS	At1g32900	Plastidial, mutants lacks amylose	Yoo and Jane (2002), Deschamps et al. (2008a, b)
SSI	At5g24300	Plastidial, amylopectin have decreased amount of DP8-12 chains	Delvalle et al. (2005), Brust et al. (2014)
SSII	At3g01180	Plastidial, Amylopectin have decreased amount of DP12-18 chains	Zhang et al. (2008)
SS3	At1g11720	Plastidial/ (m) amylopectin have increased amount of DP > 60 chains, starch-excess phenotype, and starch with higher phosphate content	Zhang et al. (2005), Szydlowski et al. (2009)
SS4	At4g18240	Plastidial, in plastoglobules/ (m) present only one large granule	Szydlowski et al. (2009), Roldan et al. (2007), Gamez-Arjona et al. (2014)
Transfers a segment of a (1→4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain			
BEI	At3g20440	Arrest of embryo development at heart stage	Wang et al. (2010)
BEII	At5g03650	Plastidial, single mutant have no apparent phenotype	Dumez et al. (2006), Brust et al. (2014)
BEIII	At2g36390	Plastidial, single mutant have no apparent phenotype	Dumez et al. (2006), Brust et al. (2014)

m, mutant phenotype

phosphorylation probed to be a crucial process for the successful completion of the breakdown process (Blennow et al. 2002; Kotting et al. 2010; Mahlow et al. 2014).

Glucan phosphorylation is the only known modification of starch that occurs in vivo with steady-state starch phosphate levels varying between species and

Table 3 *Zea mays*, *Triticum aestivum*, *Solanum tuberosum*, and *Oryza sativa* best characterized genes coding for proteins involved in starch synthesis

Name	Specie	Locus	Localization/mutant phenotype	References
Glc6P < = > Glc1P				
PGM1	*Zea mays*	ZMU89341	Cytosolic	Manjunath et al. (1998)
PGM2	*Zea mays*	ZMU89342	Cytosolic	Manjunath et al. (1998)
PGM	*Triticum aestivum*	CAC85913	Cytosolic	Davies et al. (2003)
PGM1	*Solanum tuberosum*	NP_001275333	Cytosolic, alterations in plant morphology and in carbon partitioning between sink and source organs	Fernie et al. (2002)
PGM2	*Solanum tuberosum*	NP_001275281	Plastidial/(m) near-starchless	Tauberger et al. (2000)
PGM	*Oryza sativa*	Os10g0189100	Plastidial	Tanaka et al. (2008)
PGM2	*Oryza sativa*	Os03g0712700	Cytosolic	Tanaka et al. (2008)
Glc1P + ATP < = > ADPGlc + PPi				
Bt2	*Zea mays*	NP_001105038	Bt2a cytosolic–Bt2b plastidial, endosperm/(m) starch-deficient kernel, (OE) enhanced seed weight, and starch content	Teas and Teas (1953), Bae et al. (1990), Hannah et al. (2001)
AGPSEMZM	*Zea mays*	NP_001105178	Plastidial, embryo and endosperm/(m) 50 % decreased starch content	Hannah et al. (2001), Huang et al. (2014)
AGPSLZM	*Zea mays*	DAA49237	Plastidial, leaves/(m) reduced starch content	Prioul et al. (1994), Hannah et al. (2001), Slewinski et al. (2008)
Sh2	*Zea mays*	DAA58443	Cytosolic, endosperm/(m) starch-deficient kernel; (OE) enhanced seed weight and starch content	Tsai and Nelson (1966), Bhave et al. (1990)
AGPLEMZM	*Zea mays*	NP_001105717	Plastidial, leaves and embryo	Giroux and Hannah (1994), Giroux et al. (1995)
AGPL3	*Zea mays*	DAA60137	Plastidial, low transcript level	Yan et al. (2009b)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
AGPLLZM	*Zea mays*	NP_001106017	Plastidial, leaves and endosperm/(m) reduced starch content	Yan et al. (2009b), Huang et al. (2014)
OsAGPS1	*Oryza sativa*	Os09g12660	Plastidial, endosperm	Lee et al. (2007)
OsAGPS2a	*Oryza sativa*	Os08g25734	Plastidial, leaves/ (m) reduced transitory starch content	Lee et al. (2007)
OsAGPS2b	*Oryza sativa*	Os08g25734	Cytosolic, endosperm/ (m) reduced starch biosynthesis in seeds	Lee et al. (2007)
OsAGPL1	*Oryza sativa*	Os05g50380	Plastidial, endosperm	Lee et al. (2007)
OsAGPL2	*Oryza sativa*	Os01g44220	Cytosolic, endosperm/ (m) reduced starch biosynthesis in seeds	Lee et al. (2007)
OsAGPL3	*Oryza sativa*	Os03g52460	Plastidial, leaves	Lee et al. (2007)
OsAGPL4	*Oryza sativa*	Os07g13980	Plastidial, leaves and endosperm	Lee et al. (2007)
(1,4-α-D-Glc)(n) + ADPGlc = > (1,4-α-D-Glc)(n + 1) + ADP				
GBSSI	*Zea mays*	AY109531	Plastidial, amylose-free endosperm and pollen grains	Vrinten and Nakamura (2000)
GBSSI	*Oryza sativa*	AB425323	Amylose-free endosperm	Sano (1984)
GBSSI	*Triticum aestivum*	X03935	Amylose-free endosperm	Nakamura et al. (1995)
GBSSIIa	*Zea mays*	EF471312	Plastidial	Yan et al. (2009b)
GBSSIIb	*Zea mays*	EF472248	Plastidial	Yan et al. (2009b)
SSI	*Zea mays*	AF036891	Plastidial	Knight et al. (1998)
SSI	*Oryza sativa*	AY299404	Plastidial, amylopectin have decreased amount of DP8–12 chains	Bertoft (1991), Fujita et al. (2006)
SSI	*Triticum aestivum*	AF091803	Plastidial, decrease chains of DP8–12 in amylopectin	McMaugh et al. (2014)
SSIIa	*Zea mays*	AF019296	Increased content of amylose starch and decrease chains of DP13–25 in amylopectin	Zhang et al. (2004), Yan et al. (2009a, b)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
SSIIb	*Zea mays*	AF019297	Plastidial	Hennen-Bierwagen and Myers (2013)
SSIIc	*Zea mays*	EU284113	Plastidial	Yan et al. (2009a, b)
SSIIa	*Oryza sativa*	AF419099	Higher proportion of short chains within clusters observed in amylopectin	Umemoto et al. (2002)
SSIIb	*Oryza sativa*	AF395537	Plastidial	Yan et al. (2009b)
SSIIc	*Oryza sativa*	AF383878	Plastidial	Yan et al. (2009b)
SSIIa	*Triticum aestivum*	CAB86618	Increased content of amylose starch and decrease chains of DP13–25 in amylopectin	Yan et al. (2009b), Yamamori et al. (2000)
SSIIb	*Triticum aestivum*	AB201446	Plastidial	Li et al. (1999)
SSIIc	*Triticum aestivum*	AB201447	Plastidial	Yan et al. (2009b)
SS3a(du1)	*Zea mays*	AAC14014	Plastidial/(m) increased amylose content, reduced proportion of long amylopectin chains, SSI activity increased	Wang et al. (1993a, b), Gao et al. (1998), Cao et al. (1999)
SS3b1	*Zea mays*	ABP35815		Valdez et al. (2011)
SS3b2	*Zea mays*	ABP35816		Valdez et al. (2011)
SS3	*Solanum tuberosum*	NP_001274802	Plastidial/(m) reduced amylopectin synthesis short length of amylopectin chains (DP25-35) (m) altered granule morphology	Edwards et al. (1999) Abel et al. (1996), Marshall et al. (1996)
SS3-1	*Oryza sativa*	AF432915	Plastidial, mainly expressed in leaves	Dian et al. (2005)
SS3-2	*Oryza sativa*	AY100469	Plastidial, endosperm/ (m) high amylose content, decreased long chains of amylopectin, and altered granules morphology. Increased SSI and GBSSI levels	Dian et al. (2005) Fujita et al. (2007)

(*continued*)

Table 3 (continued)

Name	Specie	Locus	Localization/mutant phenotype	References
SS3	*Triticum aestivum*	AAF8799	Plastidial, endosperm	Li et al. (2000)
SS4	*Zea mays*	NP_001123603		Schnable et al. (2009)
SS4a	*Oryza sativa*	AY373257	Plastidial, mainly expressed in endosperm	Dian et al. (2005)
SS4b	*Oryza sativa*	AY373258	Plastidial, mainly expressed in leaves	Dian et al. (2005)
Transfers a segment of a (1- > 4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain				
BEI	*Zea mays*	ZMU17897	No apparent phenotype	Jeon et al. (2010)
BEI	*Oryza sativa*	EF122471	Amylopectin have decreased amount of DP12-21 chains	Jeon et al. (2010)
BEI	*Triticum aestivum*	AF286317	Plastidial	Regina et al. (2005)
BEIIa	*Zea mays*	ZMU65948	Slight reduction in the short-chain content of leaf starch	Jeon et al. (2010)
BEIIb	*Zea mays*	AF072725	High amylose starch	Jeon et al. (2010)
BEIIa	*Oryza sativa*	AB023498	Slight reduction in the short chain content of rice endosperm starch	Jeon et al. (2010), Sawada et al. (2014)
BEIIb	*Oryza sativa*	D16201	Amylopectin have decreased amount of DP8–12 chains	Nishi et al. (2001), Sawada et al. (2014)
BEIIa	*Triticum aestivum*	AF338432	Increase in amylose content	Botticella et al. (2011)
BEIIb	*Triticum aestivum*	AY740401	Plastidial	Regina et al. (2005)

m, mutant phenotype; OE, overexpression phenotype

tissues (Blennow et al. 2000; Kotting et al. 2009). The levels of starch phosphate reported varied from a relatively high amounts in potato (*Solanum tuberosum*) tuber starch (3.4–5.8 nmol phosphate per μmol glucose) and intermediate levels in *A. thaliana* (0.8–1.4 nmol phosphate per μmol glucose) to almost undetectable amounts in cereal starches.

Phosphorylation of superficial glucosyl moieties at their C6 position by glucan, water dikinase (GWD) is the essential signal that triggers starch catabolism (Ritte et al. 2006; Mahlow et al. 2014). Subsequently, C6 phosphorylation triggers phosphorylation in position C3 through the activity of phosphoglucan, water

Table 4 Arabidopsis proteins involved in starch degradation and coding loci

Name	Locus	Localization/phenotype	References
Glucan phosphorylating enzyme – 1,4 α-D-Glc + H2O + ATP → phospho-1,4-α- D-Glc + AMP + Pi			
GWD1	At1g10760	Chloroplastic. KO/KD: *sex*, strongly reduced starch phosphorylation, plants compromised in growth	Yu et al. (2001a), Ritte et al. (2002), Baunsgaard et al. (2005), Kotting et al. (2005), Ritte et al. (2006)
PWD1	At5g26570	Chloroplastic. KO/KD: *sex*, reduced level of C3 phosphorylation of glucosyl residues, minor effect on plant development	Baunsgaard et al. (2005), Kotting et al. (2005)
GWD2	At4g24450	Cytosolic. KO: growth and starch/sugar levels similar to the wild type	Glaring et al. (2007)
Phosphoglucan phosphatase – removes a phosphate group from phosphoglucosyl residues within amylopectin			
SEX4	At3g52180	Chloroplastic. KO: *sex*, leaf accumulation of linear phosphorylated oligosaccharide, stunted plants	Zeeman et al. (1998), Kotting et al. (2009), Hejazi et al. (2010)
LSF1	At3g01510	Chloroplastic. KO: *sex*, comparable growth and rosette morphology to wild-type plants	Comparot-Moss et al. (2010), Silver et al. (2014)
LSF2	At3g10940	Chloroplastic. KO: normal starch levels, increased starch phosphorylation of glucosyl residues at C3 position	Santelia et al. (2011)
β-amylase – from the nonreducing end, catalyzes the hydrolysis of the second α(1,4)-glycosidil-bond cleaving off maltose			
BAM1	At3g23920	Chloroplastic. KO: reduced total beta-amylase activity	Sparla et al. (2006), Fulton et al. (2008)
BAM2	At4g00490	Chloroplastic. KO: not distinguishable from wild-type plants	Fulton et al. (2008)
BAM3	At4g17090	Chloroplastic. KO: *sex*, lower nighttime maltose levels. Reduced total beta-amylase activity	Fulton et al. (2008), Lao et al. (1999), Kaplan and Guy (2005).
BAM4	At5g55700	Chloroplastic. Inactive. KO: *sex*	Fulton et al. (2008)
BAM5	At4g15210	Cytosolic. KO: almost complete loss of beta-amylase activity	Laby et al. (2001)
BAM6	At2g32290	Cytosolic	Smith et al. (2004)
BAM7	At2g45880	Nuclear. BZR1-BAM transcription factor	Reinhold et al. (2011)
BAM8	At5g45300	Nuclear. BZR1-BAM transcription factor	Reinhold et al. (2011)
BAM9	At5g18670	Cytosolic	Chandler et al. (2001)

(*continued*)

Table 4 (continued)

Name	Locus	Localization/phenotype	References
α-amylase – hydrolyzates α-1,4 linkages at random location yielding glucose, maltose, maltotriose, or limit dextrin			
AMY1	At4g25000	Secreted from cell. KO: starch degradation is not altered	Yu et al. (2005), Doyle et al. (2007)
AMY2	At1g76130	KO: starch degradation is not altered	Yu et al. (2005)
AMY3	At1g69830	Chloroplastic. KO: starch degradation is not altered	Yu et al. (2005), Streb et al. (2012)
α-1,6-glucosidase/starch debranching enzyme – acts on α-1,6 linkages releasing oligosaccharides			
ISA3	At4g09020	Chloroplastic. KO: sex. Increased abundance of short chains in amylopectin. Slower rate of starch breakdown than wild-type plants	Wattebled et al. (2005), Delatte et al. (2006)
LDA	At5g04360	Plastidic, KO: indistinguishable from the wild type	Delatte et al. (2006)
α-Glucan phosphorylase – ((1,4)-α-D-glucosyl)(n) + Pi ↔ ((1,4)-α-D-glucosyl)(n-1) + α-D-glucose 1-phosphate			
PHS1	At3g29320	Chloroplastic. KO: starch degradation is not altered	Zeeman et al. (2004)
PHS2	At3g46970	Cytosolic. KO: Increase in nighttime maltose	Lu et al. (2006b)
4-α-glucanotransferase/disproportionating enzyme – transfers glucose/ α-1,4-linked glucan moiety from a donor glucan to an acceptor releasing the nonreducing end glucose/glucan moiety (reversible)			
DPE1	At5g64860	Plastidial. KO: sex. Large accumulation of malto-Oligosaccharides, increased amylose to amylopectin ratio, lower amounts of starch synthesized and degraded	Critchley et al. (2001)
DPE2	At2g40840	Cytosolic. KO: maltose excess in leaves. Increased maltose moved from the shoots to the roots	Lu et al. (2006a)

KO knockout, *KD* knockdown, *OE* overexpression

dikinase (PWD) (Ritte et al. 2006). C6 phosphorylation overlaps the non-phosphorylated structure of the amylopectin structure, but C3 phosphorylation imposes a marked steric effect that leads to a conformational shift. C6 phosphorylation by GWD, then, promotes the local hydration of the crystalline lamella and C3 phosphorylation by PWD induces a deformation of the helix. This deformation renders the substrate more accessible to the glucan hydrolytic enzymes.

Both starch-phosphorylating enzymes are effectively the first step on the degradation pathway and so they are attractive candidates for the control of flux through

Table 5 *Solanum tuberosum*, *Oryza sativa*, *Solanum lycopersicum*, *Triticum aestivum*, and *Hordeum vulgare* proteins involved in starch degradation and coding loci

Name	Specie	Locus	Localization/phenotype	References
Glucan phosphorylating enzyme – 1,4 α-D-Glc + H2O + ATP → phospho-1,4-α- D-Glc + AMP + Pi				
StGWD	*Solanum tuberosum*	AFH8838	Plastidic; KD: *sex*, drastically reduced phosphate content in leaf and tuber starches	Lorberth et al. (1998)
OsGWD1	*Oryza sativa*	Os06g0498400	KO: *sex* in leaves but limited effects on vegetative growth, 20–40 % grain yield reduction	Hirose et al. (2013)
LeGWD	*Solanum lycopersicum*	ACG69788	KO: sex in leaves, pollen germination arrest, and male sterility	Nashilevitz et al. (2009)
GWD	*Triticum aestivum*	ADG27838	KD: decrease in starch phosphate content and increase in grain size and vegetative biomass	Ral et al. (2012)
Phosphoglucan phosphatase – removes a phosphate group from phosphoglucosyl residues within amylopectin				
HvSEX4	*Hordeum vulgare*	AID16302	Chloroplastic	Ma et al. (2014)
β-amylase – from the nonreducing end, catalyzes the hydrolysis of the second α(1,4)-glycosidil-bond cleaving off maltose				
PCT-BMY1	*Solanum tuberosum*	NP_001275172	KD: *sex* in leaves	Scheidig et al. (2002)
Os BAM 1-9	*Oryza sativa*	–	OsBAM2 and OsBAM3 are plastid-targeted active β-amylase	Hirano et al. (2011)
α-amylase – hydrolyzates α-1,4 linkages at random location yielding glucose, maltose, maltotriose, or limit dextrin				
OsAMY1-1	*Oryza sativa*	P17654	Chloroplastic. KO: sex. seed germination and seedling growth markedly delayed	Huang et al. (1990) Asatsuma et al. (2005)
TaAMY3	*Triticum aestivum*		Plastidic. OE: minimal effect on the overall grain weight or the starch content	Barrero et al. (2013), Whan et al. (2014)
α-1,6-glucosidase/Starch debranching enzyme – Acts on α-1,6 linkages releasing oligosaccharides				
StISA3	*Solanum tuberosum*	AAN15319	ISA-type DBE. KO: sex in leaves	Hussain et al. (2003)
OsISA3	*Oryza sativa*	AEV92948	ISA-type DBE. Plastidial. KO: sex	Yun et al. (2011)

(*continued*)

Table 5 (continued)

Name	Specie	Locus	Localization/phenotype	References
ZPU1	*Zea mays*	O81638	Pleomorphic plastids and starch granules LD/PUL-type DBE. KO: *sex* in leaves Decreased rate of starch degradation Decreased rate of cotyledon growth	Beatty et al. (1999), Dinges et al. (2003)
HvLD	*Hordeum vulgare*	O48541	LD/PUL-type DBE Amyloplastic in aleurone cells	Kristensen et al. (1999), Vester-Christensen et al. (2010)
α-Glucan phosphorylase – ((1,4)-α-D-glucosyl)(n) + Pi ↔ ((1,4)-α-D-glucosyl)(n-1) + α-D-glucose 1-phosphate				
HvPHO1	*Hordeum vulgare*	AFP19106	Plastidic. KD: not lead to any visible phenotype, no dramatic alterations in the structure of the starch	Ma et al. (2013)
HvPHO2	*Hordeum vulgare*	ACV72276	Cytosolic	Ma et al. (2013)
SP	*Zea mays*	–	Plastidic	Mu et al. (2001), Yu et al. (2001b)
TaPHO1	*Triticum aestivum*	ACC5920	Plastidic	Tickle et al. (2009)
4-α-glucanotransferase/disproportionating enzyme – transfers glucose/ α-1,4-linked glucan moiety from a donor glucan to an acceptor releasing the nonreducing end glucose/glucan moiety (reversible)				
StDPE1	*Solanum tuberosum*		KO: slightly *sex*	Takaha et al. (1993), Lutken et al. (2010)
StDPE2	*Solanum tuberosum*		Cytosolic. KO: large amounts of maltose and starch	Lutken et al. (2010)

KO knockout, *KD* knockdown, *OE* overexpression

starch degradation. Both enzymes and their expression profile possess properties consistent with modulation of their activities over the day/night cycle (i.e., redox regulation and transcript levels shows large daily changes) (Yu et al. 2001a; Smith et al. 2004; Baunsgaard et al. 2005; Kotting et al. 2005). However, recent work presented begun to tackle this point by establishing that translational control and redox activation are of limited relevance for the control of starch turnover in *Arabidopsis* leaves (Skeffington et al. 2014).

It is worth mentioning that for an efficient digestion of the starch molecule, a glucan phosphatase activity is required to avoid the accumulation of either phosphorylated starch or phosphorylated intermediates from starch breakage (Kotting et al. 2009). This role is played mainly by the starch-excess 4 (SEX4) glucan phosphatase. Primary structure of SEX4 includes an N-terminal chloroplast transit peptide, a dual-specificity phosphatase domain (DSP), and a carbohydrate-binding module (CBM) belonging to the family 48 group (Kotting et al. 2009; Vander Kooi et al. 2010). The glucan-free SEX4 crystal structure indicated that its CBM and DSP domains interact to form binding pocket that carry out two functions: glucan binding and dephosphorylation (Vander Kooi et al. 2010). SEX4 mutations lead to an important increase of starch accumulation in *A. thaliana* leaves due to a decrease in the degradation rate and an increase in the levels of soluble phospho-oligosaccharides (Zeeman et al. 1998; Sokolov et al. 2006; Kotting et al. 2009). Besides catalyzing the dephosphorylation of amylopectin, recombinant SEX4 is able to dephosphorylate crystalline maltodextrins (Hejazi et al. 2010), starch granules isolated from *A. thaliana* leaves and phospho-oligosaccharides (Kotting et al. 2009). While phosphorylation at positions C6 and C3 is performed by two different dikinases, SEX4 is able to dephosphorylate both positions but preferentially acts on C6 position (Kotting et al. 2009; Hejazi et al. 2010). Recently, the phosphoglucan-bound crystal structure of SEX4 was resolved and the authors described its possible catalytic mechanism. In addition, the identification of two DSP domain residues involved in SEX4 site-specific activity leads to the construction of a SEX4 mutant that reversed the specificity from the C6 to the C3 position (Meekins et al. 2014).

Two SEX4-homolog enzymes have also been characterized in Arabidopsis and named as LSF1 (for Like SEX4 1)and LSF2 (for Like SEX4 2) (Comparot-Moss et al. 2010; Santelia et al. 2011). Although plants lacking LSF2 possess normal levels of starch, *lsf2*/*sex4* double mutants show a more severe starch-excess phenotype respect to *sex4* alone, suggesting that LSF2 has a partial overlapping function in starch dephosphorylation. Indeed, LSF2 exclusively dephosphorylate the C3 position of amylopectin in vitro (Santelia et al. 2011); however, unlike other starch-binding enzymes, including SEX4, LSF2 does not possess a CBM. Instead, LSF2 possesses a unique DSP active site which has a dual function: a glucan binding site and phosphatase activity (Meekins et al. 2013).

LSF1 is also a chloroplast enzyme needed for efficient starch degradation: lsf1 mutants, like sex4 mutants, contain more quantities of starch in their leaves than wild type (wt) plants during the day (Comparot-Moss et al. 2010). However, genetic and transcription profile analyses as well as biochemistry studies indicate that its function would be different from SEX4 since it is mentioned as an inactive phosphatase although it shares similar primary structure to SEX4 and contains all the essential residues required for catalysis (Comparot-Moss et al. 2010). Thereby, LSF1 functions in starch metabolism, although its precise role is unknown; however, it is possible that LSF1 may recruit starch degradative enzymes to the granule surface.

As a consequence, during starch degradation, the granule surface is destabilized by phosphorylation and then simultaneously degraded by glucan hydrolytic enzymes and dephosphorylated by SEX4 and LSF2 (Stitt and Zeeman 2012). Precisely how GWD, PWD, SEX4, and LSF2 might be regulated to limit the ATP consumption of the system while permitting an appropriate flux through the starch degradation pathway remains to be discovered.

Among the hydrolytic enzymes we found β-amylases, α-amylases, debranching enzymes, disproportionating enzymes, and α-glucan phosphorylases. The five enzymes play a concerted role in the liberation and metabolism of malto-oligosaccharides in the stroma, being β-amylases and debranching enzymes the most important.

β-amylases (BAM) are exoamylases that catalyze the hydrolysis of α1-4 D-glucosidic linkages in polysaccharides, successively removing maltose units from the nonreducing ends of the chains. BAMs cannot hydrolyze nor act close to α-1,6 branch points neither can act over phosphorylated glucans. That is the reason why dephosphorylating enzymes (discussed above) as well as debranching enzymes (DBEs, discussed later) are critical for correct starch degradation. Plant genomes encode multiple β-amylase-like proteins. In Arabidopsis, there are nine genes, which have been designated BAM1 to BAM9 to provide a unifying nomenclature (Smith et al. 2004). Of all such genes, only BAM1 and BAM3 have been directly implicated in starch degradation playing a central role in the breakdown of leaf starch (Kaplan and Guy 2005). The loss of BAM2 has little effect in all genetic backgrounds assayed and no discernible function has been assigned yet (Fulton et al. 2008). Other data suggest that BAM4 facilitates or regulates starch breakdown and operates independently of BAM1 and BAM3 revealing unexpected complexity in terms of the specialization of protein function within the β-amylase gene family (Fulton et al. 2008; Li et al. 2009; Francisco et al. 2010). The complexity of β-amylase gene family was more recently exemplified by the finding of BAM7 and BAM8 as nuclear β-amylases involved in transcriptional activation (Soyk et al. 2014).

DBEs, also called isoamylases (ISAs), hydrolyze α1-6 D-glucosidic branch linkages in amylopectin and their β-limit dextrins releasing oligosaccharides into the stroma. There are two classes of DBE in higher plants, and they are referred to as pullulanase-type DBE (PUL) and isoamylase-type DBE (ISA) (Ball et al. 1996; Beatty et al. 1999). Four genes coding for DBE proteins are conserved in higher plants, one for a PUL protein and three for ISA proteins, designated as ISA1, ISA2, and ISA3 (Deschamps et al. 2008a, b). They play a dual role in the synthesis and degradation of starch in higher plants. ISA1 and ISA2 proteins exist together in a heteromeric complex, function together, and are strongly implicated in amylopectin synthesis (Fujita et al. 1999; Hussain et al. 2003; Bustos et al. 2004; Delatte et al. 2005; Utsumi and Nakamura 2006; Takashima et al. 2007; Zeeman et al. 2010). On the other hand, PU1 and ISA3 are not strongly associated within a multimer and may function as a monomer, both preferring substrates with short outer chains, such as β-limit dextrins, suggesting that their role is primarily in starch degradation. Consistently, mutating these

genes in Arabidopsis causes a sex phenotype (Delatte et al. 2006; Streb et al. 2008; Wattebled et al. 2008).

Although BAM3 and ISA3, the major enzymes that hydrolyze the α-1,4 and 1,6 linkages of the Glc polymers that constitute the starch granule, are inhibited by oxidation in vitro (Glaring et al. 2012), there is no evidence about the importance of these proteins on the modulation or control of starch degradation in vivo.

The starch degradative scenario is completed by α-amylases (an endoamylase that hydrolyze α 1–4 D-glucosidic linkages in polysaccharides containing three or more α-1,4-linked D-glucose units), α-glucan phosphorylases (act on the nonreducing end of α-1,4-linked glucose giving Glc P using polysaccharide and phosphate as substrate), and the disproportionating enzymes.

Alpha-amylases are endo-hydrolases belonging to the GH13 family (Majzlova et al. 2013). The roles and number of isoforms vary across the plant kingdom. In Arabidopsis, three isoforms have been described: AtAMY1, AtAMY2, and AtAMY3. AtAMY3 is the only plastidic alpha-amylase, whereas AtAMY1 and AtAMY2 do not have any chloroplast targeting peptide (Yu et al. 2005).

The importance of AMY3 in transitory starch catabolism was demonstrated when AMY3 knockout mutants were combined with knockout ones of debranching enzyme and/or limit dextrinase-deficient lines. AMY3 could participate in starch breakdown in wild type leaves; however, other enzymes can compensate its deficiency. In addition, neither of the three AMY proteins are required for normal rates of starch degradation, and starch degradation is normal even when all three AMY genes are disrupted (Yu et al. 2005). Recently, Streb et al. (2012) suggested that AMY3 acts on starch granules leading to short branched glucans within the chloroplast stroma. Thus, these glucans undergo a rapid conversion into maltose and glucose by debranching enzymes (Streb et al. 2012).

In Arabidopsis, α-amylase does not have a major role in starch degradation. However, in germinating cereal seeds, different amylase isoforms are secreted from the aleurone cells and play an important role in the degradation of storage starch in the nonliving starchy endosperm (Asatsuma et al. 2005; Whan et al. 2014).

Another enzyme involved in starch breakdown is α-glucan phosphorylase (PHS). This is the only phosphorolytic enzyme involved in starch metabolism and catalyzes a reversible glucosyl transfer reaction either in the direction of glucan synthesis from Glc1P in the presence of glucan primer liberating orthophosphate (Pi) or, if Pi is abundant, in the direction of glucan phosphorolysis forming Glc1P. In both cases, the nonreducing end(s) of the glucan primer act(s) as glucosyl acceptor and donor, respectively.

The metabolic function of PHS has been a great deal of debate. One widely accepted idea, according to which the physiological function of PHS1 is to degrade rather than to synthesize glucans, is based on the observations that PHS1 has a low affinity for Glc1P and that low levels of Glc1P and high levels of inorganic phosphate are found in plant cells (Kruger and Ap Rees 1983; Preiss and Sivak 1998). On the other hand, it has been demonstrated that PHS homologs are involved in starch biosynthesis in rice endosperm and potato tuber (Satoh et al. 2008; Fettke et al. 2010; Hwang et al. 2010; Nakamura et al. 2012).

In this direction, PHS plays a probed role in the capacity of the leaf lamina to endure a transient water deficit; however, it is not required for the degradation of plant starch: complete loss of the enzyme does not cause a significant change in the overall accumulation of starch during the day or its remobilization at night in healthy plants (Zeeman et al. 2004).

Finally, we have to mention the disproportionating enzymes (DPE). DPE is an α-1,4 glucanotransferase which catalyzes the cleavage of α-1,4 glucosidic bonds of polyglucans (maltotriose or larger), transferring the glucosyl groups to the nonreducing end of another glucan chain or free glucose and releasing either glucose or a glucan chain depending on the cleavage site. The *Arabidopsis* genome encodes two 4-α-glucanotransferase-like proteins: DP1 and DP2. DP1 is a chloroplastic enzyme responsible for the metabolism of the maltotriose or short malto-oligosaccharide products of starch degradation at night, presumably generated by chloroplastic β-amylolysis since DPE1 mutants accumulate maltotriose during starch breakdown at night (Critchley et al. 2001). The other DPE protein, DPE2, is cytosolic (Lutken et al. 2010). Independent mutant lines deficient in DPE2 show a decreased capacity for both starch synthesis and degradation in leaves. Thus, DPE2 is an essential component of the pathway from starch to sucrose and cellular metabolism in leaves at night despite its cytosolic localization. Probably, its role is related with the metabolization of maltose exported from the chloroplast (Chia et al. 2004; Lu and Sharkey 2006).

In summary, the main route of starch degradation in *Arabidopsis* is via GWD and PWD, which introduce some disorder at the starch granule surface, and are followed by the action of the β-amylases, debranching enzymes, and glucan phosphatases (SEX4 and LSF2). These enzymes act progressively and in combination to produce maltose, maltotriose, and longer malto-oligosaccharides (MOS) as the major products of the initial steps of starch degradation. α-amylases, DBE, PHS1, and DPE1 complement starch degradation to produce finally maltose and glucose, the two products of starch breakdown that are exported from the chloroplast to the cytosol via distinctive specific transporters (see Fig. 1). Despite its importance for productivity, we do not yet know how the rate of starch degradation is controlled.

5 Starch-Related Enzymes in *Arabidopsis thaliana* Preserve a Modular Structure

Enzymes that catalyze the reactions of glycosidic bonds share a modular organization that includes a catalytic domain and one or more carbohydrate binding module (CBM) connected by a loosely structured chain (Henrissat and Davies 1997).

Catalytic domains are often classified with an EC number according to the International Union of Biochemistry and Molecular Biology (IUBMB) recommendations and their substrate specificities (Barrett 1995), but this classification may fail to describe structural, evolutionary, and mechanistic similitude between these enzymes (Henrissat and Davies 1997). Sequence similarity classification can take into account these aspects because they are defined by the primary sequence alone

(Henrissat and Davies 2000) and could provide a more descriptive classification. CAZy classification includes these approaches (structural and mechanistic based on sequence similarity) to create a database of all known carbohydrate-associated enzymes. However, this system often grouped enzymes of different substrate specificity into a single "poly-specific" group. Nevertheless, a family can only be defined when one of its members is characterized biochemically (Valdez et al. 2008). Still, catalytic residues could be identified in both position and function for one member of a family, and then, they can easily be inferred for all members of the family (Henrissat and Davies 2000). This classification is available on the CAZy website (http://www.cazy.org) (Lombard et al. 2014).

This common modular structuration is also conserved in numerous *Arabidopsis thaliana* starch metabolism enzymes (Table 6). A batch of starch-associated enzymes were analyzed with the NCBI Conserved Domain-search tools (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009, 2011), and the output was organized according to detected domain superfamily. Of the 40 proteins analyzed, we found 19 independent domains, many of them repeated in several degradation and synthesis enzymes.abcdefg

Table 6 shows that some domains tend to be exclusive of degradative (alpha-amyl_C2, CBM20, PPDK_N, PTPc, Glyco_hydro_77 and PDZ) or synthetic (alpha-amylase C, CBM 53, GT-A, LbH, phosphohexomutase, MRP-L20, RAP1, and tropomyosin_1) enzymes and others are present in both types of enzymes (AmyAc_family, CBM 48 and GT-B).

The domains present in the AmyAc_family are the most frequent glycoside hydrolase (GH) domain with the majority of enzymes acting on starch, glycogen, and related oligo- and polysaccharides (Janecek 1997; Janecek et al. 1997) catalyzing the transformation of α-1,4 and α-1,6 glucosidic linkages with retention of the anomeric center. In the *Arabidopsis thaliana* starch metabolic pathway, these modules are present in degradative enzymes such as ß-amylases (BAM1-9), pullulanase, and isoamylase (ISOA) as well as in synthesizing enzymes such as starch branching enzymes (SBE3, SBE2.2, and SBE2.1). These latter proteins also have an alpha/beta-barrel domain present in eight stranded α/ß barrel, interrupted by a calcium-binding domain, and a C-terminal Greek key beta-barrel domain (Strobl et al. 1998).

Alpha-amyl_C2 domains are associated to an O-glycosyl hydrolase (EC 3.2.1.) activity, which are found in the C-terminal end in numerous α-amylases and fold into a five-stranded antiparallel ß-sheet structure (Kadziola et al. 1998). They are actually present in three *A. thaliana* α-amylases (AMY1, AMY2, and AMY3) catalyzing the hydrolysis of α-1,4-glucosidic bonds of starch (Lloyd et al. 2005). The branching enzymes (BEs) catalyze the formation of α-1,6 branch points in either glycogen or starch by cleavage of the α-1,4 glucosidic linkage yielding a more reactive glycogen with an increased number of nonreducing ends.

The CAZy glycoside hydrolase family 77 contains a 4-α-glucanotransferase activity (disproportionating activity) that comprise the transfer of a (1 → 4)- α-D-glucan segment to a new position in an acceptor, which may be glucose or another (1 → 4)-α-D-glucan (Pazur and Okada 1968). This domain is present in the DPE1

Table 6 Summary of domains presents in *Arabidopsis thaliana* starch metabolism enzymes

Protein name	Description	Locus ID	UniProt ID	From	To	Domain	Function
AMY1	Alpha-amylase 1 (AMY1)	At4g25000	Q8VZ56	362	422	Alpha-amyl_C2	Degradation
AMY3	Alpha-amylase 3 (AMY3)	At1g69830	Q94A41	826	885	Alpha-amyl_C2	Degradation
AMY2	Alpha-amylase 2 (AMY2)	At1g76130	Q8LFG1	355	413	Alpha-amyl_C2	Degradation
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	799	895	Alpha-amylase_C	Synthesis
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	707	801	Alpha-amylase_C	Synthesis
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	742	800	Alpha-amylase_C	Synthesis
BAM3	Beta-amylase 3 (BAM3, CT-BMY, BMY8)	At4g17090	O23553	1	548	AmyAc_family	Degradation
BAM4	Beta-amylase 4 (BAM4, BMY6)	At5g55700	Q9FM68	1	531	AmyAc_family	Degradation
BAM6	Beta-amylase 6 (BAM6)	At2g32290	Q8L762	63	573	AmyAc_family	Degradation
BAM5	Beta-amylase 5 (BAM5, BMY1, RAM1)	At4g15210	P25853	1	498	AmyAc_family	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	332	796	AmyAc_family	Degradation
ISOA	Isoamylase 3	At4g09020	Q9M0S5	220	659	AmyAc_family	Degradation
BAM1	Beta-amylase 1 (BAM1, TR-BAMY)	At3g23920	Q9LIR6	106	541	AmyAc_family	Degradation
BAM7	Beta-amylase 7 (BAM7)	At2g45880	O80831	247	678	AmyAc_family	Degradation
BAM2	Beta-amylase 2 (BAM2, BMY9)	At4g00490	O65258	95	525	AmyAc_family	Degradation
BAM8	Beta-amylase 8 (BAM8)	At5g45300	Q9FH80	254	683	AmyAc_family	Degradation
BAM9	Beta-amylase 9 (BAM9, BMY3)	At5g18670	Q8VYW2	77	503	AmyAc_family	Degradation
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	309	724	AmyAc_family	Synthesis
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	274	689	AmyAc_family	Synthesis
SBE3 (BE1)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	381	780	AmyAc_family	Synthesis
AMY1	Alpha-amylase 1 (AMY1)	At4g25000	Q8VZ56	28	372	AmyAc_family	Degradation

AMY3	Alpha-amylase 3 (AMY3)	At1g69830	Q94A41	497	836	AmyAc_family	Degradation
AMY2	Alpha-amylase 2 (AMY2)	At1g76130	Q8LFG1	27	365	AmyAc_family	Degradation
SS3	Starch Synthase 3	At1g11720	F4IAG2	328	424	CBM 53	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	495	584	CBM 53	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	153	238	CBM 53	Synthesis
DPE2	4-alpha-glucanotransferase	At2g40840	Q8RXD9	20	120	CBM20	Degradation
DPE2	4-alpha-glucanotransferase	At2g40840	Q8RXD9	164	261	CBM20	Degradation
PWD	Phosphoglucan water dikinase (PWD1, GWD3)	At5g26570	Q6ZY51	73	164	CBM20	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	791	963	DUF3372	Degradation
BAM7	Beta-amylase 7 (BAM7)	At2g45880	O80831	65	229	DUF822	Degradation
BAM8	Beta-amylase 8 (BAM8)	At5g45300	Q9FH80	96	245	DUF822	Degradation
ISOA	Isoamylase 3	At4g09020	Q9M0S5	91	240	CBM 48 (E_set)	Degradation
PULA	Pullulanase 1	At5g04360	Q8GTR4	206	307	CBM 48 (E_set)	Degradation
SBE2.2	1,4-alpha-glucan-branching enzyme 2-2	AT5G03650	Q9LZS3	176	270	CBM 48 (E_set)	Synthesis
SBE2.1 (**BE3**)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	212	305	CBM 48 (E_set)	Synthesis
DSP4	Starch-excess 4 (SEX4, DSP4)	At3g52810	Q9FEB5	256	337	CBM 48 (E_set)	Degradation
LSF1	Like Sex four 1 (LSF1)	At3g01510	F4J117	456	536	CBM 48 (E_set)	Degradation
SBE3 (**BE1**)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	118	184	CBM 48 (E_set)	Synthesis
SBE3 (**BE1**)	1,4-alpha-glucan-branching enzyme 3	At3g20440	D2WL32	334	377	CBM 48 (E_set)	Synthesis
DPE1	4-alpha-glucanotransferase	At5g64860	Q9LV91	56	576	Glyco_hydro_77	Degradation
APL2	Glucose-1-phosphate adenylyltransferase large subunit 2	At1g27680	P55230	88	349	GT-A	Synthesis
APL3	Glucose-1-phosphate adenylyltransferase large subunit 3	At4g39210	P55231	91	352	GT-A	Synthesis

(*continued*)

Table 6 (continued)

Protein name	Description	Locus ID	UniProt ID	From	To	Domain	Function
GLGL	Probable glucose-1-phosphate adenylyltransferase large subunit	At2g21590	Q9SIK1	93	354	GT-A	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	93	353	GT-A	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	101	350	GT-A	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	212	310	GT-A	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	213	271	GT-A	Synthesis
PHS1	Alpha-glucan phosphorylase 1	At3g29320	Q9LIB2	97	956	GT-B	Degradation
PHS2	Alpha-glucan phosphorylase 2	At3g46970	Q9SD76	33	835	GT-B	Degradation
SS1	Starch synthase 1	At5g24300	Q9FNF2	143	643	GT-B	Synthesis
GBSS1	Granule-bound starch synthase 1	At1g32900	Q9MAQ0	86	582	GT-B	Synthesis
SS2	Starch synthase 2	At3g01180	Q9MAC8	302	785	GT-B	Synthesis
SS4	Starch synthase 4	At4g18240	Q0WVX5	544	1026	GT-B	Synthesis
SS3	Starch Synthase 3	At1g11720	F4IAG2	595	1039	GT-B	Synthesis
APS1	Glucose-1-phosphate adenylyltransferase small subunit	At5g48300	P55228	388	514	LbetaH	Synthesis
ADG2	Glucose-1-phosphate adenylyltransferase large subunit 1	At5g19220	P55229	390	516	LbetaH	Synthesis
APL2	Glucose-1-phosphate adenylyltransferase large subunit 2	At1g27680	P55230	386	512	LbetaH	Synthesis
APL3	Glucose-1-phosphate adenylyltransferase large subunit 3	At4g39210	P55231	389	515	LbetaH	Synthesis

GLGL	Probable glucose-1-phosphate adenylyltransferase large subunit	At2g21590	Q9SIK1	391	517	LbetaH	Synthesis
SS3	Starch synthase 3	At1g11720	F4IAG2	254	302	MRP-L20	Synthesis
LSF1	Phosphoglucan phosphatase LSF1	At3g01510	F4J117	76	118	PDZ	Degradation
PGMC	Probable phosphoglucomutase	At1g23190	O49299	7	583	phosphohexomutase	Synthesis
PGMC	Probable phosphoglucomutase	At1g70730	Q9SGC1	9	585	phosphohexomutase	Synthesis
PGMP	Phosphoglucomutase	At5g51820	Q9SCY0	70	623	phosphohexomutase	Synthesis
PWD	Phosphoglucan water dikinase (PWD1, GWD3)	At5g26570	Q6ZY51	870	1193	PPDK_N	Degradation
GWD1	α-glucan water dikinase (GWD1, SEX1)	At1g10760	Q9SAC6	1089	1397	PPDK_N	Degradation
GWD2	α-glucan water dikinase (GWD2)	At4g24450	Q9STV0	970	1276	PPDK_N	Degradation
LSF2	Phosphoglucan phosphatase LSF2	At3g10940	Q9SRK5	100	237	PTPc	Degradation
DSP4	Starch excess 4 (SEX4, DSP4)	At3g52810	Q9FEB5	101	235	PTPc	Degradation
LSF1	Phosphoglucan phosphatase LSF1	At3g01510	F4J117	293	420	PTPc	Degradation
SBE2.1 (BE3)	1,4-alpha-glucan-branching enzyme 2-1	At2g36390	O23647	34	204	RAP1	Synthesis
SS4	Starch synthase 4	At4g18240	Q0WVX5	344	461	Tropomyosin_1	Synthesis

Script curated CD-search output (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009; 2011) was used to construct this table. See text for domain description. Domains that do not have a specific function annotated are annotated as "DUF" families (domain of unknown function). Protein name refers to the most common name for a specific protein. "From" and "to" refers to the domain specific position in the amino acidic sequence. "Synthesis" and "degradation" attempt to summarize the specific role of a protein in the starch metabolism, although a protein may be playing a role in both pathways

(disproportionating enzyme) of *Arabidopsis thaliana* and folds into a (β/α) 8-barrel with a strand β4-aspartic acid (catalytic nucleophile), a β5-glutamic acid (proton donor), and a β7-aspartic acid (transition-state stabilizer) critical for the catalytic machinery (Jespersen et al. 1993). At this moment, the three-dimensional structures have been solved for only five members of the family (Cantarel et al. 2009; Levasseur et al. 2013).

Glycosyltransferases (GTs) are enzymes that synthesize oligosaccharides, polysaccharides, and glycoconjugates by transferring the sugar moiety from an activated nucleotide-sugar donor to an acceptor molecule (oligosaccharide, a lipid, or a protein). Based on the stereochemistry of the donor and acceptor molecules, GTs could be classified as either retaining or inverting enzymes (Lairson et al. 2008).

It has been described that glycosyltransferases adopt two different folds, named GT-A and GT-B (Bourne and Henrissat 2001; Breton et al. 2001). The GT-A includes diverse families of glycosyl transferases that folds into two closely neighboring β/α/β Rossmann domains, one for nucleotide binding and the other for the binding of the acceptor molecule (Charnock and Davies 1999).

The GT-B fold is present in other families (GT-3 and GT-5 families) including about glycogen or starch synthases from different organisms (Gomez-Casati et al. 2013). The first protein whose structure was described in this group of GTs was the glycogen synthase from *Agrobacterium tumefaciens* (Buschiazzo et al. 2004). It was reported that the overall fold and the architecture of the active site of the protein are remarkably similar to those of glycogen phosphorylase (Buschiazzo et al. 2004). GTB proteins have distinct N- and C-terminal β/α/β Rossmann domains that face each other and have high structural homology despite minimal sequence homology. The large cleft that separates the two domains includes the catalytic center and permits a high degree of flexibility (Lairson et al. 2008).

In *Arabidopsis thaliana*, those GT-A domains are associated mainly with the starch synthesis Glc1P adenylyltransferase enzymes (APL2, APL3, GLGL, ADG2, APS1, and ADG2). Some of the mentioned GT-A present a left-handed parallel beta-helix (LbetaH or LbH) domain, which is related with enzymes showing as diverse activity as acyltransferase, ion transport, or translation initiation (Raetz and Roderick 1995). In addition, the GT-B domains are associated with both degradative enzymes such as α-glucan phosphorylase (PHS1 and PHS2) and synthesizing enzymes such as starch synthases (SS1, SS2, SS3, SS4, and GBSS1). Interestingly, the *Arabidopsis* SS3 protein presents a domain that resembles the mitochondrial ribosomal protein subunit L20 (MRP-L20) of fungi (Garcia-Cantalejo et al. 1994), but has no assigned function in the *A. thaliana* enzyme.

The PTPc domain superfamily is defined by a dual-specificity phosphatase protein that catalyzes the dephosphorylation of phosphotyrosine peptides and oligosaccharides. This domain is present in *A. thaliana* DSP4 (SEX4), LSF1, and LSF2 and mediates the reversible phosphorylation of starch at the C6 and C3 positions of glucose moieties (Comparot-Moss et al. 2010; Meekins et al. 2014). Starch phosphorylation is the only known natural modification and is the key regulatory mechanism controlling its diurnal breakdown in plant leaves (Meekins et al. 2014). The PDZ domain is present in LSF1 and it is believed that LSF1 may be

responsible for specific protein–protein interactions through this N-terminal beta-strand structure (Harris and Lim 2001).

The phosphoglucan, water dikinase (PWD1) and the α-glucan, water dikinase (GWD1, GWD2) proteins share a domain classified in the PPDK_N superfamily which is mainly characterized by enzymes showing a pyruvate phosphate dikinase, a PEP/pyruvate binding, and a reversible conversion of ATP to AMP activity (Herzberg et al. 1996). However, in these enzymes this domain are implicated in the phosphorylation of starch polymers, being the first step on the pathway of starch degradation in Arabidopsis leaves at night (Skeffington et al. 2014).

Phosphoglucomutase enzymes play an important and diverse role in carbohydrate metabolism in organisms from bacteria to humans. The *A. thaliana* phosphoglucomutase PGMC, PGMC, and PGMP (Table 6) have a phosphohexomutase domain (superfamily) that catalyze a reversible intramolecular phosphoryl transfer of Glc1P and Glc6P via a Glc1,6bisP intermediate (Levin et al. 1999).

Finally, the SBE2.1 and SS4 strikingly present some unusual domains, such as RAP1 (rhoptry-associated protein 1) (RAP-1) and a tropomyosin_1, respectively. The first domain belongs to a family that consists of several rhoptry (parasite secretory organelle)-associated proteins specific to *Plasmodium falciparum* (Moreno et al. 2001), and the second domain (tropomyosin_1) resembles to a tropomyosin-like protein.

Because the starch granule structure exposes very few chain ends, starch-associated enzymes must have a "processive" or "multiple-attack" activity that can perform many hydrolytic events without releasing the polysaccharide chain (Henrissat and Davies 1997). Some proteins are equipped with carbohydrate-binding modules (CBMs), which are able to fulfill this task. A CBM is defined as the contiguous amino acid sequence in an active enzyme involved in carbohydrate metabolism, with the ability to bind carbohydrates (Shoseyov et al. 2006). CBMs contain between 30 and 200 amino acids and may exist as simple, double, or triple repeats in a protein. These domains are located at the N- or C-terminal end of a protein and occasionally can be found in the middle of the polypeptide chain. Currently, more than 300 putative sequences in over 50 different species have been identified and the binding domains have been classified in 71 families, based on their amino acid sequence, substrate binding specificity, and structure (see CAZY http://www.cazy.org) (Boraston et al. 2004; Lombard et al. 2014). By driving the catalytic process through a strong physical association with substrates, CBMs can increase the rate of enzymatic reactions. When CBMs are present in proteins with no hydrolytic activity, they constitute an organizing subunit of catalytic domains generating cohesive multienzymatic complexes that lose enzymatic activity when CBMs are removed from the structure (Shoseyov et al. 2006).

Based on the CAZy classification, we found three carbohydrate-binding modules families, CBM53, CBM20, and CBM48 (E_set). CBM53 and CBM20 which show a starch-binding function (Mikami et al. 1999; Valdez et al. 2008). The CBM48 family is often found in enzymes containing glycosyl hydrolase, family 13, catalytic domains and is mainly described in enzymes that degrade branched substrates as glycogen (Katsuya et al. 1998; Hudson et al. 2003).

Among the CBMs, we can highlight the starch-binding domains (SBD), which have acquired the evolutionary advantage to break the structure of their substrate when compared to the CBD, due to the presence of two binding sites for the polysaccharide (Southall et al. 1999). So far, no CBM 3D structure containing such binding sites has been reported. In view of this, such domains are unable to break down the polysaccharide structure as efficiently as the SBD do (Tormo et al. 1996; Southall et al. 1999).

While SSIII has three in tandem CBM in its N-terminal domain that belongs to the CBM53 family, the degradative enzymes (LSF1, SEX4, as well as the proteins encoded by At5g39790 and At1g27070) have only one CBM in their C-terminal domain belonging to the CBM48 family. It is worth mentioning that there is a clear evolutionary relatedness of CBM48, CBM53, and CBM20. Moreover, the alignment of the amino acid sequences of CBM20 (including some mammalian proteins, such as laforin, involved in the regulation of glycogen metabolism), CBM21, CBM48, and CBM53 has revealed only subtle differences in the polysaccharide-binding sites, showing a high degree of conservation (Christiansen et al. 2009).

6 Concluding Remarks

Starch is the most important higher plant storage carbohydrate and is made up of the glucose long chains amylose and amylopectin. Plants use starch as an energy source during the night, when leaves cannot generate sugars by photosynthesis. The polysaccharide forms complex semicrystalline structures called starch granules which accumulate within the plastids. Its metabolism involves the concerted and controlled actions of many enzymes. In most cases, multiple genes encode different isoforms of each enzyme, which may have slightly different roles depending on plant species and tissue (Kotting et al. 2010).

We have attempted a comprehensive review of the existing information on the different enzymes involved in *Arabidopsis thaliana* and other species of agronomic interest. From our point of view, to address biotechnologically starch metabolism is essential to expand the knowledge of the enzymes involved in this process. Tables 2, 3, 4, and 5 brought together the main works on this subject, describing not only the native proteins but also the plant phenotypes obtained by overexpression or deletion of specific genes. Furthermore, previous work was done in vivo where different groups have obtained modified starches (quantity and quality) through the action of different carbohydrate-binding modules of degradative microbial enzymes. Indeed, we also described the existence of such modules in some starch biosynthetic enzymes in plants. Therefore, and considering the modular structure of many enzymes involved in the metabolism of this polysaccharide, we have a section dedicated to review this point.

Recent work has focused on the role of starch as a carbon storage polymer without demonstrated regulatory activities. It is more likely that regulators of starch metabolism or signals derived from starch act as integrators of plant metabolism and growth. Gibon et al. (2009) have demonstrated a strong correlation between

starch turnover, protein content, and biomass when *Arabidopsis thaliana* Col0 plants were grown at different photoperiods (Gibon et al. 2009). Although the biomass was only very weakly correlated with individual metabolites in a recombinant Arabidopsis population, a highly significant prediction was obtained when multivariate analysis was used on the entire metabolite profile. Meyer et al. (2007) confirmed the negative link between biomass and starch (except in very short days). The simplest explanation for this negative relationship would be that large accessions maximize growth at the expense of their carbon reserves. Such a strategy would be an advantage when an excess of carbon is available, but not when carbon is in small amounts (Sulpice et al. 2009).

Moreover, starch represents an important nutrient for humans and animals and a raw material for many industrial applications. For the industry, starch represents a cheap and renewable material, with differential physicochemical properties that make it increasingly exploited in the agricultural and food sectors and in manufacturing processes (Blennow et al. 2002; Delcour et al. 2010). Starch is also used as a feedstock for first generation bioethanol production (e.g., corn, sugar cane, and cassava). The use of major food crops for energy purposes has led to the study of their applications and potential applications to obtain energy from cellulose present in plant cell walls, in addition to the study of different strategies to increase the levels of starch in plants as well as the production of starches with new attributes, in accordance with the present industrial requirements (Santelia and Zeeman 2011).

Finally, the importance of a variety of renewable biofuels has been renovated due to the increase of petroleum fuel costs and the consequences resulting from greenhouse emissions (Cheng et al. 2011). This has enhanced the interest in the study of photosynthetic algae (microalgae and macroalgae) as a possible biofuel resource because some algae species have a higher rate of biomass production compared to terrestrial plants (Dismukes et al. 2008). Graham et al. (2000) have postulated that land plants evolved from green algae belonging to the Charophyceae (Graham et al. 2000). Starch biosynthesis is unique to the Archaeplastida supergroup, comprising Chloroplastida (green algae and land plants), Rhodophyceae (red algae), and a minor group called the glaucophytes. Differences in the starch biosynthetic pathways between the archaeplastidal lineages have arisen during subsequent evolution whose starting point is the ancestral bacteria capable of synthesizing glycogen (Zeeman et al. 2010). Most notably, in green plants, starch is synthesized in the plastid compartment, whereas in red algae and in glaucophytes, its synthesis occurs in the cytosol (Shimonaga et al. 2008). Plant genomes are usually large and complex, having gene redundancy, duplications, and transposable elements among other features (Derelle et al. 2006; Armisen et al. 2008; Plancke et al. 2008). Interestingly, in the last years, several nuclear and organelle algae genomes have been sequenced (Busi et al. 2014). Because these genomes have proven to be simple, and due to the genomic, molecular, and physiological characteristics of these organisms, the unicellular green algae have become highly suitable for the study of numerous biological processes related with starch metabolism, and thus, these studies could help clarify the unknown mechanisms of starch synthesis in higher plants.

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Fungal Cultivation and Production of Polysaccharides

12

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Contents

Abstract

Many species of higher basidiomycetes have traditionally been used because of their medicinal properties. The positive effects associated to the consumption of those fungi have been mainly attributed to cell wall polysaccharides, which have important structural roles and are present throughout the entire life cycles of fungi.

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One of the most consumed and studied species native of the Americas is *Agaricus subrufescens*, a mushroom prescribed in different countries for prophylaxis and noninvasive treatment of numerous health-related disorders. Prior to the process of extraction, purification, and application of these polysaccharides, one needs to be concerned with the preservation of the specimen and production of fungal biomass. Even though basidiomata (syn. fruiting bodies, mushrooms) generally yield larger volumes of biomass when compared to the mycelium, cultivation of mycelium allows a more efficient control of the process and, therefore, is the method of choice of polysaccharide production. Mycelial biomass can be produced by solid-state fermentation (SSF) or submersed fermentation (SmF). Further separation and concentration of bioactive polysaccharides can be done by means of porous membranes, such as tangential flow nanofiltration.

Keywords
Agaricus subrufescens • Biological activity • Fungal biomass • Solid-state fermentation (SSF) • Submersed fermentation (SmF) • Nanofiltration • Polysaccharides

1 Introduction

For millennia, many species of higher basidiomycetes have been traditionally cultivated and used as sources of healthy food and medicines. The interest upon products obtained from fungi has increased during the recent years. Among the most noticeable, nutraceuticals, enzymes, and natural drugs occupy a privileged position. The active constituents found in fungi are polysaccharides, dietary fibers, oligosaccharides, triterpenoids, peptides and proteins, alcohols and phenols, and mineral elements such as zinc, copper, iodine, selenium, and iron, in addition to vitamins and amino acids (Lakhanpal and Rana 2005).

Polysaccharides are one of the main components of fungal cell walls. They have important structural roles and are present throughout the entire life cycles of fungi, commonly displaying different structural arrangements according to the fungal developmental stage. These compounds are structurally oriented, mainly by β-D-glucose monomers with high molar mass. Furthermore, different cross-linkages between the monomers from the main chain and its lateral branching give structural characteristics and specifications to these polysaccharides that have unique biological activities, which are normally species related (Cleary et al. 1999; Brown and Gordon 2001; Shu et al. 2003; Mantovani et al. 2008).

The term "nutraceutical" has been used to describe all components that can considerably act as potential dietary supplements for the prevention and treatment of various human diseases, without the troublesome side effects that frequently accompany treatments involving synthetic drugs. As such, a mushroom nutraceutical is a refined/partially defined extractive from either mycelium or fruiting body, which is consumed in a capsule or tablet form, as a dietary supplement (not as a

regular food) with potential therapeutic applications (Chang and Buswell 1996). Regular intake of nutraceuticals may enhance the immune response of the human body, thereby increasing resistance to diseases and in some cases causing regression of a disease state. This characteristic of mushrooms has been related mainly to the active polysaccharides that are known to modulate the function of the immune system, denominated immunomodulators.

Fungal species with medicinal value or nutraceutical potential include *Agaricus subrufescens*, *Cordyceps militaris*, *Ganoderma lucidum*, *Grifola frondosa*, *Hericium erinaceus*, *Lentinus edodes*, and *Pleurotus ostreatus* (Lakhanpal and Rana 2005). *Agaricus subrufescens* Peck (syn. *A. brasiliensis*) is a mushroom native of the Americas and has been prescribed in different countries for prophylaxis and noninvasive treatment of numerous pathological conditions. These include cancer, diabetes, arteriosclerosis, viruses, and other infectious diseases. Its wide range of medicinal applications is consensually attributed to the immunopotentiating effects of noncellulosic polysaccharide compounds, which are macromolecular constituents of the fungal cell wall isolated from both basidiocarps and mycelial fractions. A substantial body of experimental evidence has demonstrated an intrinsic relationship between the bioactivity of *A. subrufescens* polysaccharides and their structural features, such as the degree of β-glycosidic branches, the architecture of side chain groups, and chiefly the molecular weight, although their biochemical and functional mechanisms remain to be fully elucidated (Largeteau et al. 2011; Silveira et al. 2012). Several trademarks, patents, and publications have reported the medicinal properties of polysaccharides extracted from *A. subrufescens* and their pharmaceutical products. Among them are the cosmeceuticals, well documented and proved effective against various dermatologic disorders, which are cosmetics with biological properties used for the nourishment and improvement of the skin condition (Izawa and Inoue 2004; Uchiyama and Haramaki 2004; Kozuka et al. 2005; Hyde et al. 2010). In addition, polysaccharides from *A. subrufescens* are also important to activate keratinocytes, which are important for skin regeneration (Sugita et al. 2008).

Even though fungi (especially reproductive structures) may have all this potential, prior to the process of extraction, purification, and application of these structural polysaccharides, one needs to be concerned with the production of fungal biomass, from which the compounds can be eventually extracted. The traditional way to obtain the basidiocarps involves complex pre-cultivation stages that include selecting the right substrate for the right fungus, substrate composting, and the inoculum production. During the development of the fungus in this non-axenic substrate (colonization), contamination can take place. Aside from this problem, the process lags for a long period. Furthermore, it generally discards an important portion of the fungal biomass after basidiomata (syn. fruiting bodies, mushrooms) harvesting, the spent mushroom substrate, which is the mycelium that has colonized the substrate (Oei 1996; Eira 2003).

In that respect, several research groups, including Brazilian-based ones, have focused on improving the methods for mushroom cultivation. These both employ the traditional composting methodology, largely used for species of *Agaricus*, and

diversify existing techniques, utilizing methodologies such as Jun Cao (Urben et al. 2004; Mendonça et al. 2005; Colauto and Linde 2012a; Figueirêdo et al. 2013). Due to the potentially high fungal diversity, searches for new native species from different Brazilian regions, selecting and genetically modifying species, as well as focusing on strategies for biodiversity conservation, have also been a keen condition (Neves et al. 2005; Colauto and Linde 2012b; Sales-Campos et al. 2013). On another ground, the spent mushroom substrate has been evaluated as source of energy, enzymes for industrial applications, animal feed, and fertilizers and soil detoxifiers (Ribas et al. 2009; Gonçalves et al. 2010; Camassola et al. 2013).

In this context, studies involving the Brazilian native species *A. subrufescens* (strain UFSC 51, CBMAI 1449) have been broadly explored for fungal biomass production, both aiming at basidiocarps and mycelia. These studies have focused on determining the functional polysaccharide contents of the species, with nutritional and medicinal applications (Camelini et al. 2005; Angeli et al. 2009b; Cardozo et al. 2011, 2013a, b). For this purpose, the best cultivation conditions, both in solid and liquid substrates, as well as aspects related to the separation and purification of biotechnological active polysaccharides have been explored (Silveira et al. 2012; Camelini et al. 2013a, b).

Shorter cycles during fungal cultivation allow for large biomass amounts, which consequently increases polysaccharide production. Scale-up from laboratory to industry is common for microorganisms used for the production of antibiotics, enzymes, hormones, and antibodies and for plant cell micropropagation (Hölker et al. 2004). For the cultivation is fundamental to establish an ideal method for the preservation of the microorganism, maintaining its reproductive capabilities and a high productivity for both the biomass and the compound of interest.

Mycelial preservation allows the employment of the microorganism for biotechnological purposes over time. Based on the method of choice, risks of genetic modifications are considerably low during preservation, assuring, therefore, the physiological identity of the microorganism. Several methods are available for in vitro preservation of microorganisms, ranging from simple, such as water or mineral oil, to complex ones, such as cryopreservation in −196 °C liquid nitrogen. Even the simpler methods yield good results, especially when the viability and the genetic identity of the microorganism are periodically checked (Richter and Bruhn 1989; Croan et al. 1999).

Once the organism is preserved, it can be used for the production of biomass in axenic substrates that can be either solid or liquid, denominated solid-state fermentation (SSF) and submersed fermentation (SmF), respectively. These processes are broadly employed in the industrial production of fungal inoculants for mushroom cultivation; production of flavoring agents, polysaccharides, vitamins, enzymes, organic acids, and antibiotics; or consumption as fungal biomass in the mycelial form (Hölker et al. 2004; Couto and Sanromán 2006).

The SSF presents some advantages over the SmF method. Among them, the low water requirements during cultivation and the lack of dependency of a solid support for the development of the fungi are the most important. However, one

disadvantage is the difficulty to quantify and separate the fungal biomass and to extract only the polysaccharide of interest. Subsequently, this method is more appropriate for the obtainment of fungal biomass to be used in whole in the food industry (Hölker et al. 2004; Couto and Sanromán 2006).

Nevertheless, the SmF, a method of remarkable expansion over the last decades, allows for a more precise control of the process and less risk of contamination, aside from producing a "cleaner" mycelium. This more "pure" mycelium comes from a biomass that is easily separated from the substrate. Cleaner mycelium yields polysaccharides that are easier to isolate, allowing for singular applications, such as pharmaceutical formulations (Ito and Sumiya 2000; Hölker et al. 2004; Rossi et al. 2004).

The choice for this or that method is dependent upon several factors. Among them are those of economic importance, those related to the quality of the final product, and those concerned with the particular governmental regulations regarding the disposal of the solid or the liquid residual substrates (Hölker et al. 2004). The biological activity of the resulting product is also of concern when choosing the method. Some substrates may modify the chemical structure of the mycelial polysaccharides, altering its main chain, lateral chains, or the degree to which the branching is arranged and, therefore, may impair the molecule effectiveness (Wasser and Weis 1999; Silva et al. 2006; Mantovani et al. 2008; Lavi et al. 2010).

Therefore, in the face of the factors exposed above, the potentiality of polysaccharides produced and extracted from *A. subrufescens* for the production of nutraceuticals at a commercial level is dependent upon a series of events. These events, which need to be fully understood for the success of the enterprise, include the fungal preservation, the maximization of biomass production, and the establishment of a process that allows for an adequate and reliable polysaccharide obtainment. These are sine qua non conditions for the advancement of this field allowing for the viability of preclinical and clinical studies involving these molecules.

2 Preservation of Fungal Material

Preservation of microorganisms is important for research and industrial applications, especially due to the active biological substances they may produce. By preserving, one may also be maintaining the genetic and ecological functions of these organisms (Cameotra 2007).

In order to maintain the on-growing needs for fungal material that has medicinal applications, determining the best conditions for preserving mycelia is among the main interests for those cultivating *A. subrufescens* or other important fungal species. For many fungal species, mineral oil and distilled water are often used for short-term preservation periods (Richter and Bruhn 1989; Croan et al. 1999). On the other hand, lyophilization and cryopreservation in liquid nitrogen, which arrest growth and may compromise some metabolic processes, represent long-term preservation techniques (Croan et al. 1999; Colauto et al. 2012a, b).

Two of the main challenges in maintaining cultures of *A. subrufescens* are its low culture viability and the frequent changes in growth habits, mainly after subcultures (Neves et al. 2005). In addition, some techniques may not present desirable results, as is the case for lyophilization that proved to be inefficient for *A. subrufescens*, even when 10 % trehalose and 10 % skimmed milk are used.

Colauto et al. (2012a) showed that strains of *A. subrufescens* respond differently to cryopreservation with DMSO. Recoveries of near 40 % have been obtained for isolates maintained frozen at /196 °C in DMSO for about 1.5 years. The variation in the recovery percentage indicates that this species is sensitive to the formation of intracellular ice crystals or even to the quick expansion of the cellular volume. However, a long-term cryopreservation (4 years) was effective when sucrose or glucose was used as cryoprotectant, regardless of the freezing protocol (Colauto et al. 2012b). Therefore, the use of cryoprotectants that penetrate only the cellular wall as mono- and disaccharides is a potential candidate for cryopreservation of other basidiomycetes.

Another important aspect that needs to be taken into account is that many fungi are able to produce secondary metabolites, such as agaritine. This metabolite is particularly produced by species of *Agaricus* when growth is ceased or any growing impediment is imposed (Kondo et al. 2006). When such fungi are grown in culture conditions, these metabolites are introduced into the media. Agaritine, in turn, has a mutagenic activity and may be harmful to the species (Walton et al. 1997). Therefore, methods for the attenuation of the effects of such compounds are necessary. Activated charcoal (AC) has proven to be very helpful for the removal of several toxic compounds in hemicellulosic hydrolysates used for the cultivation of fungi (Mussatto and Roberto 2004; Chandel et al. 2007), and it is used in tissue culture to improve cell growth in plants cultivated in vitro (Teng 1997). AC should also provide proper conditions for the preservation and evaluation of several microbial characteristics in culture (Hays et al. 2005).

During a particular preservation period, it is important to investigate both the viability and the morphological characteristics of the mycelium (Clark and Anderson 2004; Marín et al. 2008). It is crucial to observe the genetic stability of the preserved mycelium. The polymerase chain reaction (PCR) and other protocols for DNA analysis, such as random amplified polymorphic DNA (RAPD) markers, have become important tools in evaluating the intraspecific variability in microorganisms (Atienzar et al. 2002; Neves et al. 2005) and in detecting DNA damages and mutations (Lee et al. 2000).

Agaricus subrufescens shows a reduction in mycelial growth after subculture, with difficulties for the recovery of the original state. In that respect, Camelini et al. (2012) have explored preservation and recovery techniques for its maintenance. Mycelium on solid media supplemented with activated charcoal was compared with culture under mineral oil and mycelial disks in sterile water, all intended for long-term storages at 8 °C. Mycelium viability, radial growth rate, and genetic stability (by RAPD analysis) were continuously evaluated for 12 months. The use of AC showed a 75 % viability in the initial and final periods. On the other hand, mineral oil showed a 50 % reduction in viability toward the end of the storage

period, while sterile water differed from the others insofar as viability was high until the 6th month, with a 50 % drop after the 10th month, and a complete loss of viability after the 12th month of storage. Overall, throughout the storage period, radial mycelium growth rate decreased. However, recovery results were improved when the media were supplemented with AC. RAPD profiles demonstrated high genetic homogeneity of the mycelia maintained under all methods (>99 %). The use of PDA in slant tubes supplemented with AC was the most efficient method for the preservation of *A. subrufescens* under cold storage and resulted in the longest period of successful storage (12 months). Furthermore, AC is important for further recovery of *A. subrufescens* without significant morphological and genetic changes.

Activated charcoal has been used to adsorb toxic compounds in fermentation media prepared from hemicellulosic hydrolysates used in the cultivation of fungal species (Mussatto and Roberto 2004; Chandel et al. 2007). AC has also been proved an efficient additive in the preservation of fungi in vitro. *Agaricus subrufescens* is the only species of *Agaricus* either harmed or killed by prolonged exposure to temperatures of 4 °C or lower (Wasser et al. 2002; Kerrigan 2005). Therefore, the employment of AC to detoxify secondary metabolites produced by fungi at lower temperatures has proven to be an important procedure for the preservation of fungi up to 12 months. Nevertheless, the main benefit may be related to the recovery of such fungi after exposure to preservation procedures.

The mycelium disks of *A. subrufescens* preserved under sterile water lost complete viability after 1 year (Camelini et al. 2012). However, Richter and Bruhn (1989) reported preservation success for up to 48 months at 5 °C for several basidiomycete species. Others were maintained for shorter periods, with a viability kept for at least 9 months. Smith et al. (1994) reported a viability of 95 % for 169 fungi maintained for 20 months under preservation. Richter (2008), on the other hand, showed an 88 % viability for 34 fungal cultures maintained for 20 years in sterile water.

An important constatation of the recovered mycelia of *A. subrufescens* maintained under all preservation techniques is the deformity of the colonies (non-radial growth, zoned, cottony like, etc.). These deformities, however, were not observed when the media for fungal recovery were supplemented with AC (Camelini et al. 2012). The lack of deformities and overall characteristics of the mycelia was also reported by Wasser et al. (2002) when cultivating the fungus in the absence of AC. Teng (1997) reported that the incorporation of AC in leaf cell suspension cultures increased the number of regenerated sporophytes, even in media free of growth regulators, preventing abnormal morphogenesis and, therefore, normalizing cell development.

Most fungi can be stored at temperatures between 4 °C and 12 °C and subcultured at intervals of 6–8 months. This procedure can induce senescence in fungi, i.e., a progressive loss of mycelial growth potential, culminating in mutation and complete interruption of growth, leading to fungal death (Maheshwari and Navaraj 2008).

DNA mutations in fungi can be induced by radiation (Lee et al. 2000), genotoxins (Delmanto et al. 2001), preservation (Clark and Anderson 2004), and

senescence (Maheshwari and Navaraj 2008). Morphological mutations can be detected in the dikaryotic phase, as the one found in *L. edodes* by Shimomura and Hasebe (2006). The mutant dikaryon was readily distinguishable from the normal dikaryon by its irregularly branched, short, and sparse aerial hyphae, together with a drastically slower growth.

Camelini et al. (2012) showed deformed sectional colonies and slow mycelial growth for *A. subrufescens* recovered in media without AC, independent of the method of preservation. However, the RAPD analysis showed high genetic similarity on the mycelium collected at the 4th, 8th, and 12th month for all techniques of preservation and recovery when compared to the mycelium before preservation. The greater genetic similarity of the preserved mycelium increased the homogeneity of the isolated cultures. Even though the low variability is not desirable for genetic diversity (Llarena-Hernandez et al. 2013), it is necessary for the quality control during the cultivation of *A. subrufescens* to produce polysaccharides with biotechnological industrial applications. On the other hand, the genetic distance between fruiting bodies and mycelium observed for most basidiomycetes is possibly due to the differentiation of reproductive and vegetative phases in the life cycles of these fungi. The dikaryon is the predominant vegetative structure that, under appropriate conditions, is responsible for meiosis and, therefore, mushroom formation. In the dikaryon state, the two haploid gametic types of nuclei are maintained indefinitely in paired association. A potential complication with the long-term culture of dikaryons in vegetative phase is the possibility of genetic exchange between the paired haploid nuclei. In these events, a dominant deleterious mutation in one nucleus is followed by a compensatory mutation in the other, resulting in an increasing linear growth rate of the mycelium (Kamada et al. 1993; Clark and Anderson 2004; Torralba et al. 2004).

The RAPD technique has been largely used to detect the genetic variability of pathogenic and nonpathogenic microorganisms (Steindel et al. 1993; Oborník et al. 2000; Neves et al. 2005; Gasser 2006). This method is cheap, simple to perform, and suitable to detect DNA mutations, which can be important for evaluating the genetic stability in microorganisms during in vitro storage (Mariano et al. 2007). The use of AC for the preservation and recovery of *A. subrufescens* is easy, quick, and inexpensive and does not result in significant morphological and genetic changes in the mycelium stored for periods of up to 1 year.

3 Polysaccharide Production by Solid-State Fermentation

The interest over different products obtained from microorganisms has recently increased. Among the products of significance, many fungal sub- or by-products are listed, such as enzymes, natural drugs, and nutraceuticals (Moore and Chiu 2001; Couto and Sanromán 2005; Ghorai et al. 2009; Baños et al. 2009). These additives can be obtained by either specific extraction or concentration, used primarily by the industrialized sector, or by the utilization of whole organisms or parts of their recovered biomass.

Two main processes are generally employed for the production of fungal biomass: solid-state fermentation (SSF) and submersed fermentation (SmF). For many of the biotechnological fungal products of interest, such as enzymes and secondary metabolites, SSF has been chosen (Baños et al. 2009; Saqib et al. 2010). This substrate promotes best growing conditions for the fungi as well as specific stability for the compounds they produce (Couto and Sanromán 2006; Singhania et al. 2009). Moreover, the SSF processes are cheaper, especially when the main substrate employed is composed of raw materials (Castilho et al. 2000).

For the production of edible mushrooms, which more often have been used as nutraceuticals, the substrate of choice is that of a mix of lignocellulolytic wastes, such as straw and bran, which may have passed through a composting process prior to use. Both stages are SSF based. This methodology usually presupposes long times before the fruiting bodies are obtained, considerably increasing the costs of production (Scrase 1995; Stamets 2000), and is dependent on a trustful and reliable source of spawn.

The SSF system is also employed to produce spawn on sterilized cereal grains, which in turn are used as inoculum for the production of edible and medicinal mushroom species (Scrase 1995; Smith et al. 2002). SSF is generally the method of choice for the production of spawn around the world. This is also true for *A. subrufescens* (Stamets 2000; Mendonça et al. 2005). Spawn can be produced on many different cereal grains such as rye, wheat, and rice. The rehydration of the grains is crucial for the production of spawn. Several methods can be employed to rehydrate the grains, and each will depend on the type of grain and the final desirable moisture. The grain should normally have around 45–50 % moisture content, depending on the type of grain. Higher moisture contents can potentially break the integrity of the grain cell walls, altering the fungus metabolic capabilities and increasing the risks for contamination (Oei 1996).

Inoculation of the substrate can be done by placing the mycelium (in agar) together with the grains. This substrate is then incubated observing the fungus temperature requirements. After the mycelium has grown and colonized the grains (after approximately 14 days), this mother spawn can be used to inoculate new fresh grains, known as the grain-to-grain transfer technique. For some fungi, the addition of gypsum ($CaSO_4$) and limestone ($CaCO_3$), generally on a 1:4 ratio, is suggested to have positive effects, both on the structure of the substrate and on the substrate pH stability (Scrase 1995; Oei 1996; Stamets 2000).

Even though the production of spawn is the main purpose for this application, the fungal biomass could be alternatively processed and consumed as nutraceutical, such as in the elaboration of foods obtained through biotechnological processes. The bioconversion of grain into fungal biomass is important to improve the grain nutritional quality. This bioconversion increases the grain protein contents (all the essential amino acids), the types of polysaccharides produced (glucans, chitin, etc.), vitamins, among others nutritional aspects (Moore and Chiu 2001).

One good example of this biological transformation relates to *A. subrufescens*, a species that can successfully grow on cereal grains and is an important medicinal

fungal species with confirmed biological activities. This biological activity is attributed to cell wall polysaccharides, determined by Mizuno et al. (1999) and Camelini et al. (2013a) to be glucomannans with a main chain of β-(1 → 2)-mannans and β-(1 → 3)-glucan side branches. This fungus has been properly adapted to grow in liquid substrates, with several studies proposing its experimental optimization in SmF for the production of mycelial biomass and exopolysaccharides (Lin and Yang 2006; Fan et al. 2007; Hamedi et al. 2007). Examples of experimental optimization for several fungal species in SSF for the production of industrially important products have been proposed (Prakash et al. 2008; Baños et al. 2009). However, for *A. subrufescens*, few are the citations that report the use of SSF, due probably to the barriers inherent to this methodology (Dalla-Santa et al. 2011; Camelini et al. 2013a).

Biomass separation is one of these impediments. For most fungi, it definitely represents a challenge in SSF. Biomass separation is essential for kinetic studies of fungi (Singhania et al. 2009). Some indirect methods have been proposed to estimate biomass, such as glucosamine, ergosterol, protein, and DNA contents, as well as dry weight and changes in CO_2 evolution (Ooijkaas et al. 1998). Ergosterol, a compound well recognized for its biological activity, has been used to estimate fungal biomass percentages in SSF and proved suitable to determine the mycelial growth parameters of *A. subrufescens* (Dalla-Santa et al. 2011). All of these techniques, however, are subject to their own limitations.

Some methodologies have been proposed for polysaccharide quantification, such as high-performance liquid chromatography with refractive index detection (HPLC-RI). Examples that use this technique include the work of Nie et al. (2005), who used purified tea glycoprotein as calibration standards for the determination and comparison of glycoprotein in tea samples collected from different species, varieties, and geographic locations. Vendrell-Pascuas et al. (2000) developed a method for the quantitative determination of inulin in meat processed products containing this type of additive. This method extracted the inulin with hot water, followed by a hydrolysis with inulinase and the determination of released fructose by HPLC-RI.

Following the same trend, Camelini et al. (2013a) proposed HPLC-RI with a gel permeation column to quantify the main polysaccharide of *A. subrufescens* on SSF. Those authors have shown that polysaccharide molecules from *A. subrufescens* have around 310 kDa, representing the highest-molecular-weight polysaccharide obtained from the mycelium of that fungus. In addition to the main polysaccharide, a 25 kDa molecule has been detected. This structure was successfully removed by an α-amylase, suggesting the presence of a starch fraction on the extract, interfering significantly with the sample quantification. The purified polysaccharide used as standard showed a symmetric single peak (310 kDa), suggesting that the polymer is homogeneous. The main feature of the gel column for the analysis of polysaccharides lies in that polysaccharide and its degradation products can be determined simultaneously. The calibration curve was linear over the standard polysaccharide concentration ranging from 0.187 to 3 mg/mL, with an R^2 of 0.9997, after an analytical validation of the method.

With the objective of increasing fungal polysaccharide contents, SSF has been employed for the production of nutraceuticals by the fungus *A. subrufescens* on pretreated wheat grains (Camelini et al. 2013a). Among the important factors evaluated, time of cooking, time of resting after grain cooking, consequently grain moisture, substrate pH, temperature of incubation, and initial inoculum amount are the most significant.

Wheat grains provide essential nutrients used for fungal development, such as carbon and nitrogen. According to Van der Borght et al. (2005), whole wheat grain can be a good source of soluble starch and other polysaccharides, such as arabinoxylans, glucans, cellulose, and arabinogalactan peptides, proteins (gluten and non-gluten), and lipids. Soluble starch has also been suggested as a good carbon source for biomass and exopolysaccharide production by *A. subrufescens* in SmF (Hamedi et al. 2007).

The quantity of water in a SSF must be sufficient for fungal growth and enough for the diffusion of solutes, gas, and nutrients (Gervais and Molin 2003). Camelini et al. (2013a) report that grain cooking and resting time have significant effects on the grain moisture content and on the levels of glucomannan produced by *A. subrufescens*. High moisture contents tend to increase glucomannan production. The maximum average production was achieved when the grains had moisture close to 46 %. However, the highest polysaccharide production was not observed at the highest moisture content. Wheat grains generally open at moisture contents higher than 50 %, breaking the integrity of the grain cell walls and decreasing fungal polysaccharide production. Another factor contributing significantly with the glucomannan production is the concentration of calcium sulfate and calcium carbonate. These compounds modulate the pH, showing that high concentrations had negative effects on the production of such polysaccharides.

The addition of calcium sulfate and calcium carbonate directly on the grain surface is frequently used as a regular practice in spawn production (Scrase 1995; Oei 1996). Calcium is also used as a supplement in other solid substrates for mushroom production (Royse and Sanchez-Vazquez 2003). Royse and Sanchez-Vazquez (2003) have shown $CaCO_3$ to have great and $CaSO_4$ to have average to low modulation effects on the substrate pH. However, Camelini et al. (2013a) found that both sources of calcium modulate the pH as well as the glucomannan production in fungi: $CaSO_4$ acted decreasing the pH, while $CaCO_3$ resulted in higher pH levels. Grains without any calcium source had an initial pH of 6.4, nearly the same as observed for grains supplemented with 1.5 % $CaSO_4$ and 0.5 % $CaCO_3$ (pH 6.5). In general, better results for glucomannan production were obtained when no supplement was added or when up to 0.25 % $CaCO_3$ has been added to the mix. The addition of $CaCO_3$, even in small amounts such as that depicted above, is important to maintain the physical structure of the substrate (Oei 1996). Gadd (1995) showed that calcium ions play an important role in the regulation of the apical mycelia growth as well as in the formation of hyphal branches, corroborating the positive effects promoted by this micronutrient.

Lin and Yang (2006) obtained the highest mycelium yield of *A. subrufescens* in SmF with an initial pH of 6.06 and an incubation temperature of 27 °C.

Fan et al. (2007) observed that a pH of 6.1 and a temperature of 30 °C were the best setting points for increases in exopolysaccharide production. Other results show maximal biomass yield and exopolysaccharide production to happen at pH 5.6 and 6.9, with incubation temperatures of 24.6 °C and 20 °C, respectively (Hamedi et al. 2007).

The inoculum density is also important for the submersed cultivation of *A. subrufescens*, affecting both the fungal physiology and growth (Lin and Yang 2006). As for SSF, Camelini et al. (2013a) found that both the inoculum amount and incubation temperatures had significant negative effects on the production of glucomannans. The interaction between the two factors had also a significant effect on the polysaccharide estimation. The results from those authors demonstrate that the inoculum amount necessary for the best production is around 10 %, while the best temperature is around 27 °C. In this condition, the predicted value of glucomannan production would be close to 6.89 %, which is the maximal value obtained in this study. This experiment was conducted near the best moisture content (45 %) for polysaccharide production. Adequate moisture contents are important, since water mass transfer between the fungal tissue and the substrate is related to other parameters, such as temperature and aeration (Gervais and Molin 2003). The static condition adopted in these experiments has been critical for the definition of the initial inoculum amount, allowing for a fast colonization of the grains, as well as for proper oxygen availability (aerobic metabolism) and prevention of CO_2 saturation. In high moisture content, less dry weight substrate is filled into the bottles. In addition, there is less air space between the substrate particles. Ohga (1990) demonstrated that air spaces saturated with high moisture contents may slow the gas exchange from deep within the interior of the bottle toward the surface.

Respiration also produces water, in which an endothermic evaporation phenomenon will be the major energetic factor for heat dissipation (Gervais and Molin 2003). A great limitation of the SSF system is the difficulty to remove excess heat due essentially to a low thermal conductivity. Therefore, forced air is used for temperature control (Raghavarao et al. 2003). Hence, the SSF of *A. subrufescens* on static conditions should have an incubation temperature lower than that for SmF. Smaller amounts of the initial and scale-up substrates, as those used by Camelini et al. (2013a), are more easily controlled, allowing for improved determinations of the optimum temperature parameters.

Several models of bioreactors have been employed for SSF. The majority of them are stirred-tank or aerated bioreactors. Bioreactors are classified based on the type of aeration or the mixed system employed. Four basic types are known: trays, packed bed, horizontal drum, and fluidized bed (Singhania et al. 2009). These bioreactor designs have their own advantages and disadvantages. One major limitation imposed by the SSF method is the difficulty to remove extra heat generated by the metabolism of the fungus, essentially due to the low thermal conductivity presented by solid substances. Therefore, forced air through the body of the bioreactor is essential to dissipate the extra heat and allow for a proper temperature control. Another concern relates to the effect that aeration may bring to the

cultivation. Forced air reduces humidity, which, in turn, creates potential for desiccation of the biomass, with consequent loss of viability (Raghavarao et al. 2003).

Solid-state fermentation is an inexpensive process to obtain large quantities of biomass required for the production of foods containing fungal polysaccharides. Glucomannans of *A. subrufescens* in a SSF system can be consumed as whole biomass through the elaboration of functional foods. Product recovery and purification processes are, however, expensive. Hence, the application of the proposed procedure in the elaboration of functional foods will convey a considerable reduction in the production costs, allied to an increment of the ingestion of important confirmed bioactive compounds (Mizuno et al. 1999; Couto and Sanromán 2006; Singhania et al. 2009; Silveira et al. 2012).

4 Polysaccharide Production by Submersed Fermentation

Even though basidiomata generally produce larger volumes of biomass when compared to the mycelium, and consequently larger amounts of polysaccharides, cultivation of fungal mycelium allows for a more efficient control of the process and, therefore, is the method of choice (Smith et al. 2002). In that respect, alternative approaches that increase productivity, such as submersed cultivation, have been under investigation. Such methods are preferred since they allow for a more precise control of most of the fungal growing conditions, such as nutritional and physical–chemical factors, yielding uniform, potent, and standardized polysaccharide productions (Fazenda et al. 2008), mainly for the pharmaceutical area.

There are two kinds of bioreactors for cultivation under the SmF system. From the two, aerated ones are the most utilized in the industry (Crueger and Crueger 1990). In fact, the majority of the bioreactors employed under aerobic conditions in the industrial sector are conventional stirred-tank bioreactors. The remaining are those without a mechanical agitation, such as the airlift and the bubble column bioreactors (Chisti 1989). Nevertheless, stirred tanks are not the most suitable bioreactors for the cultivation of certain microorganisms. The degree of stirring required to reach the ideal mass transfer in many cases can damage the cells. Moreover, the agitation required by these bioreactors may imply high-energy consumption. Additionally, in the majority of the bioprocesses, the cultivation must remain free of contaminants for long periods, requiring the installation of complex mechanical seals. Conventional bioreactors are then more expensive and less resistant than the ones without mechanical agitation (Nienow 1998; Garcia-Ochoa and Gomez 2009; Choy et al. 2011).

In an airlift bioreactor, the volume of the liquid is divided into two different zones. Only one of these zones receives the injection of air. The difference in terms of gas holdup between the aerated (riser) and the non-aerated zones (downcomer) creates a difference in the liquid density between these two regions and, as a result, promotes the circulation of the fluid, reducing, therefore, the potential for damages to the cells. Gas holdup and the speed of circulation of the liquid phase, whose

source and values depend on the gas flow, are among the most important parameters during the operation of a bioreactor. These parameters interfere, for example, in the liquid circulation dynamics by reducing the speed of the rising air bubbles (Chisti 1989; Chisti et al. 1995). Aside from this, liquid circulation has an effect on the turbulence, which will have a counter effect on the coefficient of heat transfer, on the gas–liquid mass transfer, and on the shearing rate force to which the microorganisms are submitted (Chisti and Moo-young 1988). The small rising air bubbles increase the oxygen transference rates, and this current, created by this ascending flow, homogenizes the overall shearing rates through the entire bioreactor, stimulating, therefore, biomass production (Kunamneni et al. 2007).

On the other hand, the formation of *pellets* can be an alternative to avoid variations in the density of the liquid media during airlift cultivations, since many microorganisms produce and excrete exopolysaccharides that increase viscosity, modifying the transference rates of oxygen to the media (Prosser and Tough 1991; Rossi et al. 2002). Rossi et al. (submitted) have shown that airlift bioreactors are suited for such cultivations, since they promote an efficient development of the fungi while reducing the counter effects produced by the shearing forces observed in other bioreactors (such as stirred tanks). Airlift bioreactors are also more suitable for the cultivation of filamentous fungi since they promote sufficient mass transfer necessary for the growth of the fungi. Aside from those benefits, one can also explore the surplus of polysaccharides extruded to the liquid media by the fungi, proving to be an extra source of such bioactive molecules (Hamedi et al. 2007).

In that respect, a pneumatic 5 L airlift bioreactor, with external circulation (Fig. 1), was employed to study the behavior of *A. subrufescens* grown under the SmF system (Camelini et al. 2013b; Rossi et al. submitted). The unit was equipped with a heat exchange device located in the downcomer. Even though it is difficult to estimate the dissolved oxygen critical concentration for fungi that grow in pellets, a value of 45 % saturation was assumed for this study. Thus, the dissolved oxygen (DO) concentration was kept above the critical level throughout the entire cultivation period while operating at specific airflow rates of 0.35 vvm. Once the temperature was stabilized, the electrode was calibrated in the culture medium and a sample run performed. The isolate was cultured in modified MNM medium (Marx 1969) with 16 g/L glucose, with temperature maintained at 25 $\pm$ 1 °C, and with pH set at 5.8 and monitored. Settings were determined as to perform a batch cultivation, which was maintained until the full exhaustion of the main carbon source. Samples were taken to determine residual glucose and total biomass. The fungus formed relatively uniform, 4 mm Ø pellets reaching a final biomass concentration of 5.5 g/L with glucose biomass (gx/gs) conversion ($Y_{X/S}$) of 36 %, obtained when 0.5 g/L of initial inoculum was employed. Average cell growth-specific velocity was 0.4/day and biomass productivity around 0.76 g/(L.day). In this condition, almost the entire carbon source was depleted at the end of the experiment, suggesting that this is the factor to be utilized whenever calculations for the production planning and culture media are to be performed. Considering an initial biomass concentration of 0.5 g/L, it is possible to perform an entire process within 1 week of operation. A reduction to 5 days is seen when the initial biomass

Fig. 1 Pneumatic (5 L) airlift bioreactor (Rossi et al. submitted)

concentration is close to 1.0 g/L, which is generally difficult to achieve in the scale-up process when employing the fungal species utilized in this study.

Cultivation periods of 5–7 days are considered exceptional when dealing with fungi from the phylum *Basidiomycota*, which may take up to 2 months when growing in solid media (Fazenda et al. 2008). On the other hand, it is important to maintain the cultivation within this period, especially when processes with controlled systems are carried out. Increasing the time of cultivation will unvaryingly create conditions where the mycelium will get fixed in the bioreactor interfaces, such as the sensor's and other gadget's prominences, potentially interfering with the overall readings (Rossi et al. 2002).

For industrial purposes, the initial quantity of inoculum can be obtained either by producing it in a second bioreactor that fed a parallel cultivation or by continuous cultivation regimes. However, inoculum concentrations above 1.0 g/L, generally chosen to reduce the time of cultivation, are not recommended, since the inoculum homogenization may be compromised otherwise. Fragmentation, which is necessary to open the clusters of biomass and stimulate small fungal growing pockets, causes the intracellular material to be extruded to the growing media. These compounds can include both genetic material and metabolic intermediaries. These may suffer intrinsic reactions upon reaching the oxygen-rich environment of the medium and produce toxic substances, which invariably interfere negatively in the fungus growing kinetics and viability (Rossi et al. submitted).

Although approaches to optimize the cultivation of *A. subrufescens* in a bubble column bioreactor have been tested, such as the one proposed by Kawagoe et al. (2004), average productivity was eight times lower (0.095 g/(L.day)) than that obtained in an airlift bioreactor (0.76 g/(L.day)), even at similar airflow rates (Camelini et al. 2013b). Aside from having a superior performance over bubble column bioreactors (Chisti 1989), the results of the former study may have been impaired by the reduced quality of the initial inoculum. High performances are more likely to be reached when the fungal inocula are less than 15 days old, and fragmentation has been done in a way as to allow for minute cores to be formed. Small fragments associated to the right conditions (type of bioreactor, pH, airflow, etc.) allow for pellets that will have a final diameter of 5 mm or less, which is fundamental to avoid oxygen limitation to the fungi. The results from Kawagoe et al. (2004) corroborate the above conclusions, since they determine that higher aeration rates are essential for better fungal growth.

Aside from the productivity aspect, production costs are important components when dealing with biotechnological products. This aspect is crucial to determine the feasibility of the process and involves all types of costs, which includes operational (production), fixed, and general industrial expenses. The costs associated with the purchase of raw materials represent anything from 30 % to 80 % of the production costs. Supplies, which are not directly related to operation, account for 15 % of those costs. The funds necessary for the physical portion of the investment, which include buildings, equipment, etc., are directly dependent on the process performed, since it may require the purchase of bioreactors, centrifuges, homogenizers, and ultrafilters, among others (Kalk and Langlykke 1986). Consequently, the price of the final product will be a direct result between the conjugations of all these costs. Cost estimates are important to determine, among all available options, including SSF and SmF, which will yield the best proceeds for the money to be invested.

Examples of such calculation costs are available in the literature. For the production of lipase from *Penicillium restrictum*, Castilho et al. (2000) showed a fivefold increase in production costs when the fungus was grown on SmF, in comparison to SSF. One alternative used by the industrial sector to overcome these higher costs observed in the SmF is the use of hydrolyzed agro-industrial residues. These include sugarcane bagasse, brewery residues, and wheat bran (Pandey et al. 2000; Mussatto and Roberto 2006; Dobrev et al. 2007). Additionally, the transformation of these compounds into microbial biomass also reduces the volumes of residues passive of conversion prior to their disposal into the environment.

5 Extraction and Concentration

Most methods available today for the extraction of polysaccharides are considerably time- and energy consuming and require a large amount of organic solvents for polysaccharide precipitation, resulting in the critical problem of solvent recovery and environmental pollution. The extraction of polysaccharides has generally been done in water using different temperatures (Table 1).

Table 1 Biological activities of polysaccharides obtained from *A. subrufescens* Peck (syn. *A. brasiliensis* Wasser) and the related species *A. blazei* Murrill sensu Heinemann

Species' given name/ material analyzed	Strain/origin	Isolated polysaccharide	Isolation/extraction method	Biological activity	References
Agaricus brasiliensis fruiting bodies	Strain KA21, Brazil	1,6-β-Glucan	Extraction in hot water (121 °C) followed by ethanol precipitation	In vivo (mice) antitumoral activity (solid sarcoma 180)	Hashimoto et al. (2006)
	Not mentioned	1,6-β-Glucan	Extraction in hot water followed by ethanol precipitation and centrifugation	In vitro antigenotoxic activity on human peripheral lymphocytes	Angeli et al. (2006)
	Not mentioned	1,4-α-1,6-β-Glucan	Subsequential extractions with ethanol at boiling point, extraction in hot water (100 °C) and ammonium oxalate, dialysis	Enhances the in vivo (mice) candidacidal activity of murine macrophages	Martins et al. (2008)
	Strain UFSC 51 (mature stages), Brazil	1,6-β-Glucan with traces of 1,3- and 1,2-β-glucans	Extraction in hot water (100 °C), ethanol precipitation, dialysis, and DEAE-cellulose column chromatography	In vitro antigenotoxic activity on HepG2 cells	Angeli et al. (2009b)
	Strains ABL 99/25, 99/28, 99/29, and 00/30, Brazil	1,6-β-Glucan	Subsequential extractions with ethanol at boiling point, extraction in hot water (100 °C) and ammonium oxalate	In vivo (mice) antitumoral (Ehrlich adenocarcinoma) activity	Pinto et al. (2009)
	Brazil	1,6-α-Fucogalactan	Chloroform–methanol extraction followed by ethanol precipitation	In vivo (mice) antinociceptive activity	Komura et al. (2010)

(*continued*)

Table 1 (continued)

Species' given name/ material analyzed	Strain/origin	Isolated polysaccharide	Isolation/extraction method	Biological activity	References
	Strain M7700, the Netherlands	1,6-β-Glucan, 1,6-1,4-α-glucan, and mannogalactan	Extraction in hot water followed by ethanol precipitation	In vitro immunomodulatory activity on THP-1 cells	Smiderle et al. (2011)
	Strain KA21, Japan	1,6-β-Glucan with traces of 1,3-β-glucan	Extraction in hot water followed by ethanol precipitation	In vitro immunostimulatory activity on murine splenocytes and bone marrow-derived dendritic cells	Yamanaka et al. (2012)
	Strain UFSC 51, Brazil	1,6-1,3-β-Glucan	Extraction in hot water (100 °C), ethanol precipitation, and dialysis	In vitro cytotoxic activity on A549 cells	Cardozo et al. (2013a)
	Strain M7700 produced in Brazil from mycelia, originally from Belgium	1,6-β-Glucan	Extraction in hot water (100 °C), ethanol precipitation, freeze thawing	In vitro immunostimulatory activity on human THP-1-derived macrophages	Smiderle et al. (2013)
	Strain M7700 produced in the Netherlands from mycelia, originally from Belgium	1,6-β-Glucan	Extraction in hot water, ethanol precipitation, dialysis, and pronase incubation	In vitro PBMC immunostimulatory and antioxidative effect	Kozarski et al. (2014)
Agaricus brasiliensis mycelia	Strain UFSC 51, mycelia cultured in Brazil	Sulfated 1,2-β-gluco-1,3-mannan	Extraction in hot water (100 °C), ethanol precipitation, dialysis, and chemical sulfation	In vivo (mice) anti-herpes simplex virus activity	Cardozo et al. (2013b)

Agaricus blazei fruiting bodies	Not mentioned	1,4-α-1,6-β-Glucan	Subsequential extractions with ethanol at boiling point, hot water (100 °C) and ammonium oxalate, dialysis	In vivo (mice) antitumoral activity	Fujimiya et al. (1998)
	Brazil	1,6-β-Glucan	Extraction in hot water (121 °C), ethanol precipitation, and NaOH extraction	In vivo (mice) antitumoral (sarcoma 180) activity	Ohno et al. (2001)
	Not mentioned	1,3-1,6-β-Glucan	Extraction in hot water followed by ethanol precipitation and centrifugation	In vitro antigenotoxic and antimutagenic activity on HepG2 cells	Angeli et al. (2009a)
	Strain UFSC 51 (mature stages), Brazil	1,6-β-Glucan with traces of 1,3- and 1,2-β-glucans	Extraction in hot water (100 °C), ethanol precipitation, dialysis, DEAE-cellulose column chromatography	In vitro antigenotoxic activity on HepG2 cells	Angeli et al. (2009b)
	Brazil	1,4-α-1,6-β-Glucan–protein complex	Extraction in hot water (100 °C), ethanol precipitation, and centrifugation	In vivo (mice) antitumoral (sarcoma 180) activity	Gonzaga et al. (2009)
	China	1,3-β-Glucan	Ethanol extraction, hot (75 °C) water extraction, dialysis, DEAE–Sepharose column fractionation	In vitro inhibition of metastasizing capacity of cancer cells (HT-29)	Liu et al. (2010)

(*continued*)

Table 1 (continued)

Species' given name/ material analyzed	Strain/origin	Isolated polysaccharide	Isolation/extraction method	Biological activity	References
	China	1,6-β-Glucan	Not mentioned	In vivo (rat) stimulation of wound skin recovery	Sui et al. (2010)
	China	Backbone: 1,6-β-D-galactopyranosyl, 1,6-β-D-glucopyranosyl, and 1 → 3,6-β-D-glucopyranosyl	Ethanol extraction, hot water (75 °C) extraction, ethanol precipitation, DEAE–Sepharose column fractionation	In vivo (mice) Th1 immunity-stimulating adjuvant	Cui et al. (2013)
		Terminals: (1→)-β-L-fucopyranosyl, (1→)-β-L-arabinofuranose, and (1→)-β-D-glucopyranosyl			
	Brazil	1,4-α-1,6-β-Glucan–protein complex	Extraction in hot water (100 °C), ethanol precipitation, centrifugation	In vitro chemopreventive activity on HepG2 cells	Da Silva et al. (2013)
Agaricus blazei mycelia	Not mentioned	1,2-β-Gluco-1,3-mannan	Extraction in hot water, ethanol precipitation, DEAE–Sepharose column fractionation	In vivo (mice) antitumoral (sarcoma 180) activity	Mizuno et al. (1999)

Following production, extraction and concentration of bioactive polysaccharides can be done by means of porous membranes (Charcosset 2006). Membrane separation has often been used in industrial and pilot scales for size-based separations, with the advantage of high-throughput screening. Furthermore, it is environmentally friendly, and it is especially suitable for isolation of molecular-weight-specific polysaccharides with particular biological activities (Xiao et al. 2007). Silveira et al. (2012) report the employment of membrane technology for the fractionating of polysaccharides from *A. subrufescens* obtained from the main current methods of fungal biomass production, fruiting bodies, and SmF mycelium, in order to determine an optimal mass separation approach for further application at the industrial level. Both the microfiltration and ultrafiltration membranes were capable of separating high-molecular-weight polysaccharides from both extracts. As expected, higher retention levels were achieved when the nominal porosity of membranes decreased, regardless of the source of extracts. All polysaccharide components were detected in the nanofiltration membrane modules, producing greater retention yields. Therefore, this system represents an efficient alternative for scaling-up processes.

Among the membrane separation systems available, tangential flow filtration, also known as cross-flow filtration, is an option. This method allows for longer nonstop operational periods, reducing the needs for constant interruptions for cleaning, guaranteeing, therefore, higher productivity and procedural confidence. The system operates based on a feeding stream mode where the liquid phase, under pressure, passes through a membrane disposed parallel to the fluid current. A percentage of the fluid goes through the membrane every time there are components in the liquid phase that are smaller than the membrane pores. The portion that passes through the membrane is denominated permeate. The current applied will be the driven force to remove materials that have not been permeated by the membrane. This current is called retentate (Van Reis and Zydney 2007).

The use of membranes presents some advantages over alternative technologies. These include the use of low temperatures, low energy consumption, and reduced environmental impact, especially due to the reduction in the use of solvents and maintenance of the compounds' intrinsic properties (Xu and Wang 2005; Mello et al. 2010; Hsieh et al. 2014).

Following the studies of Silveira et al. (2012), Camelini et al. (2013b) developed a process to separate polysaccharides extracted from *A. subrufescens* grown in airlift bioreactors. The main objectives of that study were to produce large volumes of fungal biomass; extract the polysaccharide contents, both from the biomass and the culture media; and employ tangential flow nanofiltration to concentrate and purify these bioactive substances. Dehydrated fruiting bodies, purchased at a local market, were used to compare polysaccharide amounts with those from the mycelium and the culture media (Figs. 2a, b). The nanofiltration experiments were performed in a tangential flow filtration pilot plant using a polyvinylidene fluoride (PVDF) membrane in a spiral configuration (HL2521, GE Osmonics®, Philadelphia, USA), with molecular-weight cutoff (MWCO) ranging between 150 and 300 Da and filter area of 0.9 m^2. The operating conditions used in the experiments

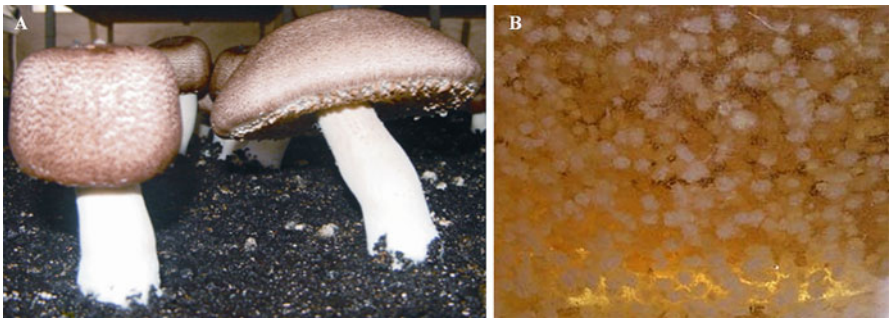

Fig. 2 *Agaricus subrufescens* (strain UFSC 51, CBMAI 1449, Brazil). (**a**) Fruiting bodies. (**b**) Mycelia growing in liquid media

followed temperatures of 35 ± 1 °C (water bath), tangential velocity of 0.68 m/s (obtained from the flow proportional to the membrane transversal area), and transmembrane pressure of 6 bar, until reaching a volumetric reduction factor (VRF) for each extract. Total and high molar mass polysaccharides as well as soluble solids were determined in the retentate for the three extracts. High molar mass polysaccharides with molecular weights of 617.93 ± 12.01 and 309.89 ± 2.22 g/mol were identified in the extracts obtained from the fruiting bodies and the mycelium, respectively. In the culture medium, two different polysaccharides were identified (20 ± 2.01 and 201 ± 3.01 g/mol). Polysaccharides were not detected in the permeate. However, there was a small amount of soluble solids in the permeate, possibly due to the polysaccharide hydrolysis into monosaccharides and due to the residual glucose and minerals from the culture media. In that respect, nanofiltration has contributed to reduce the initial extract volumes and contributed to concentrate total polysaccharides, especially those with high molar masses. Additionally, the permeate flow, the influences of temperature and pressure, and the resistance to the permeate flow during filtration were also evaluated. Nanofiltration has yielded polysaccharide increases of 85 %, 82 %, and 92 % in the extracts from fruiting bodies, mycelium, and liquid media at VRFs of 5, 6, and 7, respectively. Nevertheless, these increases were not proportional to the increases in VRF. A reduction in the permeate flow was observed during filtration, and it was compensated by increases in pressure and temperature.

Nonetheless, the membrane performance can be reduced during the processing caused by permeate flux decline as a function of time. Three phenomena can interfere with this reduction: concentration polarization, polarized gel layer, and fouling. All these factors impose high transport resistances throughout the membranes, and the extension that will affect the process depends on the membrane type and the type of fluid fed (Noble and Stern 1995; Mulder 2000). Camelini et al. (2013b) showed that the highest resistance to the permeate flux was caused by polarization due to concentration (polarized gel layer), reaching values of 88 % for the culture media. Maximal resistance caused by the membrane reached values of 40 % for the extract from the fruiting bodies. On the other hand, resistance

caused by fouling was responsible for less than 3.5 %. In conclusion, nanofiltration is efficient to concentrate these functional compounds extracted from *A. subrufescens* and can, therefore, be applied in different biotechnological areas.

Even though pressure and temperature can compensate reductions in the permeate flux, it was observed that higher pressures (6–7 bars) did not increase the flux linearly. Generally, higher pressures, while in the presence of soluble solids, cause a compacting effect over the membrane and induce the formation of a polarized gel layer, considerably increasing pore obstruction (Salgado et al. 2013). Temperature increases (25–45 °C) resulted in up to 30 % flux increases in the extracts. According to Mello et al. (2010), this result may be attributed to the effect of temperature in the viscosity of the solution. Furthermore, the temperature has an effect on the polymeric chain mobility of the membrane, making the polymeric material more flexible and, therefore, allowing for larger elements to permeate (Goosen et al. 2002). The temperature variation in the feeding process must be carefully monitored to avoid compound degradation and losses and at the same time facilitate the parameters for fluid processing and transportation (Michelon et al. 2014). As a rule of thumb, the temperature limit must be established as a function of the membrane's thermoresistance and the economic aspects related to energy consumption. In industrial processes, where time may translate into effectiveness, increasing temperatures can be a feasible and practical approach to reduce processing time. However, the use of higher temperatures will raise energy consumption and costs, aside from stimulating the degradation of thermosensitive compounds.

According to Cheryan (1998) and Chacón-Villalobos (2006), the solids present in the feed are carried to the membrane surface by convection, forming a concentration gradient. This phenomenon is responsible for altering the flow when compared to that of pure water, since it causes a counter flux of solutes toward the feeding as a function of diffusion. When the concentration of particles close to the filtering surface reaches and exceeds solubility limits, there will be jellification (gel layer) of the solution. This is caused by precipitation as a function of macromolecular saturation. This layer, so-called polarized gel layer, causes a reduction in the permeate flux (Noble and Stern 1995). When there is a deposition and accumulation of solids in the surface and/or inside the membrane pores, an irreversible phenomenon, called fouling, will occur. This phenomenon is directly related to the characteristics of the membrane and the solute–solute and solute–membrane interactions within the membrane, leading to a reduction in the effective pore size (Cheryan 1998; Mulder 2000; Van Reis and Zydney 2007).

It is important to mention that the resistance caused by polarization due to concentration can be alleviated by altering the operational conditions such as pressure, feeding temperature, and tangential velocity. In such manner, it is possible to detect higher permeate fluxes with the same feeding regime if the operational settings are altered. Fouling, on the other hand, is an irreversible phenomenon that increases the costs of operation and reduces permeation, requesting, therefore, higher working pressures and frequent chemical washings that can, potentially, reduce the membrane half-life. Furthermore, it invariably increases energy costs (Listiarini et al. 2009).

Camelini et al. (2013b) found that resistance caused by fouling alone was responsible for less than 3.5 % of the total resistance for the polysaccharide extracts. This is a remarkable result since this is the main problem faced by nanofiltration of such materials (Mänttäri et al. 2000). Inorganic and organic materials present in water typically cause fouling in nanofiltration membranes. These materials stick to the surface and pores of the membrane, resulting in the deterioration and lack of performance (reduced membrane flux) with a consequent increase in energy costs and membrane replacement (Al-Amoudia and Lovitt 2007). Therefore, reducing fouling will alleviate a second major problem associated with this technology, especially for industrial processes, which is the cleaning and recovery of the membranes.

6 Structure and Biological Activity

Polysaccharides are natural polymers found in all living organisms. In the biosphere, they represent an abundant group of macromolecules, such as cellulose and starch in plants, glycogen in animals, and glucans in fungi. These molecules, located mainly in the cell wall of fungi, may be composed of more than 75 % of carbohydrates, including predominantly glucans and mannans. In addition to acting as a sustenance element to the hyphae, some polysaccharides form an extracellular sheath around the mycelium, providing support for the adhesion of lignin-degrading enzymes, an indirect source of hydrogen peroxide, aside from protecting the cells from dehydration. Furthermore, polysaccharides may regulate extracellular glucose concentration (Gompertz et al. 2002; Da Silva et al. 2006).

The primary structure of a polysaccharide is defined by its sugar composition, the configuration of glycosidic bonds, the sequence of monosaccharides, the chain length, as well as the nature, number, and location of attached noncarbohydrate groups. Moreover, the monosaccharide polymerization can occur in different positions of the sugar units, generating a wide variety of linear or branched chains with great structural variability and different functions (Da Silva et al. 2006; Hu et al. 2013). The secondary structure of the molecule can also vary, depending on the conformation of its components, the molecular mass, and the inter- and intrachain interactions (Paulsen 2002). Modification processes, such as sulfation, naturally occurring in marine organisms and in the extracellular matrix of vertebrates, also contribute to the structural diversity of polysaccharides (Kirkwood 1974).

The structure of polysaccharides is species and strain related, and is correlated to many factors, such as location and time of collection, life cycle phase, method of cultivation, isolation, and drying. Processing may drastically affect the original chemical structure of polysaccharides altering their degree of branching, molecular weight, viscosity, and solubility, which may cause change in their biological properties. In fact, all the phases involved in the preparation of polysaccharides point toward the fact that each isolated batch is heterogeneous in nature. In this sense, the characterization of polysaccharides is necessary for ensuring their efficacy and safety and, because of its complexity, represents a challenge for industrial processing (Hu et al. 2013; Giavasis 2014).

7 An Example from the *Agaricus subrufescens* Species

In the last years, nomenclatural taxonomists have segregated *Agaricus blazei* Murrill sensu Heinemann as a different species from *A. subrufescens* Peck. In 2002, Wasser et al., based on the morphology of the fruiting bodies (size, pileal surface, type of pileal covering, presence of cheilocystidia, etc.) and the size of the spores, suggested the name *Agaricus brasiliensis* for a species of fungi collected in Brazil, very similar in nature to *A. blazei* and *A. subrufescens* (Wasser et al. 2002). In 2005, using biological and phylogenetic methods, Kerrigan showed that a cultivated specimen of *A. brasiliensis* from Brazil and a wild specimen of *A. subrufescens* from the USA (California) were in fact the same thing and, therefore, should belong to the same species name. This in turn illegitimated the name *Agaricus brasiliensis* as a later homonym (Kerrigan 2005, 2007). There are still disputes about the proper used name for this or that species within this complex, and because of this dispute, the comparison of data may be, somewhat, compromised. In this chapter, we refer to *A. subrufescens* as a synonym of *A. brasiliensis*, and, since some specimens might have been misidentified as *A. blazei*, studies on fungi coined under that name have also been considered.

Polysaccharides exhibit low toxicity to mammals and display numerous pharmacological properties. To illustrate some of these properties, Table 1 visits and presents the biological activities displayed by structurally different polysaccharides obtained from *A. subrufescens* and the related species *A. blazei*. The most common method of extraction and isolation consisted of hot water followed by ethanol precipitation. Additional fractionation using column chromatography or centrifugation has also been applied.

The main polysaccharide isolated from the fruiting body of *A. subrufescens* is the (1 → 6)-β-D-glucan, potentially with side chains. This polysaccharide is especially related to immunomodulatory, antitumoral, and antigenotoxic activities. In contrast, the majority of the mycelial polysaccharide isolated from *A. subrufescens* was found to be a (1 → 3)-β-D-gluco-(1 → 2)-β-D-mannan. This glucomannan was chemically sulfated to obtain a derivative (MI-S), which presented promising in vitro (Cardozo et al. 2011) and in vivo (Cardozo et al. 2013b) antiherpetic activities. The chain configuration of the polysaccharides has not changed after sulfation. However, the process reduced the molecular weight of the original polymer probably as a result of the sulfation conditions (acidic medium and high temperatures) (Lanteri 1978; Cardozo et al. 2013a).

The structural differences in polysaccharides obtained from the mycelium and from fruiting bodies of the same species may be related to the structural dynamic of the fungal cell wall, subjected to modifications at different stages in the life cycle, due to apical (mycelium) and lateral (fruiting bodies) growth. One good example is the glucan–glycosaminoglycan complex present in the cell walls of *A. bisporus*. In that species, the fruiting body polysaccharide has a lower number of connections, increasing flexibility and allowing reorientation of the wall, axial elongation, and insertion of new polymers, up to the maximum expansion reached by the fruiting body. On the other hand, the mycelial polysaccharides have more branched leading chains, which provide a greater rigidity of the cell wall (Mol and Wessels 1990).

8 Polysaccharides and Antiviral Activity

The antiviral activity has been displayed by polysaccharides of various sources, such as plants (Jassim and Naji 2003), lichens (Olafsdottir and Ingólfsdottir 2001), bacteria (Matsuda et al. 1999), seaweeds (Smit 2004), and fungi (Leung et al. 2006). The lists include activity against many viruses, comprising human herpesvirus types 1 (HHV-1) and 2 (HHV-2) (Eo et al. 2000; Liu et al. 2004), human immunodeficiency virus (HIV) (Jassim and Naji 2003), hepatitis B virus (Lee et al. 2002), cytomegalovirus, influenza virus (Kanekiyo et al. 2005), and coxsackievirus B3 (Lee et al. 2010).

Considering only the sulfated versions of the molecules, antiviral activity has been reported for HHV-1, HHV-2, and HHV-6 (Liu et al. 2004; Talarico et al. 2004; Zhu et al. 2004; Naesens et al. 2006), dengue virus (Qiu et al. 2007), HIV (Talyshinsky et al. 2002), cytomegalovirus, respiratory syncytial virus, influenza virus, and adenovirus (Witvrouw and De Clercq 1997). According to Ghosh et al. (2009), many of the polysaccharides studied in the last 20 years exhibited activity against more than one species of virus, showing their broad spectrum of antiviral activity. The primary mechanism of antiviral activity of sulfated polysaccharides has to do with the molecule's anionic features, allowing them to interact with the positive charges present in the viral envelope and/or on the cell surface, inhibiting the adsorption, penetration, and fusion of the virus with the host cells (Eo et al. 2000). Moreover, in vivo antiviral mechanisms via indirect stimulation of the innate and adaptive immune response have also been verified (Hayashi et al. 2008).

Table 2 summarizes studies on the in vitro antiviral activity of polysaccharides, extracts, or fractions from *A. subrufescens* and *A. blazei*. Some extracts or fractions have showed antiviral activities against western equine encephalitis (WEE) virus (Sorimachi et al. 2001), herpes simplex virus type 1 (HSV-1) (Sorimachi et al. 2001; Bruggemann et al. 2006), bovine herpesvirus 1 (BoHV-1) (Bruggemann et al. 2006), and poliovirus 1 (PV-1) (Faccin et al. 2007). However, their chemical composition was not determined, which compromises structure–activity discussions and result comparisons.

Two different polysaccharides, (1 → 6)-(1 → 3)-β-D-glucan (FR) and β-(1 → 6)-α-(1 → 4)-glucan–protein complex (PLS), were isolated from the fruiting bodies of *A. subrufescens* (Table 2). The FR was obtained through hot (100 °C) water extraction, followed by 95 % ethanol precipitation, centrifugation, and dialysis (5 kDa cutoff membrane) (Cardozo et al. 2013a). Differently, the PLS was obtained by some additional steps from the previous method, including hot (100 °C) water extraction, neutralization with NaOH to pH 7, 100 % ethanol precipitation in the presence of NaCl, centrifugation, and clarification with 30 % hydrogen peroxide, followed by a second extraction with ethanol (Gonzaga et al. 2005). Besides variances in glucan chain configuration, differences in protein content [FR = 1.76 % (w/w), PLS = 25.3 %] and molecular weight [FR = 609 kDa, PLS = 97.7 kDa] may explain the diverse antiherpetic activities displayed by these two compounds. Yamamoto et al. (2013) found an anti-HSV-1 activity

Table 2 Studies on in vitro antiviral activities of polysaccharides, extracts, or fractions obtained from *A. subrufescens* (syn. *A. brasiliensis*) and *A. blazei*

Species (origin)	Extraction	Tested sample	Fractionation with ethanol (%)	Chemical composition	In vitro antiviral activity							References
					WEE virus	PV-1	HSV-1	HSV-2	BoHV-1	Method	Mechanism of action	
A. blazei (Japan)	Hot water (50 °C)	Fruiting bodies fractionated with ethanol (100 μg/mL)	–	Not determined	++	NI[a]	NI[a]	Not tested	Not tested	Cytopathic effect (postinfection treatment)	Not determined	Sorimachi et al. (2001)
			17		+	NI[a]	NI[a]					
			29		++	NI[a]	NI[a]					
			38		++	NI[a]	NI[a]					
			44		+++	NI[a]	++					
			50		+++	NI[a]	+					
		Mycelia fractionated with ethanol (100 μg/mL)	–	Not determined	++	NI[a]	NI[a]					
			17		+	NI[a]	NI[a]					
			29		++	NI[a]	NI[a]					
			38		++	NI[a]	NI[a]					
			44		+++	NI[a]	++					
			50		+++	NI[a]	+					
A. brasiliensis (Brazil)	Water (25 °C)	Fruiting bodies fractionated with ethanol (PLS)	–	Not determined	Not tested	EC_{50} = 992.9 μg/mL	Not tested	Not tested	Not tested	PA T0 treat.[b]	Not determined	Gonzaga et al. (2005), Faccin et al. (2007)
			46	Not determined		EC_{50} = 187.6 μg/mL						
	Hot water (100 °C)	Fruiting bodies fractionated with ethanol (PLS)	100	Glucan–protein complex β(1,6)- and α(1,4)-glucans		EC_{50} = 97.2 μg/mL						
A. blazei (Brazil)	Water (25 °C)	Fruiting bodies	-	Not determined	Not tested	Not tested	% of inhibition at 100 μg/mL = 47.3 ± 0.09	Not tested	% of inhibition at 100 μg/mL = 20.9 ± 0.3	PA PI treat.[c]	Virucidal	Bruggemann et al. (2006)
A. blazei (Brazil)	Hot water (100 °C)	Fruiting bodies fractionated with ethanol	100	Glucan–protein complex β(1,6)- and α(1,4)-glucans (PLS)	Not tested	Not tested	Not tested	Not tested	EC_{50} = 160 μg/mL	PA T0 treat.[a]	Not virucidal. Did not inhibit virus attachment	Gonzaga et al. (2005), Angeli et al. (2006), Minari et al. (2011)
A. blazei (Brazil)	Hot water (? °C)	β-Glucan from fruiting bodies	?	Glucan–protein complex β(1,6)- and α(1,4)-glucans (PLS)				Not tested	EC_{50} = 140 μg/mL			

(*continued*)

Table 2 (continued)

Species (origin)	Extraction	Tested sample	Fractionation with ethanol (%)	Chemical composition	In vitro antiviral activity							References
					WEE virus	PV-1	HSV-1	HSV-2	BoHV-1	Method	Mechanism of action	
A. brasiliensis (Brazil)	Hot water (100 °C)	Fruiting bodies fractionated with ethanol	100	Glucan–protein complex β(1,6)- and α(1,4)-glucans (PLS)	Not tested	Not tested	EC_{50} = 454 μg/mL	Not tested	EC_{50} = 634 μg/mL	PA T0 treat.[a]	Not virucidal. Did not inhibit virus attachment	Gonzaga et al. (2005), Yamamoto et al. (2013)
							% of inhibition at 800 μg/mL = 83.6		% of inhibition at 800 μg/mL = 69.2	PA PI treat.[b]		
		Sulfated derivative of PLS (SPLS)	100 % + sulfation	Glucan–protein complex C4 sulfated at β(1,6)-glucan and C6 sulfated at α(1,4)-glucan			EC_{50} = 346 μg/mL		EC_{50} = 830 μg/mL	PA T0 treat.[a]		
							NI[c]		% of inhibition at 800 μg/mL = 28.6	PA PI treat.[b]		
		Carboxymethylated derivative of PLS (CPLS)	100 % + centrifugation	Carboxymethylated β(1,6)-glucan–protein complex			NI[c]		NI[c]	PA T0 treat.[a]		
										PA PI treat.[b]		
		Fraction 1		Glucan–protein complex β(1,6)- and α(1,4)-glucans			NI[c]			PA T0 treat.[a]		
							% of inhibition at 800 μg/mL = 20			PA PI treat.[b]		
		Fraction 2		Glucan–protein complex β(1,6)- and α(1,4)-glucans[d]			–			PA T0 treat.[a]		
							% of inhibition at 800 μg/mL = 20			PA PI treat.[b]		
		Fraction 3		Glucan–protein complex β(1,6)- and α(1,4)-glucans[d]			% of inhibition at 800 μg/mL = 35.9		EC_{50} = 674 μg/mL	PA T0 treat.[a]		
							% of inhibition at 800 μg/mL = 32.3		% of inhibition at 800 μg/mL = 77.4	PA PI treat.[b]		
		Fraction 4		Glucan–protein complex β(1,6)- and α(1,4)-glucans[d]			NI[c]		NI[c]	PA T0 treat.[a]		
										PA PI treat.[b]		

A. brasiliensis (Brazil)	Hot water (100 °C)	Mycelia fractionated with ethanol (MI)	95 % + centrifugation	β(1,2)-(1,3) glucomannan	Not tested	Not tested	NI[ce]	NI[ce]	Not tested	PA T0 treat.[a]	–	Cardozo et al. (2011)
							NI[ce]	NI[ce]		PA PI treat.[b]	–	
		Sulfated derivative of MI (MI-S)	95 % + centrifugation + sulfation	Carbons C3, C4, and C6 sulfated at β(1,2) mannan + carbons C2, C4, and C6 sulfated at β(1,3)-glucan	Not tested	Not tested	EC_{50} = 1.24 ± 0.05 μg/mL	EC_{50} = 0.39 ± 0.17 μg/mL	Not tested	PA T0 treat.[a]	Not virucidal. Inhibition of adsorption, penetration, protein expression, and cell-to-cell spread	
							EC_{50} = 5.50 ± 0.58 μg/mL	EC_{50} = 4.30 ± 0.36 μg/mL		PA PI treat.[b]		
A. brasiliensis (Brazil)	Hot water (100 °C)	Fruiting bodies fractionated with ethanol FR	95 % + centrifugation	β(1,6)-(1,3)-glucan	Not tested	Not tested	NI[ce]	NI[ce]	Not tested	PA T0 treat.[a]	–	Cardozo et al. (2014)
							NI[ce]	NI[ce]		PA PI treat.[b]	–	
		Sulfated derivative of FR (FR-S)	95 % + centrifugation + sulfation	Carbons C4 and C6 sulfated at β(1,6)-glucan and at β(1,3)-glucan		Not tested	EC_{50} = 1.43 ± 0.06 μg/mL	EC_{50} = 0.62 ± 0.08 μg/mL	Not tested	PA T0 treat.[a]	Not virucidal. Inhibition of adsorption, penetration, and cell-to-cell spread	
							EC_{50} = 6.74 ± 0.01 μg/mL	EC_{50} = 4.62 ± 0.38 μg/mL		PA PI treat.[b]		

[a]Plaque assay time zero treatment (simultaneous treatment)
[b]Plaque assay postinfection treatment
[c]No inhibitory activity
[d]Reduced α(1,4)-glucan content
[e]Maximum tested concentration (1 mg/mL)

under simultaneous (EC_{50} = 454 μg/mL) and postinfection (83.6 % of inhibition at 800 μg/mL) treatments with PLS, using a viral plaque number reduction assay. Nonetheless, neither anti-HSV-1 nor anti-HSV-2 activity under these two treatments was found for FR at the maximum tested concentration (1 mg/mL) (Cardozo et al. 2014).

Both polysaccharides were chemically modified to produce their respective sulfated derivatives FR-S and SPLS. FR-S had had no detectable protein content and lower molecular weight (127 kDa) in comparison to the non-sulfated version (Cardozo et al. 2013a). The introduction of sulfated groups on FR resulted in a sulfated derivative (FR-S) with promising in vitro antiherpetic activity (EC_{50} < 6.74 μg/mL), acting by inhibition of HSV-1 and HSV-2 adsorption, penetration, and cell-to-cell spread (Cardozo et al. 2014). In a different way, SPLS did not demonstrate much improvement on antiviral activity after sulfation in the simultaneous treatment (EC_{50} = 346 μg/mL) and had no activity in the postinfection treatment (Yamamoto et al. 2013). The variable antiviral effects and mechanism of action demonstrated by these two polysaccharides are primarily related to their structural differences, not excluding variances related to the fungus isolate and modifications in the methodology used to determine the antiviral effects.

9 Conclusions

In the face of the factors exposed throughout this chapter, the potentiality of polysaccharides produced and extracted from *A. subrufescens* for the production of nutraceuticals and medicines at a commercial level is dependent upon a series of events. These events, which need to be fully understood for the success of the process, include the fungal preservation, the maximization of biomass production, and the establishment of a process that allows for an adequate and reliable polysaccharide obtainment. These are sine qua non conditions for the advancement of this field allowing for the viability of preclinical and clinical studies involving these molecules.

For the preservation and recovery of *A. subrufescens*, the use of activated charcoal is an easy, quick, and inexpensive method that does not result in significant morphological and genetic changes within a 1-year storage period. This method may also be suitable for the preservation of other species and lineages of filamentous fungi.

Solid-state fermentation is a feasible process to obtain large quantities of biomass required for the production of foods containing fungal glucomannans. The parameters for the production of glucomannans on wheat grains have been, over the years, optimized. Based on these results, additional research is currently in progress, aiming at a formulated product with nutritional contents and safety information.

For SmF, pneumatic systems based on airlift bioreactors, which have a simplified construction design, have shown remarkable results when utilized for the production of fungal cells such as those from *A. subrufescens*. The system proved

to be very efficient when compared to agitated tanks, even when important parameters, such as pH, were left uncontrolled. These results can be potentially improved when some parameters, such as the use of hydrolyzed residues in the culture media, are optimized. Experimental results are important to guide the planning and operational settings, in order to achieve higher productivities. Among these settings, the definition of the type and amount of culture media and inoculum is particularly important. In this respect, nanofiltration has contributed to reduce the initial extract volumes, permitting the concentration of total polysaccharides, especially those with high molar masses.

Biotechnological processes involving fungi must focus on selecting the right species for the products of interest. In addition, the method of cultivation, the identification of the compounds of interest, and the method of extraction, purification, and final processing are crucial for successful results. For species of *Agaricus*, a fair amount of studies has focused on important molecules proved to have nutraceutical and medicinal values, especially the polysaccharides, which are important components of fungal cell walls. Polysaccharides are components produced by certain species of fungi with proved antiviral, antitumoral, and immunomodulatory, among others, activities. This is a promising and very attractive field that will probably rampage in future research projects around the world, aiming at obtaining natural products that can easily and safely be employed to treat a wide range of diseases.

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Starch and Nanoparticle

13

Alain Dufresne

Contents

Abstract

Nanotechnology and renewable bio-based materials are driving forces for the development of new materials that combine innovative properties and sustainability. Starch is an abundant, natural, renewable, and biodegradable polymer produced by many plants as a source of stored energy. Because of the multiscale structure of starch granules consisting of alternating crystalline and amorphous concentric layers, the controlled acid hydrolysis treatment of native starch disrupts this organization and releases crystalline platelet-like particles with nanoscale dimensions. This chapter intends to provide a comprehensive overview of their preparation, characterization, properties, and applications. The preparation and morphology of starch nanocrystals are exposed as well as strategies intended to improve the existing current methods. The properties of ensuing aqueous suspensions and reported surface chemical modifications are also presented. The preparation of polymer nanocomposites reinforced with starch nanocrystals and their properties is then reviewed.

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1 Introduction

Fossil energy depletion and growing environmental concerns have brought up increasing interest in bio-based eco-efficient and high-technology materials. In this context, the development of products made of sustainable or renewable non-petroleum-based polymeric materials has become an active research area that has received much attention. This is mainly driven by the public's growing demand for more environmental friendly materials. Unfortunately, the thermomechanical performance of most renewable polymers is generally inferior compared to traditional petroleum-based polymers. The composite materials concept is one solution for creating renewable materials that match or exceed the performance of commonly used petroleum-based engineering polymers. Combining bio-based/renewable polymers with renewable reinforcements could address the property-performance gap between renewable and traditional synthetic polymers.

Starch is a natural, renewable, and biodegradable polymer produced by many plants as a source of stored energy. It is the major carbohydrate reserve in plant tubers and seed endosperm, and it is found in plant roots, stalks, crop seeds, and staple crops such as rice, corn, wheat, tapioca, and potato (Buléon et al. 1998). Starch is therefore a promising material because of its versatility, low price, availability, and numerous industrial applications. It is interesting to note that Romance languages, unlike Germanic languages and English, make a distinction between the two main sources of starch: for example, *amidon* for cereal-derived starch and *fécule* for tuber-derived starch in French (http://ec.europa.eu/agriculture/eval/reports/amidon/chap1.pdf). In 2000, the world starch market was estimated to be 48.5 million tons, including native and modified starches, but also the large volume of starch that is converted into syrups for direct use as glucose and isoglucose and as substrates in the form of very high-dextrose syrups (known as starch hydrolysates) for fermentation into organic chemicals, including ethanol (http://ec.europa.eu/agriculture/eval/reports/amidon/chap1.pdf). The value of the output is worth € 15 billion per year, explaining the interest of the industrialists and researchers seeking new properties of high-value application. The major categories to be considered while mapping the starch processing industry are food and nonfood products. The use of starch in food products includes food processing and beverages. Nonfood products of starch include paper, glue, thickening agent, and stiffening agent, among others.

Starch is biosynthesized as semicrystalline granules with varying polymorphic types and degrees of crystallinity. The amorphous regions are more susceptible to hydrolysis and, under controlled conditions, may be dissolved, leaving the rigid crystalline regions intact. It was reported that the acid hydrolysis of native starch granules releases platelet-like nanoscale highly crystalline residues. The potential of

nanotechnology and nanocomposites in various sectors of research and application is promising and attracting increasing investment. By definition, materials having at least one linear dimension in the range from approximately 1 to 100 nm are called nanomaterials. As the size of a particle is decreasing down to the nanometer scale, important changes occur. Both the specific surface area and total surface energy increase. Moreover, starch nanoparticles display a highly reactive surface with plenty hydroxyl groups. When blended with a polymeric matrix, ensuing nanocomposites show unique properties, because of the nanometric size effect, compared to conventional composite even at low filler content. Indeed, nanofillers have a strong reinforcing effect, and studies have also shown their positive impact in barrier effect. However, for decades, studies have been conducted with nonrenewable inorganic fillers. Increasing environmental concerns have led to investigating the potential uses of renewable resources for such application. It has brought two scientific fields together, viz., nanotechnologies, which allow the development of innovative and efficient materials, and biomaterial processing, with the use of renewable raw materials for more environmentally friendly and sustainable solutions.

2 Occurrence/Sources

In plants, starch accumulates in many different photosynthetic or non-photosynthetic tissues. It accumulates during the day in plant cell leaf cells and breaks down at night to achieve a more or less constant supply of sucrose to the non-photosynthetic tissues. It is therefore the major energy reserve of higher plants. The starch industry extracts and refines starches by wet grinding, sieving, and drying. After its extraction from plants, starch occurs as a flour-like tasteless and odorless white powder insoluble in cold water. This powder called native starch consists of microscopic granules with diameters depending on the botanical origin, ranging from 2 (for wheat and rice) to 100 μm (for potato), and with a density around 1.5 g cm^{-3} (Buléon et al. 1998).

2.1 Starch Composition

The composition of starch was originally determined by studying the residue of its total hydrolysis by β-amylase. It mainly consists of a combination of two glucosidic macromolecules, namely, amylose and amylopectin (Fig. 1). In most common types of starch, the amylopectin content ranges between 72 % and 82 %, while the amylose content ranges between 18 % and 28 %. However, some mutant types of starch have very high amylopectin content (99 % for waxy maize), and some very high amylose content (up to 70 % and more for amylomaize). Other trace elements associated with starches correspond to three categories of materials, viz., (i) particulate material, mainly composed of cell-wall fragments; (ii) surface components such as proteins, enzymes, amino acids, and nucleic acids, removable by extraction procedures; and (iii) internal components, mainly composed of lipids.

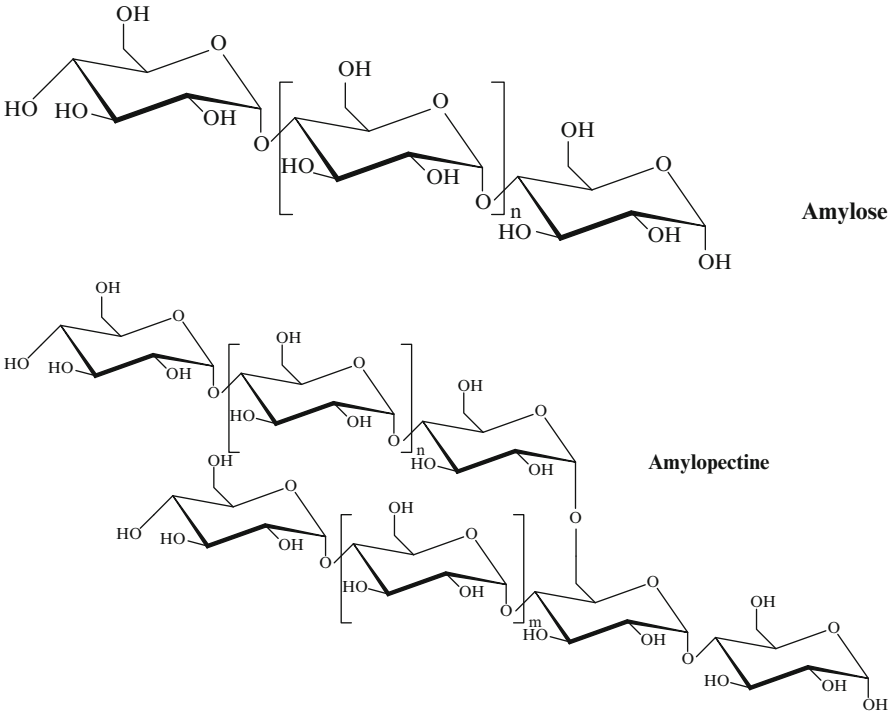

Fig. 1 Chemical structure of amylose and amylopectin

Amylose is essentially a linear polymer consisting of glucose units linked by α-(1→4) glycosidic bonds, slightly branched by α-(1→6) linkages. It has a degree of polymerization up to DP 6000 and has a molar mass of 105–106 g mol^{-1}. The chains can easily form single or double helices. Amylopectin is a highly branched polymer consisting of relatively short branches of α-D-(1→4) glycopyranose that are interlinked by α-D-(1→6) glycosidic linkages. It is a larger molecule and differs from amylose in that branching occurs, with an α-1,6 linkage every 24–30 glucose monomer units. Each amylopectin molecule contains up to two million glucose residues in a compact structure. The molecules are oriented radially in the starch granule, and as the radius increases, so does the number of branches required to fill up the space, with the consequent formation of concentric regions of alternating amorphous and crystalline structure.

2.2 Multiscale Structure of Starch Granule

As many living organisms, starch granule displays a multiscale structure as shown in Fig. 2. It consists of the (a) starch granule (2–100 μm), into which we find (b) growth rings (120–500 nm) composed of (d) blocklets (20–50 nm) made of

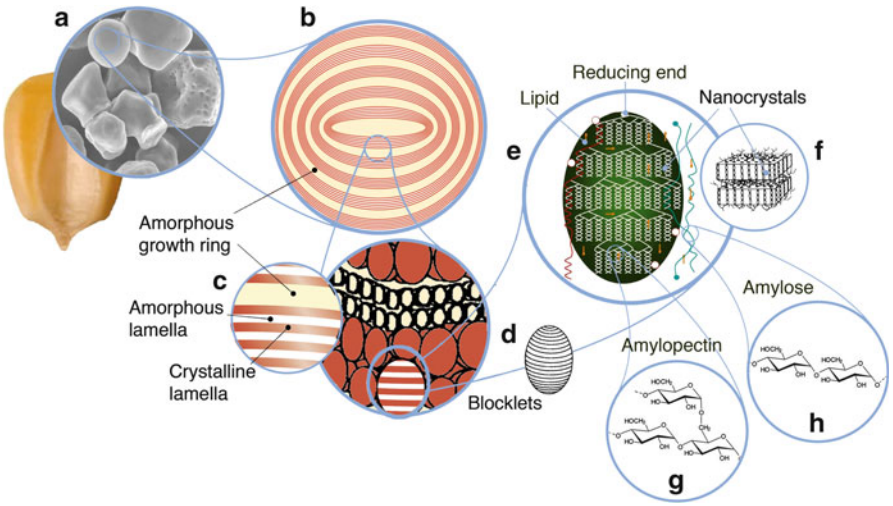

Fig. 2 Starch multiscale structure: (**a**) starch granules from normal maize (30 μm); (**b**) amorphous and semicrystalline growth rings (120–500 nm); (**c**) amorphous and crystalline lamellae (9 nm), magnified details of the semicrystalline growth ring; (**d**) blocklets (20–50 nm) constituting unit of the growth rings; (**e**) amylopectin double helixes forming the crystalline lamellae of the blocklets; (**f**) nanocrystals, other representation of the crystalline lamellae called starch nanocrystals when separated by acid hydrolysis; (**g**) amylopectin's molecular structure; (**h**) amylose's molecular structure (0.1–1 nm) (Reproduced with permissions from LeCorre et al. (2010))

(c) amorphous and crystalline lamellae (9 nm) (Gallant et al. 1997) containing (g) amylopectin, and (h) amylose chains (0.1–1 nm). The blocklet concept was developed in the 1930s (Katz 1930).

When observed under a microscope and polarized light, starch shows birefringence. The refracted characteristic "Maltese cross" corresponding to the crystalline region is typical of a radial orientation of the macromolecules. The so-called onion-like structure of starch granule that consists more or less concentric growth rings is composed of alternating hard crystalline and soft less ordered shells growing by apposition from the hilum (center of the granule). The thickness of the combined repeated crystalline and amorphous lamellae is 9 nm regardless of the starch botanical origin (Gallant et al. 1997).

Native starches contain between 15 % and 45 % of crystalline material (Zobel 1988). Depending on their X-ray diffraction pattern, starches are categorized in three crystalline types referred to as A, B, and C. A type is characteristic of cereal starches (wheat and maize starch). B type is typical of tuber and amylose-rich cereal starches. C type is characteristic of leguminous starches and corresponds to a mixture of A and B crystalline types. V type is observed during the formation of complexes between amylose and a complexing molecule (iodine, alcohols, cyclohexane, fatty acids, etc.). The appearance of starch X-ray diffraction pattern depends on the water content of granules during the measurement. The more starch is hydrated, the thinner the diffraction pattern rings up to a given limit. Water is

therefore one of the component of the crystalline organization of starch. The crystalline-to-amorphous transition occurs at 60–70 °C in water, and this process is called gelatinization.

3 Preparation of Starch Nanocrystals

Nanosize particles can be prepared from microscopic starch granules following different strategies. Consequently, ensuing nanoparticles have different properties, crystallinity, and morphology (LeCorre et al. 2010). For instance, starch nanoparticles were prepared by precipitating an aqueous gelatinized starch solution within ethanol as the precipitant (Ma et al. 2008). Nanoscale starch particles with a spherical or oval shape and diameters in the range of 10–20 nm were obtained by complex formation with other components involving mainly amylose rather than amylopectin (Kim and Lim 2009). However, the complex contained a large portion of amorphous material so that its selective removal by enzymatic hydrolysis was needed, resulting in an extremely low yield of nanoparticles.

The method for producing nanofibrillated cellulose (NFC) has also been transferred for producing starch colloids (Liu et al. 2009). Diluted slurry of high amylose corn starch was run through a Microfluidizer for several passes (up to 30). The particle size of the sample obtained from more than ten passes was below 100 nm with a yield close to 100 %, and the gel-like suspension remained stable for more than 1 month. However, the ensuing starch colloids were obtained from breaking down both amorphous and crystalline domains, rendering amorphous nanoparticles after ten passes.

Therefore, the most convenient and most investigated procedure for preparing starch nanoparticles has been acid hydrolysis yielding highly crystalline nanoparticles or starch nanocrystals.

3.1 Acid Hydrolysis Process of Starch Granules

In starch industry, acid hydrolysis process is a chemical treatment largely used to prepare glucose syrups. The preparation at room temperature of a low molecular weight acid-resistant fraction of potato starch after hydrolysis with a 15 % (w/v) sulfuric acid (H_2SO_4) suspension during 30 days was reported by Nägeli 140 years ago (Nägeli 1874). The ensuing insoluble fraction would be known as Nägeli amylodextrin. Later, Lintner reported a 40-day hydrolysis process with a 7.5 % (w/v) hydrochloric acid (HCl) suspension of potato starch at 30–40 °C to produce a high molecular weight starch suspension called “lintnerized starch” (Lintner 1886). More recently and by analogy with the acid-induced extraction of cellulose nanocrystals from cellulosic fibers, Dufresne et al. reported a method for producing a material which was called “microcrystalline starch,” consisting of agglomerated particles of a few 10 nm in diameter (Dufresne et al. 1996). This procedure consisted in hydrolyzing potato starch granules (5 wt%) in a 2.2 N HCl suspension for 15 days.

A detailed study of the HCl-induced degradation of granular starch from different origins was reported (Robin et al. 1975). The higher susceptibility to acid of amylopectin-rich waxy maize compared to other cultivars was attributed to the fact that hydrolysis severed branched molecules more readily than linear molecules. Moreover, the kinetics of lintnerization showed a two-stage profile for the hydrolysis of starch granules. For shorter times, the hydrolysis kinetics was fast, whereas for longer times, it was slow. It was explained by the fact that acid attacked amorphous areas more rapidly than the crystalline ones, less accessible to H_3O^+ ions. The critical time corresponding to fast/slow hydrolysis conditions depends on the botanical origin of starch (Singh and Ali 2000; Jayakody and Hoover 2002). The hydrolysis of the granules appeared to be governed by two factors: reactivity and accessibility. It was also reported that the hydrolysis process is faster when using HCl rather than H_2SO_4 (Muhr et al. 1984). Temperature is another parameter that catalyzes the hydrolysis reaction, but it should obviously remain below the gelatinization temperature of starch in acidic medium. The gelatinization process corresponds to an irreversible swelling and solubilization phenomenon occurring when native granules are heated above 60 °C in excess water. As for temperature, the acid concentration favors the hydrolysis kinetics. However, its upper limit is restricted (around 2.5–3 N for HCl) since above this critical acid concentration, granule gelatinization occurs (Robin 1976).

3.2 Morphology and Structure of Starch Nanocrystals

The amorphous regions of the starch granule act as structural defects which are susceptible to acid attack, and, under controlled conditions, they may be removed, releasing crystalline regions intact. The shape and dimensions of starch nanocrystal can be directly accessed by microscopic observations. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) can be used. Nevertheless, AFM is well known to overestimate the size of the nanocrystals due to the tip-broadening effect. SEM is useful to perform a rapid examination, but the resolution is simply insufficient for more detailed information. Field emission SEM (FESEM) can be used to improve the spatial resolution and minimize sample charging and damage. Transmission electron microscopy (TEM) is the best-suited method to identify individual nanocrystals and determine their morphology.

A detailed investigation on the structure of acid-hydrolyzed waxy maize starch fragments was reported (Putaux et al. 2003; Putaux 2005). TEM observation of such fragments using HCl and H_2SO_4 is shown in Figs. 3 and 4, respectively. The insoluble residue from HCl hydrolysis contains polydisperse and more or less individualized platelet nanocrystals corresponding to the lamellae formed by the association of amylopectin side branches into parallel arrays of double helices (Fig. 3). The 5–7 nm thick lamellae are still connected by α(1→6) linkages after 2 weeks of hydrolysis and can be seen edge on (Fig. 3a) but also flat on the carbon grid (Fig. 3b, c). As the hydrolysis is progressing up to 6 weeks (Fig. 3d), more α(1→6) branching points located in the interlamellar areas are severed, and the

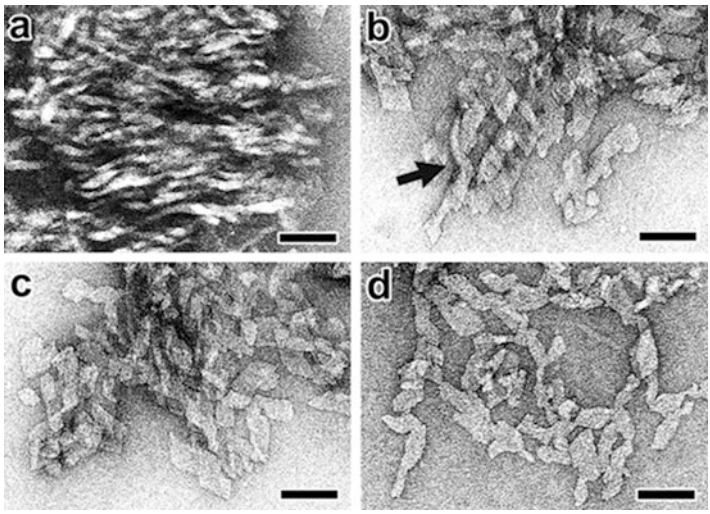

Fig. 3 TEM micrographs of negatively stained waxy maize starch samples: (**a–c**) fragments of waxy maize starch granules after 2 weeks of 2.2 N HCl hydrolysis at 36 °C. In (**a**) a lamellar organization is clearly revealed with the platelets lying parallel to the incident electron beam. In (**b**) and (**c**) parallelepipedal platelets are seen lying flat on the carbon film. The *arrow* in (**b**) indicates a pyramidal stack of crystals. (**d**) Nearly individual waxy maize starch nanocrystals obtained after 6 weeks of hydrolysis (scale bars: 50 nm) (Reproduced with permissions from Putaux et al. (2003))

platelets are thus observed in planar view. Marked 60–65° acute angles and constituting parallelepipedal blocks with a length of 20–40 nm and a width of 15–30 nm are observed.

A similar morphology was observed for H_2SO_4-hydrolyzed nanocrystals resulting from the disruption of waxy maize starch granules as shown in Fig. 4 (Angellier et al. 2004). Nanoplatelets were generally observed in aggregates of 1–5 μm (Fig. 4a) as confirmed by laser granulometry (Angellier et al. 2005a). Different organizations were also reported as observed in Fig. 4b–d. X-ray and electron diffraction showed that these nanoplatelets retain the crystalline A type of the parent granules (Putaux et al. 2003).

The influence of the botanical origin and amylose content of native starches on the morphology of starch nanocrystals was also investigated (LeCorre et al. 2011a). Starch nanocrystals were prepared from normal maize, high amylose maize, waxy maize, potato, and wheat starch, covering three botanical origins, two crystalline types, and three ranges of amylose content (0 % , 25 %, and 70 %) for maize starch. Different types of crystalline nanoparticles were obtained with a thickness ranging between 4 and 8 nm and diameter from about 50 to 120 nm depending on the source. For the same amylose content, maize, potato, and wheat resulted in rather similar size and crystallinity of nanocrystal, proving the limited influence of the botanical origin. For the same botanical origin (maize), differences in size were more significant indicating the influence of the amylopectin content, and thicker

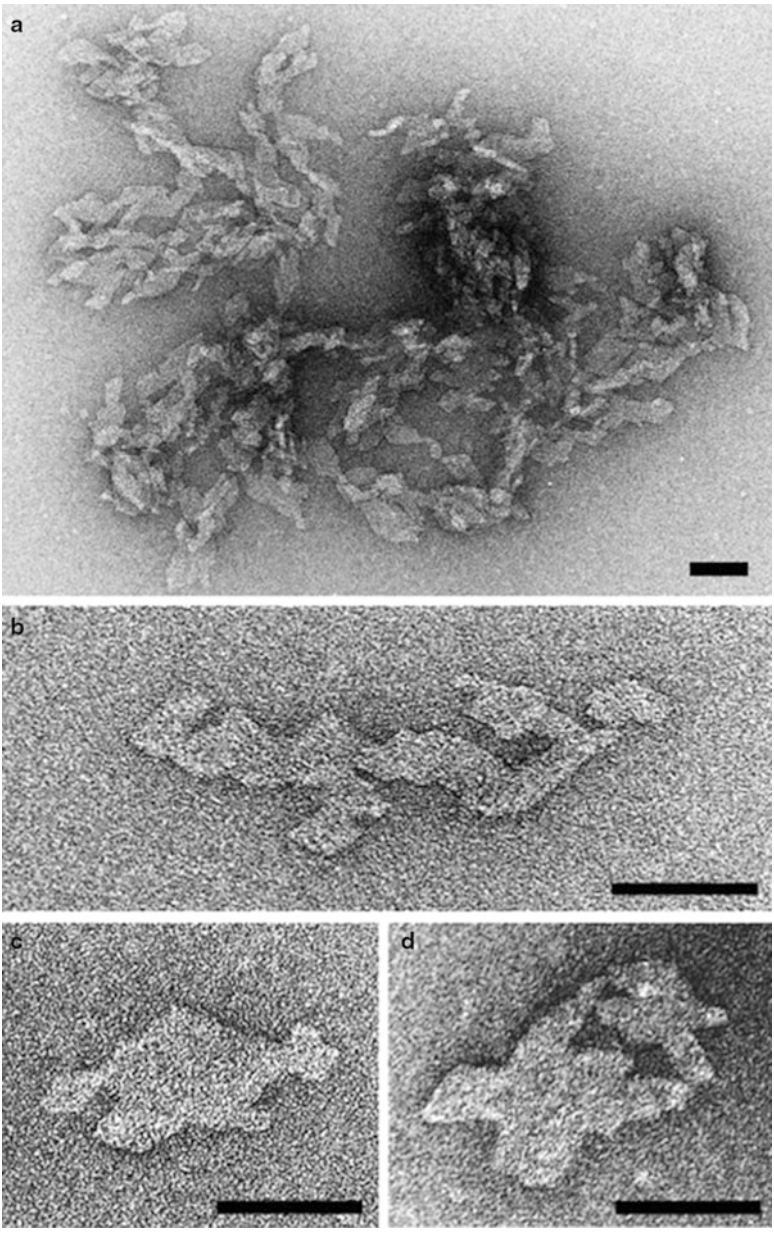

Fig. 4 TEM micrographs of negatively stained starch nanocrystals obtained by 3.16 M H_2SO_4 hydrolysis of waxy maize starch granules during 5 days, at 40 °C, 100 rpm and with a starch concentration of 14.69 wt% (optimized conditions): (**a**) aggregates of nanocrystals and (**b–d**) organizations of nanoplatelets (scale bar: 50 nm) (Reproduced with permissions from Angellier et al. (2004))

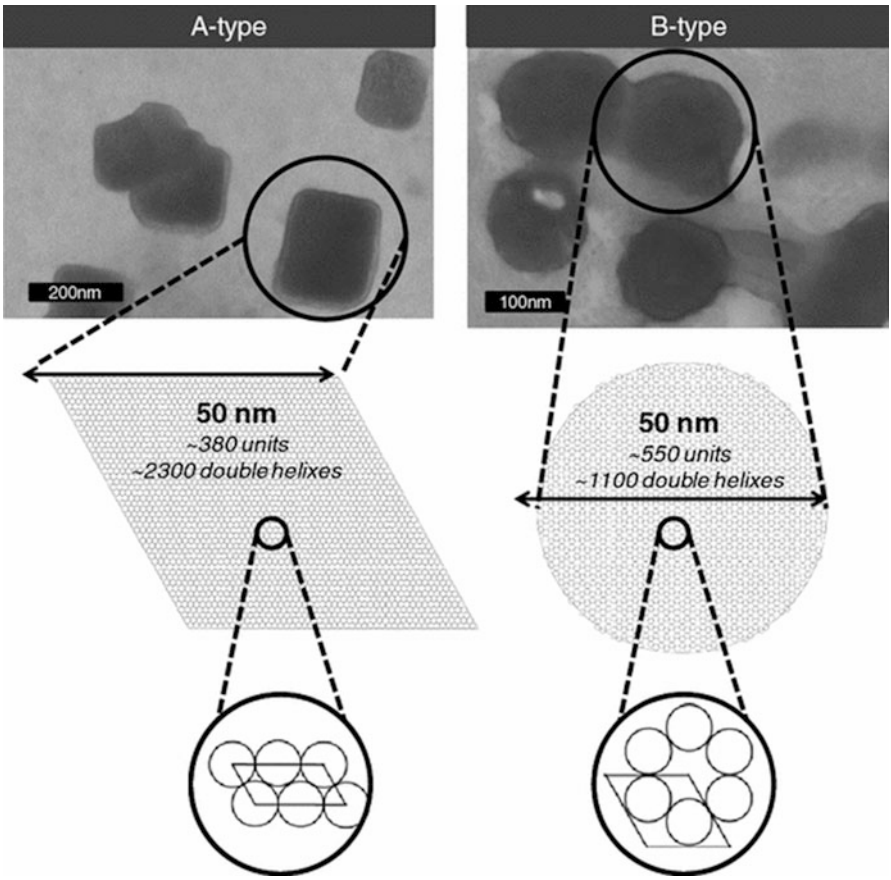

Fig. 5 Double helixes packing configuration depending on crystalline type and corresponding picture of starch nanocrystals from waxy maize starch (A type) and high amylose starch (B type) (Reproduced with permissions from LeCorre et al. (2011a))

nanocrystals were obtained for higher amylose content starches. It was also observed that nanocrystals produced from A-type starches rendered square-like particles, whereas nanocrystals produced from B-type starches rendered round-like particles (Fig. 5). This was explained by the different packing configurations of amylopectin chains for A- and B-type starches.

A detailed characterization of the molecular content of A-type nanocrystals prepared by mild sulfuric acid hydrolysis of waxy maize starch granules was reported (Angellier-Coussy et al. 2009). Two major groups of dextrins were found in the nanocrystals and were isolated. Each group was then structurally characterized using β-amylase and debranching enzymes (isoamylase and pullulanase) in combination with anion-exchange chromatography. One of these had a degree of polymerization (DP) of 14.2, which in the double-helical structure corresponds to a length of 5 nm and to the thickness of the crystalline lamellae

within the starch granule. This clearly indicated that the nanocrystals correspond to the crystalline lamellae present in native starch granules. As the nanocrystals were described by parallelepipedal blocks with a length of 20–40 nm and a width of 15–30 nm (Putaux et al. 2003), it was assumed that between 150 and 300, double-helical components are making up these crystalline domains. Further analysis indicated that roughly half of the dextrins in the nanocrystals were branched molecules, which was far more than previous investigations suggested. It was also concluded that they were equally distributed between populations of high and low molecular weights, respectively. Taking into account the length of these branches and the thickness of the platelets, it was likely that the majority of the branching points were found at the reducing-end surface of the nanocrystals, whereas the rest were located at the nonreducing side.

Because the acid hydrolysis process preferentially dissolves the amorphous domains of the starch granule leaving the crystalline zones intact, the degree of crystallinity (ratio between the mass of crystalline domains and the total mass of nanocrystal) of starch nanocrystals should be theoretically close to 100 %. However, incomplete removal of amorphous regions and less ordered surface chains may result in a lower degree of crystallinity. The values commonly reported in the literature are within the range 45–50 % (Lin et al. 2012). However, higher values such as 79 % after 10 days of hydrolysis have also been reported (Duan et al. 2011).

3.3 Acid Hydrolysis Conditions

In earlier studies on the degradation of starch granules in acidic medium, no attention was paid on the duration and yield of the process. However, these parameters should be considered before any potential application of starch nanocrystals. Long duration (40 days) and low yield (0.5 wt%) were reported for the acid hydrolysis of native starch granules (Battista 1975). The process was improved by performing periodic stirring of the suspension, and the duration of the acid hydrolysis treatment has been reduced to 15 days using a 5 wt% starch suspension and 2.2 M HCl (Dufresne et al. 1996).

The effect of five different factors on the selective sulfuric acid hydrolysis of waxy maize starch granules was investigated using a response surface methodology to comprehensively optimize the preparation of aqueous suspensions of starch nanocrystals (Angellier et al. 2004). The selected predictors were temperature, acid concentration, starch concentration, hydrolysis duration, and stirring speed. The objective of the study consisted in determining the operative conditions leading to the smallest size of insoluble residue with the shortest time and with the highest yield. The optimized preparation of aqueous suspensions of starch nanocrystals was achieved after 5 days with 3.16 M H_2SO_4 hydrolysis at 40 °C, 100 rpm and with a starch concentration of 14.69 wt% with a yield of 15.7 wt%. Ensuing nanoparticles had the same shape as those obtained from the classical procedure after 40 days of HCl treatment, with a yield of 0.5 wt%. This procedure continues to serve for most studies as the standard recipe for the preparation of starch nanocrystals. Confirming

the effect of acid concentration, it was shown that when hydrolysis was carried out with 2.2 M HCl, nanoparticles with the minimum size were obtained after 50 days, whereas this occurred just after 24 days using 3.7 M HCl (Jivan et al. 2013). It was also observed that the stronger acidity not only shortened the time required to obtain the minimum size for particles but also resulted in tinier crystals.

A further study showed that starch nanocrystals are produced from a very early stage of the acid hydrolysis treatment opening the door to new opportunities for the optimization of the process (LeCorre et al. 2011b). Microscopic observations of the insoluble residue were performed at a different stage of hydrolysis. From day 1, both granule fragments with a hollow center and starch nanoparticles were observed, indicating that starch nanocrystals were formed, at least, after 24 h of H_2SO_4 hydrolysis and that consequently, at any time including final suspension, both microscaled and nanoscaled particles can be found and coexist. It suggested that some nanocrystals might turn to sugar by the end of the batch production process explaining the low yields. This study clearly showed the need for a continuous production and extraction process of starch nanocrystals. In the same study (LeCorre et al. 2011b), differential centrifugation has been tested as an isolation process for separating these two kinds of particles but did not seem fitted for fractionation due to hydrogen bonding and different densities within starch granules.

The possibility of separating starch nanocrystals from microparticles in a continuous extraction technique was investigated (LeCorre et al. 2011c). Filtration of the hydrolyzed residues using a microfiltration unit equipped with ceramic membranes to assess the cross-flow membrane filtration potential of starch nanocrystal suspensions was therefore conducted. Contrary to frontal filtration, cross-flow filtration allowed isolation without modifying the crystalline structure of starch nanocrystals and did not favor their aggregation. The proposed microfiltration process is shown in Fig. 6.

The process parameters were monitored, and the properties of feed, permeate, and retentate were investigated. Cross-flow filtration was proved to be an efficient continuous operation for separating starch nanocrystals from the bulk suspension and non-fully hydrolyzed particles whatever the ceramic membrane pore size (0.2–0.8 μm). Analysis on permeate showed not only that collected nanoparticles were more crystalline than feed but also that mostly B-type particles were produced during the first day of hydrolysis. Based on this observation and as an attempt to establish a predictive model for the optimal parameter setting for preparing starch nanocrystals in 1 day, a statistical experimental design and a multi-linear regression method analysis were performed (LeCorre et al. 2012a).

The effect of an enzymatic pretreatment of starch granules before the acid hydrolysis aiming in reducing its duration was also investigated (LeCorre et al. 2012b). This pretreatment was performed to create pit holes at the surface of native granules without damaging the crystalline structure of starch and therefore to create pathways expected to facilitate and make more homogeneous the acid penetration during the subsequent acid hydrolysis treatment. Three amylases have been selected for the study, namely, α-amylase, β-amylase, and glucoamylase,

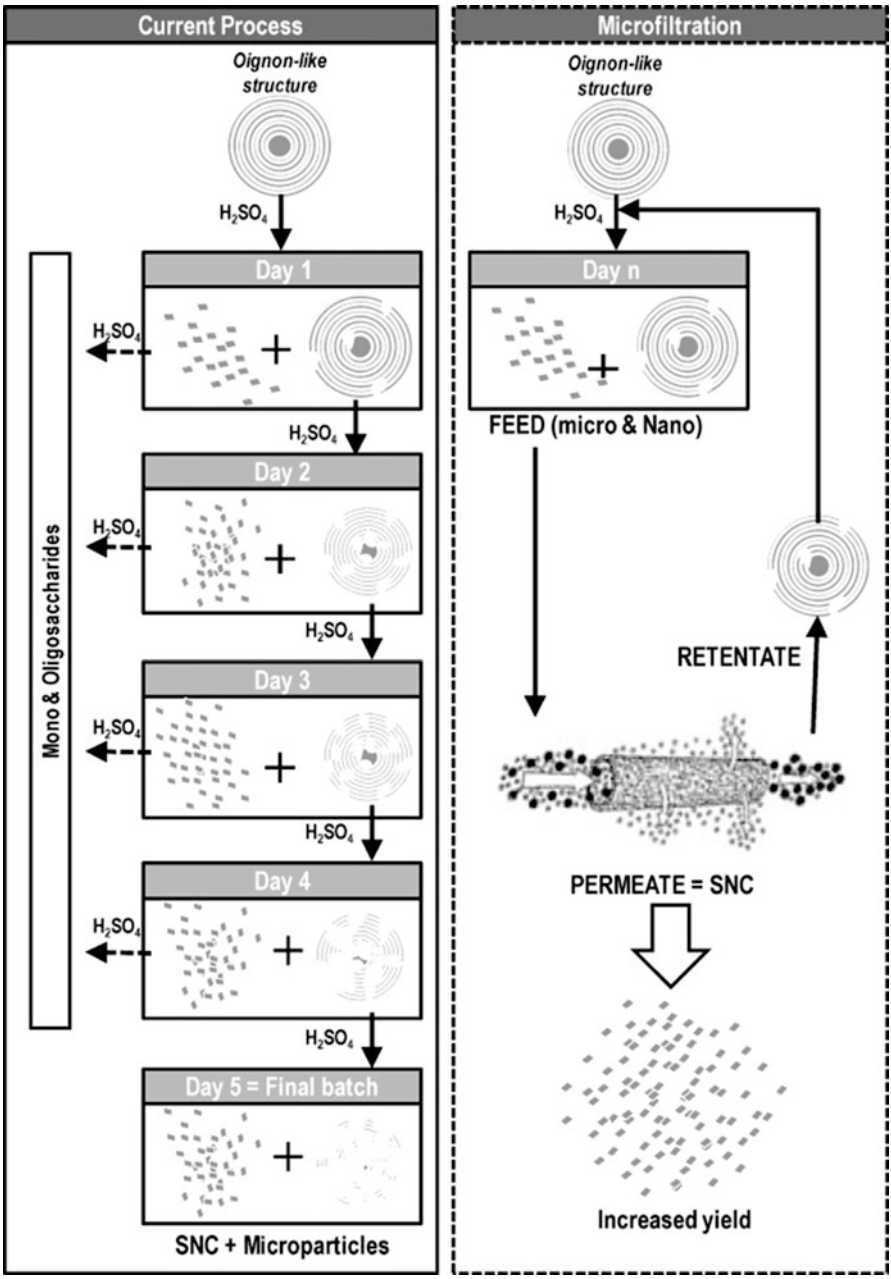

Fig. 6 Schematic comparison between the current preparation process involving the progressive production of starch nanocrystals as evidenced in LeCorre et al. (2011b) and the proposed microfiltration process (Reproduced with permissions from LeCorre et al. (2011c))

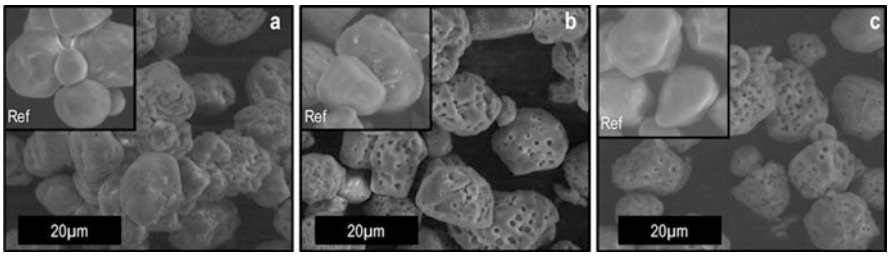

Fig. 7 Scanning electron micrographs of waxy maize starch granules after 2 h of enzymatic hydrolysis pretreatment with (**a**) α-amylase, (**b**) β-amylase, and (**c**) glucoamylase under optimal conditions (Reproduced with permissions from LeCorre et al. (2012b))

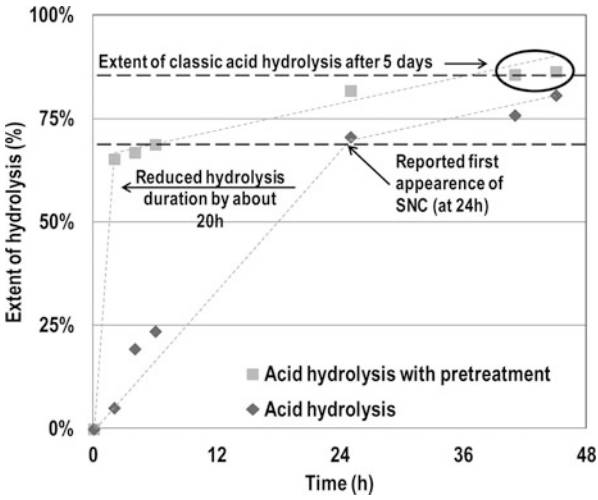

Fig. 8 Kinetics of sulfuric acid hydrolysis of non-pretreated (filled lozenges) and pretreated (*gray squares*) waxy maize starch (Reproduced with permissions from LeCorre et al. (2012b))

which correspond to three different action mechanisms. It was observed that the latter was the most efficient for producing microporous starch (Fig. 7) while keeping intact the semicrystalline structure of starch. Upon 2 h pretreatment of waxy maize starch granules, the extent of acid hydrolysis currently reached in 24 and 120 h (5 days) was reached in only 6 and 45 h, respectively, as shown in Fig. 8. Similar crystallinity and crystallite size were reported for starch nanocrystals prepared from pretreated starch in 45 h than for unpretreated starch in 120 h.

3.4 Thermal Properties of Starch Nanocrystals

The most investigated potential application for starch nanocrystals is as nanofiller for the processing of polymer bionanocomposites. However, the processing step raises the question of the potential melting of starch nanocrystals at high temperatures. A study was conducted to assess the maximum processing temperature for starch

nanocrystals in the dry state and in excess water (LeCorre et al. 2012c). The influence of the botanical origin, amylose content, and crystalline type of native starch used to produce these nanocrystals on the thermal properties of the nanoparticles was also investigated. Five types of starches (waxy maize, normal maize, high amylose maize, potato, and wheat) and their corresponding starch nanocrystals were characterized by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).

It was shown that native starches showed as expected only one thermal transition that shifted to higher temperatures when decreasing the water content, whereas nanocrystals showed two transitions (LeCorre et al. 2012c). In excess water, the first peak was attributed to the first stage of crystallites melting (unpacking of the double helixes) and the second transition to the second stage of crystallites melting (unwinding of the helixes). B-type crystallinity starch nanocrystals gained more stability than A-type nanocrystals as they consist of more rigid crystallites. In the dry state, the peaks were attributed to crystallite melting, with a direct transition from packed helixes to unwound helixes. The presence of two peaks was attributed to the heterogeneity in crystallite quality. Limited influence of the amylose content of starch was reported.

Important information was provided from this study for the processing conditions of starch nanocrystal-based nanocomposites. It was concluded that starch nanocrystals can be used in wet processes, such as coating, keeping temperatures lower than 80–100 °C, and in dry processes at temperatures below 150–200 °C. Cross-linking of starch nanocrystals with sodium hexametaphosphate (SHMP) was found to hinder the phase transitions of the nanoparticles upon heating (Jivan et al. 2013).

3.5 Sustainability of Starch Nanocrystals

Being a bio-based material is not enough for starch nanocrystals to be sustainable. Their production and application processes need to have limited impacts on the environment, society, and economy. Indeed, sustainability was first coined by the Brundtland Commission (formally the World Commission on Environment and Development of the United Nations in 1983) and was defined as the "social and economic advance to assure human beings a healthy and productive life, but one that did not comprise the ability of future generations to meet their own needs" (Bruntland 1987). More recently, life cycle assessment (LCA) was standardized by the International Organization for Standardization (ISO) and largely contributed in assessing environmental impacts across all stages of production, use, and waste management for different materials.

The "environmentally sensitive" steps involved in the preparation of starch nanocrystals have been identified using life cycle assessment (LCA), and different scenarios have been proposed and compared according to different environmental impacts (LeCorre et al. 2013). A comparison to its main competitor, i.e., organically modified nanoclay (OMMT), was also proposed in this study. From an LCA point of view, the production of starch nanocrystal requires less energy than the

extraction of OMMT, but global warming and acidification indicators were higher than for OMMT. However, starch nanocrystals are renewable and biodegradable contrary to OMMT which contribute to nonrenewable energy and mineral depletion. Recommendations for the scaling-up of starch nanocrystal production process were made, and the main concern deals with the extensive use of land and water. It was suggested to:

1. Use noncultivated low-water-consuming naturally occurring starch sources, such as amaranth and some tubers, to limit acidification and eutrophication linked to starch cultivars.
2. Include less-water- and less-energy-consuming extraction and washing processes such as microfiltration, as described in LeCorre et al. (2011c).
3. Reach higher yield than current process.

4 Aqueous Dispersions of Starch Nanocrystals

The preparation of starch nanocrystals by acid hydrolysis treatment yields a diluted colloidal aqueous dispersion at the end of the process. The stability of this suspension depends on the dimensions of the dispersed particles and their size polydispersity and surface charge. The surface charge can be controlled through the nature of the acid used for the hydrolysis treatment. Indeed, HCl-prepared nanocrystals display an almost charge-free surface, whereas H_2SO_4 reacts with the nanocrystal surface hydroxyl groups via an esterification process inducing the grafting of anionic sulfate ester groups ($-OSO_3^-$). The presence of these randomly distributed negatively charged surface ester groups on the surface of the nanoparticle results in the formation of a negative electrostatic layer covering the nanocrystals and promotes their dispersion in water. The higher stability of H_2SO_4-prepared nanoparticles results therefore from an electrostatic repulsion between individual or aggregated nanoparticles.

Acid hydrolysis with HCl results in less stable suspensions as shown in Fig. 9 for waxy maize starch nanocrystals (Angellier et al. 2005a). It was clearly observed that the use of H_2SO_4 instead of HCl allows reducing the possibility of agglomeration of starch nanoparticles and limits their flocculation in aqueous medium. The kinetics of sedimentation of the nanoparticles was evaluated by small-angle light-scattering experiments performed on 3.4 wt% H_2SO_4-prepared starch nanocrystal aqueous suspensions (Angellier et al. 2005b). The results showed that there was no sedimentation of the nanocrystals for a period of at least 12 h. Nevertheless, the intensity of scattered light slightly was found to increase, revealing that starch nanocrystals tend to aggregate in aqueous medium but not sufficiently to induce a sedimentation phenomenon.

The dispersibility of starch nanocrystals in aqueous medium can also be controlled by adjusting the pH of the suspension. In a recent study, the effect of the pH of the dispersion on zeta potential, size distribution, and aggregation behavior of H_2SO_4-prepared starch nanocrystals was investigated (Wei et al. 2014). As the pH

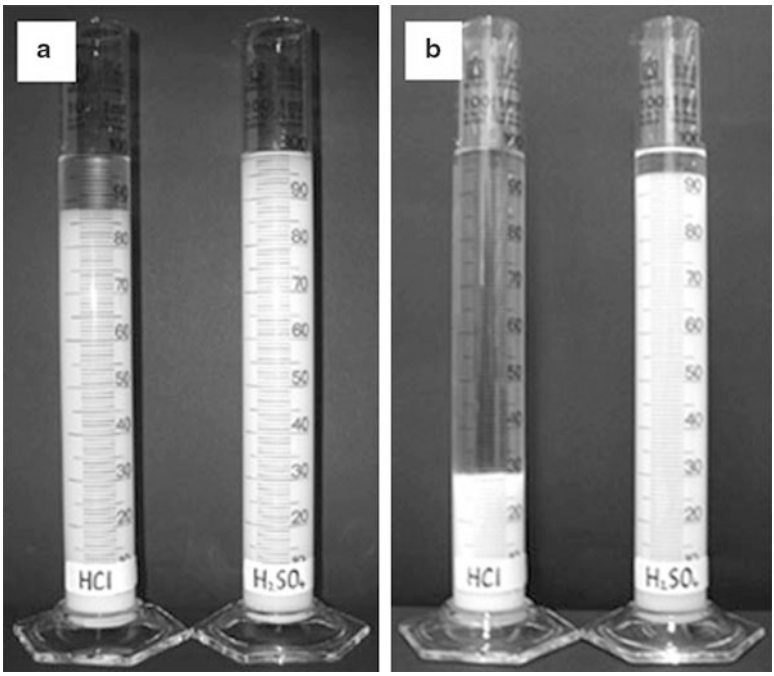

Fig. 9 Comparison of the sedimentation properties of HCl (*left* tube)- and H_2SO_4 (*right* tube)-hydrolyzed starch nanocrystals suspended in water after (**a**) 5 min and (**b**) 60 min (Reproduced with permissions from Angellier et al. (2005a))

of the dispersion increased, the zeta potential decreased, and aggregated platelet-like nanocrystals (1.5 μm) changed to monodispersed spherical-like nanoparticles (50 nm). Based on the results, it was suggested that stable starch nanocrystal aqueous suspensions could be obtained by adjusting the pH of the dispersion in the range 7.44–9.45. Improved dispersibility in water of starch nanocrystals obtained from H_2SO_4 hydrolysis of waxy maize starch was also observed upon cross-linking with sodium hexametaphosphate (SHMP) (Ren et al. 2012). The cross-linking reaction was performed in water at temperatures below the gelatinization temperature of starch and did not disrupt the crystalline structure of the nanocrystals. Stable and uniform starch nanocrystal aqueous suspensions were obtained.

The application of particles as a long-term stabilizer for the preparation of particle-stabilized emulsions, usually referred to as Pickering emulsions, is important for the development of novel food, cosmetic, and pharmaceutical products. Starch nanocrystals are surface active particles, and their food compatibility as well as complete biodegradability allows their use as particle emulsifier in food, cosmetics, and pharmaceuticals. The emulsifying ability of starch nanocrystals was investigated for oil-in-water emulsions consisting of 50 vol% paraffin liquid and equal concentration of water (Li et al. 2012). Stable emulsions were obtained when

the nanoparticle content relative to water was above 0.02 wt%. Both the size of the droplets and creaming decreased for increasing starch nanocrystal concentrations. The emulsions were very stable to coalescence over months, and the creaming was completely inhibited with 6.0 wt% starch nanocrystals, possibly because of the formation of an emulsion gel structure. When heating the emulsion above the gelatinization temperature of starch nanocrystals, it completely phase-separated.

5 Chemical Modification of Starch Nanocrystals

The high specific surface area resulting from their nanoscale dimensions coupled with the high density of surface hydroxyl groups allows targeted surface chemical modification of starch nanocrystals to introduce virtually any desired surface functionality. The reactive hydroxyl group content present on the surface of platelet-like starch nanocrystals was estimated to be ca. 14 % of the total amount available, i.e., 0.0025 mol.g^{-1} of starch nanocrystal (Angellier et al. 2005c). Different grafting strategies have been reported in the literature to graft either small molecules or polymers on the surface of starch nanocrystals as shown in Table 1. Mild reaction conditions should be applied to limit the reaction to the surface and

Table 1 Surface chemical modification of starch nanocrystals

Starch nanocrystal	Reagent	Objective of the modification	Reference
Corn	Polystyrene	Amphiphilic	Song et al. 2008
	Acetic anhydride	Hydrophobization	Xu et al. 2010
Pea	Microwave-assisted ROP of PCL	Blending with PLA	Yu et al. 2008
		Blending with PCL	Chang et al. 2009
Potato	$Sn(Oct)_2$-catalyzed ROP of PCL	Medical applications	Namazi and Dadkhah 2008
Waxy corn	Octanoyl, nonanoyl, decanoyl chloride	Hydrophobization	Namazi and Dadkhah 2010
Waxy maize	Alkenyl succinic anhydride	Dispersion in dichloromethane	Angellier et al. 2005c
	Phenyl isocyanate		
	Stearic acid chloride	Hydrophobization	Thielemans et al. 2006; Alila et al. 2011
	Poly(ethylene glycol) methyl ether		Thielemans et al. 2006
	Poly(tetrahydrofuran)	Compatibilization with polymer matrices	Labet et al. 2007
	Poly(ethylene glycol) monobutyl ether		
	Polycaprolactone		Labet et al. 2007; Habibi and Dufresne 2008

ROP ring-opening polymerization, *PCL* polycaprolactone, *PLA* polylactic acid, *$Sn(Oct)_2$* tin (II) octoate

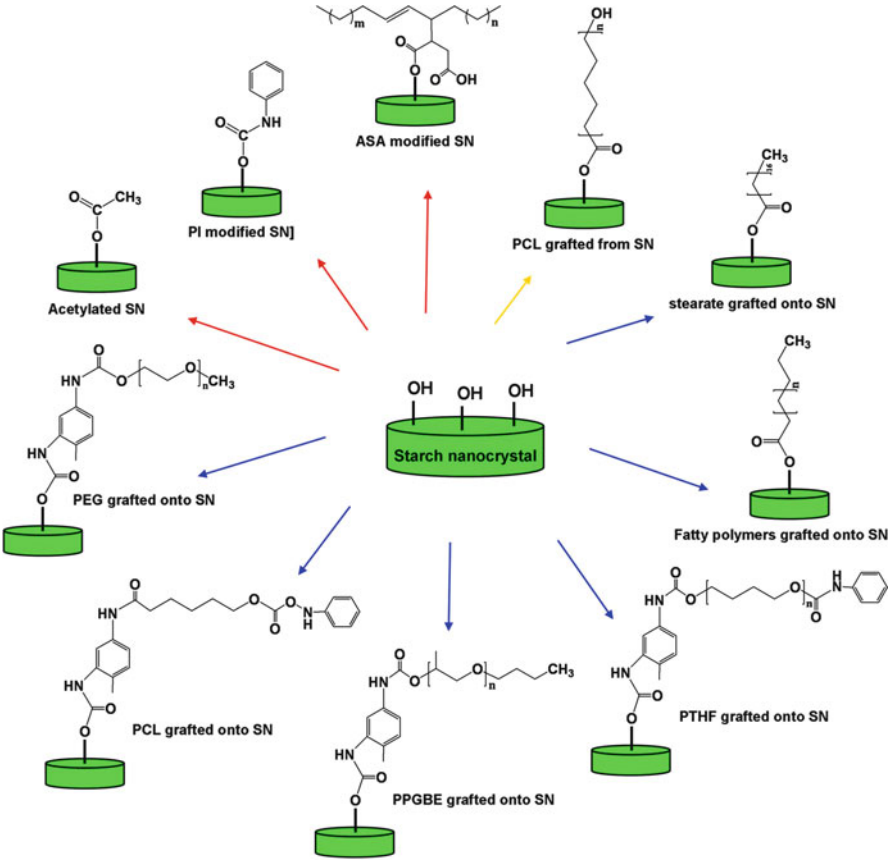

Fig. 10 Common chemical modifications of starch nanocrystals. *PTHF* poly(tetrahydrofuran), *PPGBE* poly(propylene glycol) monobutyl ether (Reproduced with permissions from Lin et al. (2012))

retain the original morphology of starch nanocrystals. The common surface chemical modifications of starch nanocrystals are schematically depicted in Fig. 10.

The surface of waxy maize starch nanocrystals was modified with alkenyl succinic anhydride (ASA) and phenyl isocyanate (PI) (Angellier et al. 2005c). The reaction was conducted in toluene/(dimethylamino)pyridine medium to avoid hydrolysis of ASA. Modified nanoparticles were characterized by Fourier transform infrared (FTIR) spectroscopy and X-ray photoelectron spectroscopy (XPS), contact angle measurements, TEM, and X-ray diffraction analysis. The lower polarity of the modified nanocrystals was also demonstrated by a simple experiment (Fig. 11). The pristine and modified nanoparticles were mixed with two immiscible solvents having both different polarities and densities (water and dichloromethane were chosen for the test) and the visual inspection of the mixture indicated with which solvent they are best wetted. It was observed that unmodified starch nanocrystals

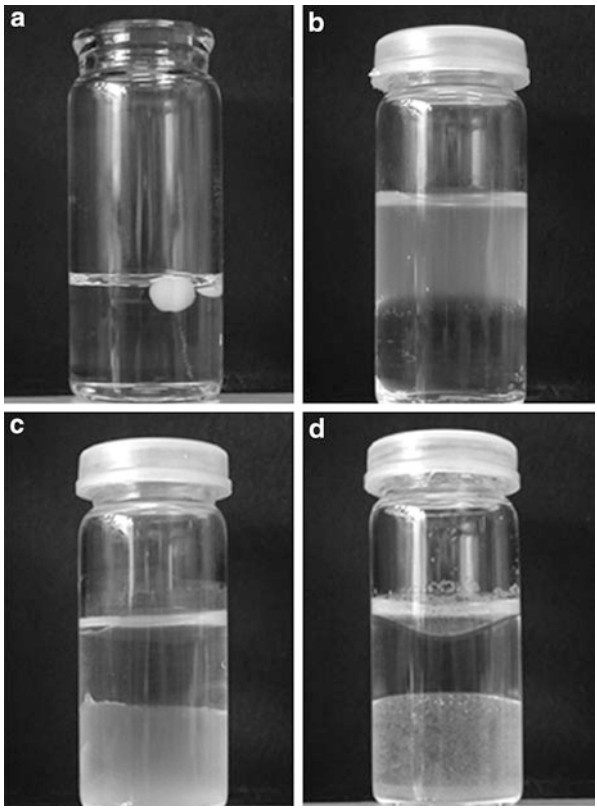

Fig. 11 Wettability tests: (**a**) a drop of an aqueous suspension of waxy maize starch nanocrystals in dichloromethane, (**b**) migration of unmodified starch nanocrystals in distilled water, migration of (**c**) ASA-modified and (**d**) PI-modified starch nanocrystals modified with (**c**) ASA and (**d**) PI in dichloromethane (Reproduced with permissions from Angellier et al. (2005c))

remained in the water medium whereas modified nanoparticles migrated towards the dichloromethane phase (Fig. 11).

The surface chemical modification of starch nanocrystals with longer chains can yield some extraordinary possibilities. Indeed, the grafted chains can act as binding sites for active agents in drug delivery systems or for low toxins in purifying and treatment systems. Moreover, for composite applications, the grafted chains may be able to interdiffuse upon heating to form the polymer matrix resulting in near-perfect stress transfer at the interface because of the covalent linkage between the reinforcement and the matrix.

Stearic acid chloride and poly(ethylene glycol) methyl ether (PEGME) were successfully grafted on the surface of starch nanocrystals (Thielemans et al. 2006). Extensive crystallization of the stearate moieties grafted to the starch nanoparticle surface, forming a crystalline hydrophobic shell around the hydrophilic starch nanocrystal, was observed. On the contrary, strong interactions between the grafted PEGME chains and the starch particle limited PEGME crystallization. These interactions caused the PEGME chains to align themselves with the nanocrystal surface. Both modifications, however, exhibit a large effect on the individualization

of the nanocrystals because of reduced hydrogen bonding and polar interactions between the individual particles.

These stearate-modified starch nanocrystals were employed as adsorbents for the removal of a wide range of dissolved aromatic compounds from water (Alila et al. 2011). The grafted stearate long chains enhanced the adsorption capacity of aromatic organic compounds on the nanometric substrate, which ranged between 150 and 900 μmol g^{-1}, and the maximum adsorption amount reached 100 mg g^{-1}. The adsorption isotherms were accurately described by the Langmuir model, and the adsorption kinetics followed a two-step process with first pure adsorption of the aromatic compounds onto the surface of the nanoparticles followed by a diffusion of the compounds into the layer of surface chains grafted onto the starch nanocrystals. Furthermore, the feasibility of using these nanoparticles in continuous flow mode processes was confirmed using a fixed-bed column setup. The fixed-bed column could also be regenerated by washing with ethanol and was found not to exhibit any loss in adsorption capacity over multiple adsorption-desorption cycles.

Even longer polymeric chains such as polycaprolactone (PCL) were grafted on the surface of starch nanocrystals using "grafting onto" (Labet et al. 2007; Habibi and Dufresne 2008) and "grafting from" (Xu et al. 2010; Yu et al. 2008; Namazi and Dadkhah 2008) approaches. Polystyrene was also grafted on starch nanocrystals using a "grafting from" strategy (Li et al. 2012). It was systematically verified that the crystalline structure of the nanoparticles was not changed after grafting and that it only occurred on the surface. The surface coating of the nanoparticles allowed dispersion in organic solvents and compatibilization with apolar polymeric matrices. Amphiphilic starch nanocrystals prepared by the graft copolymerization of starch nanocrystals with styrene were well dispersed both in polar and nonpolar solvents (Li et al. 2012). Moreover, microscopic observations of modified starch nanocrystals showed the individualization of nanoparticles. The grafting efficiency of PCL chains onto the surface of starch nanocrystals decreased with the length of the polymeric chains, as expected (Labet et al. 2007). However, the PCL content for grafted nanoparticles was higher than 50 wt% as determined from elemental analysis.

6 Starch Nanocrystal-Reinforced Polymer Nanocomposites

By adding conventional high modulus reinforcements into a polymer, usually the modulus and the strength of the composite are improved, while the ductility and the impact strength are decreased. In the case of well-dispersed nanoparticles, the modulus and strength can be improved without significant change in ductility since they do not create high stress concentrations due to their nanosize. However, to improve the mechanical properties of the host material and take advantage of this property, special care needs to be paid to the processing on the nanocomposite. Indeed, it is well known that the macroscopic mechanical properties of heterogeneous materials depend on the specific behavior of each phase and the composition (volume fraction of each phase) but also on the morphology (spatial arrangement of the phases) and the interfacial properties.

6.1 Processing of Starch Nanocrystal-Reinforced Polymer Nanocomposites

Different polymeric matrices have been associated with starch nanocrystals to prepare nanocomposite materials. Some systems reported in literature are collected in Table 2. The first investigation was performed using a copolymer of styrene and

Table 2 Polymer nanocomposites obtained from starch nanocrystals and polymeric matrix

Polymer	Starch nanocrystal	Processing technique	Reference
Carboxymethyl chitosan	Waxy maize	Casting/ evaporation	Duan et al. 2011
NR	Waxy maize	Casting/ evaporation	Angellier et al. 2005b, d, 2006a; Mélé et al. 2011; LeCorre et al. 2012d
	Amylomaize		LeCorre et al. 2012d
	Normal maize		
	Potato		
	Wheat		
	Potato		Bouthegourd et al. 2011
PCL	Waxy maize	Casting/ evaporation	Habibi and Dufresne 2008
PHO	Pea	Casting/ evaporation	Dubief et al. 1999
PLA	Pea	Casting/ evaporation	Xu et al. 2010
Poly(BuMA)	Waxy maize	Casting/ evaporation	BelHaaj et al. 2013
Poly (S-co-BuA)	Potato	Freeze-drying/ hot-pressing	Dufresne et al. 1996; Dufresne and Cavaillé 1998
	Waxy maize		Angellier et al. 2005a
Pullulan/ sorbitol	Waxy maize	Casting/ evaporation	Kristo and Biliaderis 2007
PVA/glycerol	Pea	Casting/ evaporation	Chen et al. 2008a
Starch/glycerol	Waxy maize	Casting/ evaporation	Angellier et al. 2006b; Garcia et al. 2009, 2011
Starch/sorbitol	Waxy maize	Casting/ evaporation	Viguié et al. 2007
WPU	Potato	Casting/ evaporation	Chen et al. 2008b
	Pea		Yu et al. 2008; Wang et al. 2010; Zou et al. 2011
SPI/glycerol	Pea	Freeze-drying/ hot-pressing	Zheng et al. 2009

NR natural rubber, *PCL* polycaprolactone, *PHO* poly(β-hydroxyoctanoate), *PLA* polylactic acid, *Poly(BuMA)* poly(butyl methacrylate), *Poly(S-co-BuA)* poly(styrene-co-butyl acrylate), *PVA* polyvinyl alcohol, *WPU* waterborne polyurethane, *SPI* soy protein isolate

butyl acrylate (poly(*S*-*co*-BuA)) matrix in latex form (Dufresne et al. 1996). This aqueous dispersion was mixed with the aqueous suspension of starch nanocrystals, and the mixture was freeze-dried and hot-pressed. Following works generally involved a casting/evaporation method because of its simplicity and good dispersion level of the nanoparticles in water that is preserved when processing the material in aqueous medium. Therefore, most polymeric matrices consisted of water-soluble polymers such as carboxymethyl chitosan (Duan et al. 2011), pullulan (Kristo and Biliaderis 2007), polyvinyl alcohol (PVA) (Chen et al. 2008a), gelatinized starch (Angellier et al. 2006b; Garcia et al. 2009, 2011; Viguié et al. 2007), and soy protein isolate (SPI) (Zheng et al. 2009) or polymers in the latex form (aqueous polymer dispersion) such as natural rubber (NR) (Angellier et al. 2005b, d, 2006a; Mélé et al. 2011; LeCorre et al. 2012d; Bouthegourd et al. 2011), poly(β-hydroxyoctanoate) (PHO) (Dubief et al. 1999), poly(*S*-*co*-BuA) (Dufresne et al. 1996; Dufresne and Cavaillé 1998; Angellier et al. 2005a), and waterborne polyurethane (WPU) (Yu et al. 2008; Chen et al. 2008b; Wang et al. 2010; Zou et al. 2011). However, care must be taken regarding the processing temperature to avoid gelatinization of starch nanocrystals. For instance, for the processing of thermoplastic starch reinforced with starch nanocrystals, the temperature of gelatinized starch was decreased to 40 °C before adding starch nanocrystals (Angellier et al. 2006b; Viguié et al. 2007).

To improve the nanoparticle-polymer interface and promote individualization of starch nanocrystals, in situ one-pot miniemulsion polymerization reaction of butyl methacrylate (BuMA) in the presence of starch nanocrystals was conducted (BeHaaj et al. 2013). It imparted a high degree of binding between the nanoparticles and the polymer particles. It was shown that starch nanocrystals were not sufficient to stabilize the monomer droplets, but provided a synergetic stabilization effect when used together with a cationic surfactant, reducing thus the required surfactant amount by a factor of 4. For starch nanocrystal-reinforced polycaprolactone (PCL) (Habibi and Dufresne 2008) and polylactic acid (PLA) (Xu et al. 2010), the surface chemical modification of the nanocrystals was performed to allow their dispersion in dichloromethane, a solvent for both PCL and PLA, that was used as processing liquid medium.

6.2 Mechanical Properties of Starch Nanocrystal-Reinforced Polymer Nanocomposites

To evaluate the mechanical reinforcing effect of starch nanocrystals in a polymeric matrix, mechanical measurements in both the linear (dynamic mechanical analysis – DMA) and nonlinear range (tensile tests) can be performed. In the pioneering work on potato starch nanocrystal-reinforced poly(*S*-*co*-BuA), a high reinforcing effect of the filler was observed by DMA, especially above the glass transition temperature (Tg) of the matrix (Dufresne et al. 1996; Dufresne and Cavaillé 1998). This reinforcing effect was later confirmed by most authors for different polymeric systems. For instance, Fig. 12 shows the DMA curves

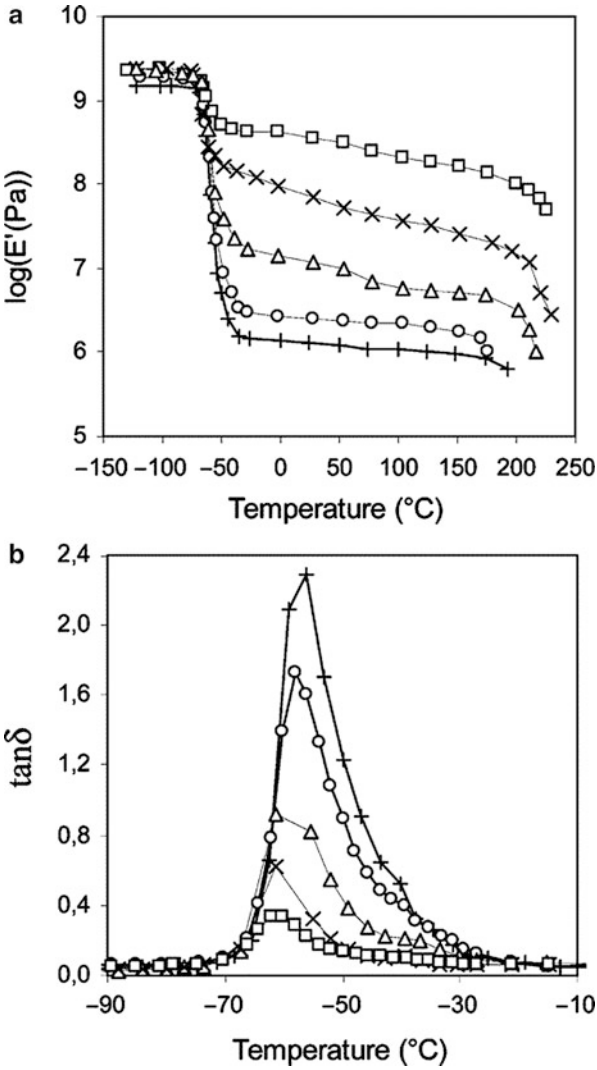

Fig. 12 Logarithm of the storage tensile modulus E′ (**a**) and tangent of the loss angle tan δ (**b**) vs temperature at 1 Hz for NR films waxy reinforced with 0 (+), 5 (○), 10 (Δ), 20 (×), and 30 wt% (□) maize starch nanocrystal (Reproduced with permissions from Angellier et al. (2005d))

obtained for waxy maize starch nanocrystal-reinforced NR (Angellier et al. 2005d). A continuous increase of the storage modulus was observed, mainly above the Tg of the matrix, when adding the nanoparticles (Fig. 12a). The evolution of the loss angle (Fig. 12b) displays a peak located in the temperature range of the glass transition of the NR matrix. A decrease of the magnitude of the peak when increasing the starch nanocrystal content is observed. It is ascribed to both the decrease of the number of mobile units participating to the relaxation process and decrease of the magnitude of the modulus drop associated with Tg.

Obviously, the magnitude of the relative reinforcing effect depends on the intrinsic mechanical properties of the neat polymer. A higher reinforcing effect is

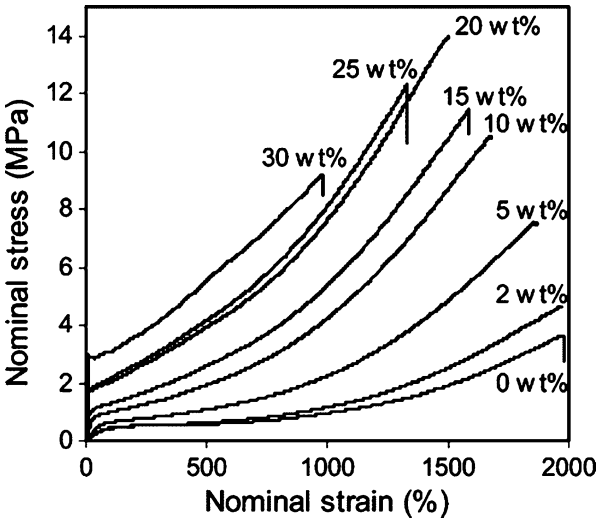

Fig. 13 Typical nominal stress vs nominal strain curves of waxy maize starch nanocrystal-reinforced NR nanocomposite films. The starch nanocrystal contents are indicated in the figure (Reproduced with permissions from Angellier et al. (2005d))

generally observed when using a soft polymer matrix (amorphous rubbery polymer) than when using a semicrystalline or amorphous glassy polymer.

Nonlinear tensile tests are more classically used to investigate the mechanical behavior of polymeric materials. The stiffness or tensile modulus can be determined from the initial slope of the stress-strain curves, and ultimate properties such as strength and strain at break correspond to the experimental data associated with the break of the sample. The introduction of starch nanocrystals within a polymeric matrix generally induces an increase of both the tensile modulus and strength, whereas the strain at break decreases. Typical stress-strain curves for waxy maize starch nanocrystal-reinforced NR are shown in Fig. 13. However, a decrease of the reinforcing capability of starch nanocrystals has been reported in some studies for higher filler contents because of self-aggregation within the polymeric matrix (Chen et al. 2008a; Zheng et al. 2009).

The reinforcing effect of starch nanocrystals to NR was compared to the one provided by other fillers such as clays, organoclays, carbon black, fly ash, and chitin nanocrystals (Angellier et al. 2005d). Starch nanocrystals were not as competitive as organoclays. They displayed a lower tensile modulus but higher ultimate properties (strength and elongation at break) than chitin nanocrystals because of the higher aspect ratio of the latter. However, starch nanocrystals presented better mechanical properties than fly ash and carbon black. It was shown that the addition of only 10 wt% starch nanocrystals to NR induced a reinforcing effect similar, in terms of stiffness, to the one observed with 26.6 wt% carbon black while maintaining a higher elongation at break.

Starch is a hydrophilic material, and therefore the performance of starch nanocrystals can be altered in moist atmosphere. The effect of moisture content was investigated for NR-based materials, and the stiffness of the nanocomposite was found to decrease when increasing the water content (Angellier et al. 2005d).

For some systems, an increase of the glass transition temperature (Tg) of the matrix when increasing the starch nanocrystal content was observed, attributed to the existence of an interphase of immobilized matrix material in contact with particle surface (Kristo and Biliaderis 2007; Angellier et al. 2006b; Viguié et al. 2007). The formation of this interphase resulted from favorable filler/matrix interactions. Interestingly, a considerable slowing down of the recrystallization (retrogradation) of the thermoplastic starch matrix upon storage in humid atmosphere was observed when adding starch nanocrystals (Angellier et al. 2006b; Viguié et al. 2007). For this system, the reinforcing effect was more significant than in NR because of strong interactions between the filler and amylopectin chains from the matrix and possible crystallization at the filler/matrix interface.

Waterborne polyurethane (WPU) was reinforced with starch and cellulose nanocrystals obtained by acid hydrolysis of waxy maize starch granules and cotton linter pulp, respectively (Wang et al. 2010). A synergistic effect was observed when adding 1 wt% starch and 0.4 wt% cellulose nanocrystals with a significant improvement in tensile strength, Young's modulus, and tensile energy at break without significant loss for the elongation at break. The mechanical performance was found to be higher than for individual filler, but it is worth noting that the total filler content was different. In the ternary system, the formation of much jammed network consisting of nanoparticles with different geometrical characteristics was suggested to play an important role in the enhancement of the cross-linked network. Moreover, strong hydrogen bonding interactions between the nanoparticles and between the nanoparticles and the hard segments of WPU matrix were suspected to improve the mechanical properties.

6.3 Effect of Chemical Modification on Mechanical Performance

Surface chemical modification of starch nanocrystals allows broadening the range of polymeric matrices that can be used in association with them through the possibility of processing composite materials from an organic solvent instead of aqueous dispersions. It consists in transforming the polar hydroxyl groups from the surface of starch nanocrystals into moieties capable of enhancing interactions with nonpolar media and polymers.

The mechanical performance of ASA- and PI-modified starch nanocrystal-reinforced NR was investigated and compared to the one of unmodified nanoparticle-based systems (Angellier et al. 2005d). The chemical modification of the nanoparticles was found to lower their reinforcing capability. This unusual behavior was ascribed to hindered interactions between chemically modified particles resulting from their coating with the grafting agents. However, it is worth noting that the nanocomposite films were obtained by casting/evaporation from an aqueous or toluene mixture, respectively, for unmodified and modified nanoparticles. On the contrary, both a higher modulus and elongation at break were reported when PCL-decorated starch nanocrystals were used in a PCL matrix,

compared to unmodified nanoparticles (Habibi and Dufresne 2008). For both systems, nanocomposite films were obtained from the same processing conditions by casting/evaporation from dichloromethane. Obviously, strong self-aggregation of the unmodified nanocrystals in dichloromethane was the source of the poor mechanical properties of the nanocomposites prepared from these particles. It was observed that the addition of 5 wt% PCL-grafted starch nanocrystals in a PLA matrix induced simultaneous enhancements of the strength and elongation at break (Xu et al. 2010). The grafted rubbery PCL component was supposed to improve the flexibility of PLA chains.

6.4 Reinforcing Mechanism of Starch Nanocrystals

A comprehensive study was performed to try to understand the reinforcing mechanism of starch nanocrystals in a non-vulcanized NR matrix through the development of a phenomenological modeling approach (Mélé et al. 2011). Nonlinear dynamic mechanical experiments highlighted the significant reinforcing effect of starch nanocrystals and the occurrence of the Mullins and Payne effects. Two models have been used to predict the Payne effect assuming the preponderance of either filler-filler (Kraus model) or matrix-filler (Maier and Goritz model) interactions. With the Maier and Goritz model, it was demonstrated that the phenomena of adsorption and desorption of NR chains on the nanofiller surface governed the nonlinear viscoelastic properties, even if the formation of a percolating network for filler contents higher than 6.7 vol% (i.e., around 10 wt%) was evidenced by the Kraus model.

6.5 Swelling Properties of Starch Nanocrystal-Reinforced Polymer Nanocomposites

The swelling process and its kinetics allow quantifying the capacity of a linear or branched polymer to dissolve or of a cross-linked polymer to swell in different liquid or vapor media. The interaction of polymeric materials with solvents can be an issue from a technological point of view because the dimensions and physical properties of the material may change with the penetration of solvent molecules into the specimen.

Obviously, the effect of starch nanocrystals which are hydrophilic by nature on the swelling property of the nanocomposite system strongly depends on the nature of the matrix (polar or apolar) and swelling liquid (hydrophilic or hydrophobic). The addition of starch nanocrystals to poly(*S*-*co*-BuA) (Dufresne and Cavaillé 1998) or NR (Angellier et al. 2005b; LeCorre et al. 2012d) was found to increase the swelling of the material by water most probably because of the hydrophilic nature of starch and hydrophobic nature of the matrix. The coefficient of diffusion of water increased as well but showing two well-defined regions. Below a critical starch nanocrystal concentration, the evolution of the diffusion coefficient was

relatively low, whereas it was more significant above it. This behavior change was assumed to be due to the establishment of a starch nanocrystal network through strong hydrogen linkages between nanoparticle clusters but also to favorable interactions between the NR matrix chains and the filler. The critical concentration was assumed to be around 20 wt% and 10 wt%, for poly(*S*-*co*-BuA) and NR, respectively.

Then, the percolation threshold is an important parameter that is assumed to govern the swelling properties of the nanocomposite. For starch nanocrystal-reinforced NR, the percolation threshold of the nanoparticles was observed around 15 wt% from electrical conductivity measurements (Bouthegourd et al. 2011). Moreover, the diffusion coefficient of water for NR-based nanocomposite films was found to be correlated with the composition of starch and to decrease when increasing the initial amylose content of native starch (LeCorre et al. 2012d). It was attributed to the presence of amorphous or less organized and therefore less bonded amylose chains in starch nanocrystals prepared from higher amylose content starch. This amorphous material was supposed to not participate to the formation of a diffusing network but to participate to the water-sorption process explaining why similar equilibrium water uptake values were reached.

For soy protein films, a slight increase of the water uptake was reported for increasing starch nanocrystal contents (Zheng et al. 2009). On the contrary, a decrease of the water uptake was observed for sorbitol-plasticized pullulan (a hydrophilic system) when adding starch nanocrystals particularly at high filler loading levels (Kristo and Biliaderis 2007). Again, it was ascribed to the formation of a three-dimensional network of nanoparticles that was able to restrict the swelling of the polar matrix. The decrease in the water uptake of PVA (Kristo and Biliaderis 2007) and carboxymethyl chitosan (Duan et al. 2011) upon starch nanocrystal addition was attributed to the crystallinity of the nanoparticles and strong interfacial interactions between both polar components in the nanocomposite film. On the contrary, for glycerol-plasticized thermoplastic starch, the composites reinforced with starch nanocrystals were found to absorb more water than the neat matrix (Garcia et al. 2009). It was ascribed to a relocalization of glycerol around the nanoparticles leading to more hydroxyl groups in the matrix able to interact with water molecules.

When using an apolar matrix and performing swelling experiments in a hydrophobic liquid medium, restriction of the liquid absorption is expected when adding hydrophilic starch nanocrystals. Indeed, swelling of the NR matrix by toluene was found to decrease when adding starch nanocrystals, and the toluene diffusion coefficient decreased strongly for low filler contents and more progressively above 10 wt% (Angellier et al. 2005b). For low nanocrystal contents, a correlation between the toluene-sorption behavior and calculated specific surface area of nanocrystals obtained from different botanical origin starches was observed (LeCorre et al. 2012d). The higher the theoretical specific surface area, the higher the toluene uptake, and the lower the diffusivity. For higher starch nanocrystal contents, this phenomenon was not observed. It was supposed to be due to aggregation at higher filler contents.

6.6 Barrier Properties of Starch Nanocrystal-Reinforced Polymer Nanocomposites

Barrier properties using bio-based materials are becoming increasingly advisable in our society to develop environmentally friendly materials for different applications such as packaging. Indeed, most materials used for food packaging are practically nondegradable petrochemical-based polymers, representing a serious environmental problem. The main reason for their use is due to their easiness of processability, low cost, and excellent barrier properties. However, given their platelet-like morphology and highly crystalline nature, starch nanocrystals were suspected, as nanoclays do, to create a tortuous diffusion pathway for penetrant molecules. Nevertheless, few reports investigated the barrier properties of starch nanocrystal-reinforced nanocomposites.

For NR films, a continuous and significant reduction of the permeability to water vapor and oxygen was reported when adding starch nanocrystals up to 30 wt% (Angellier et al. 2005b). A substantial 40 % decrease of the water vapor permeability (WVP) value was also observed for cassava starch films plasticized with glycerol when adding only 2.5 wt% of waxy maize starch nanocrystals (Garcia et al. 2009). However, when using a glycerol-plasticized waxy maize starch matrix, a close association between starch nanocrystals and glycerol-rich domains was suspected to explain the unexpected increase of the WVP value upon adding starch nanocrystals (Garcia et al. 2011). A similar decrease of the WVP value was reported for carboxymethyl chitosan films when adding starch nanocrystals as shown in Fig. 14 (Duan et al. 2011). For sorbitol-plasticized pullulan films, no significant differences were observed in WVP values when adding up to 20 wt% starch nanocrystals (Kristo and Biliaderis 2007). Nevertheless, above this critical value, a significant decrease of WVP was reported. On the contrary, a detrimental effect of starch nanocrystals on the WVP of NR films was reported (LeCorre et al. 2012d). However, it is worth noting that in this study, WVP was measured

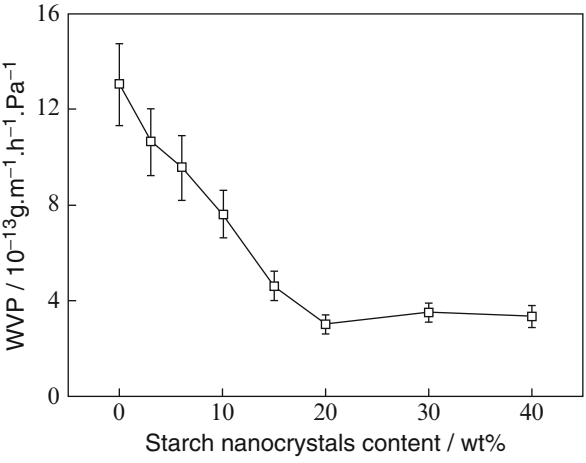

Fig. 14 Water vapor permeability of carboxymethyl chitosan nanocomposite films with different starch nanocrystal contents (Reproduced with permissions from Duan et al. (2011))

under tropical conditions (38 °C, 90 %RH), and it was suggested that the hydrophilic nature of starch nanocrystals was predominant.

Starch nanocrystals were also reported to be promising bio-nanofillers for improving barrier properties of bio-based coated papers (LeCorre et al. 2014). The nanoparticles have been introduced in a starch-based coating, and the properties of the ensuing coating color and final coated paper have been investigated. Coating colors containing starch nanocrystals showed higher viscosity but were still processable. It was shown that the nanoparticles can resist studied drying processes without melting and that their addition to the coating color decreased WVP and compensated some of the loss of mechanical properties due to the use of the water-based coating.

6.7 Drug Carrier Systems

Biomedical applications represent a niche market but with high added value for many materials. A "green" method of ionic cross-linking through Ca^{2+}, forming semi-interpenetrating polymeric networks (semi-IPN), was used to develop starch nanocrystal-reinforced pH-sensitive alginate microsphere-controlled release system for drug delivery (Lin et al. 2011). Rod-like cellulose and chitin nanocrystals were also investigated in this study. The presence of polysaccharide nanocrystals in alginate-based microspheres showed more consistent swelling patterns, higher encapsulation efficiency, and promising sustained release profiles of the drug. It was ascribed to the improvement of the stability of the cross-linked network structure and enhancement of mechanical strength, mainly for higher aspect ratio cellulose and chitin nanorods, of nanocomposite microspheres. Moreover, because of the restriction effect of rigid nanocrystals, the free diffusing routes of the drug molecules were increased and endowed the microspheres improved drug loading and sustained release profiles.

7 Conclusions

There is a growing interest in both the nonfood usage of renewable resources and nanosized particles. Polysaccharides are probably promising candidates to meet this demand since they are low-cost, abundant, and renewable materials. This chapter provides a comprehensive overview of recent advances published in the field of starch nanocrystals. This field is at the moment less reported in the literature than nanocellulose which is now growing very fast. However, there is no doubt that increasing works start to be done and will be done in the near future on starch nanoparticles. Starch consisting of crystalline and amorphous domains is a possible candidate for organic nanofillers because the amorphous domains can be removed under certain conditions such as controlled acid hydrolysis. Ensuing nanosized crystalline particles, usually known as starch nanocrystals, can be used as reinforcing nanofillers in polymer nanocomposites, thereby creating opportunities

to develop high-performance and highly durable engineering materials from a renewable resource that could be suitable for potential consumer, structural, and biomedical applications.

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Polysaccharide Production by Submerged Fermentation

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Contents

Abstract

This chapter describes the importance of the polysaccharides obtained from different natural sources as well as their natural occurrence. An overview on the different biological activities of the polysaccharides obtained from fungi, bacteria, and algae is provided. The potential of microbial organisms, especially microfungi and bacteria, as well as macromycete fungi, during production of bioactive polysaccharides at large scale is recognized. The features of the submerged fermentation technology are disclosed considering that this cultivation technique has played and continues playing a crucial role in the industrial production of different polysaccharides with diverse biological activities. Main topics related to the production of these compounds by submerged fermentation making emphasis in the production of fungal and bacterial polysaccharides are briefly described as well. Finally, some words on the significance of the research and development on production of bioactive polysaccharide are presented.

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1 Introduction

Polysaccharides are compounds widely distributed in the nature. They are present in bacteria, fungi, algae, plants, and animals. These natural biological polymers are nontoxic and have different functions, the best known of which is to be an energy reserve. They are also structural molecules as cellulose in plants, β-glucans in microorganisms (fungi and bacteria), and chitin in arthropods. In addition, these biomolecules are essential for the metabolism of living beings along with proteins and polynucleotides. They also play an important role in cellular communication, adhesion, and molecular recognition for immune system, fertilization, pathogenesis prevention, and blood coating, among others (Yang and Zhang 2009; Zong et al. 2012; Öner 2013).

Currently, people seek foods that are not only nutritive, safe, nice in sight, and with good taste but also offer health benefits like treatment and prevention of diseases. Research advances have allowed the identification of biologically active compounds of different nature in foods like peptides, carbohydrates, polyphenols, carotenoids, phytosterols, fatty acids, etc. (Mazza 2000; Webb 2007; Cheung 2008; Aluko 2012). In particular, a group of polysaccharides have gained a special interest not only because they are an important energy source in human diet but also because they exhibit special features: their chemical structures with glycosidic linkages make them resistant to the digestion; they can be used as prebiotics; they show diverse biological activities such as antitumor, anticarcinogenic, immunomodulatory, protective against mutagens, and hypoglycemic activities, among others; and they can be used in food, cosmetic, agricultural and pharmaceutical industries (Izydorczyk et al. 2005; Mantovani et al. 2008; Brown and Williams 2009; Yang and Zhang 2009; Patel et al. 2010; Lee et al. 2012; Zong et al. 2012; El Enshasy and Hatti-Kaul 2013; Öner 2013; Zhang et al. 2013).

Submerged fermentation is the most employed procedure for the production of polysaccharides. This is explained by the facility to manipulate and optimize the operating parameters during this type of fermentation (Xiao et al. 2010). Submerged fermentation has been used to cultivate different bacteria and fungi in order to obtain intracellular and extracellular polysaccharides exhibiting diverse properties and biological activities (Suárez and Nieto 2013).

The objective of this chapter is to describe the importance of the polysaccharides obtained from different natural sources and provide main topics related to the production of these compounds by submerged fermentation and their applications in food and pharmaceutical industries.

2 Polysaccharides

The polysaccharides, also named glycans, are polymers condensed from a large number of monosaccharides. These monosaccharides or simple derivatives from them can be obtained by complete hydrolysis using acids or specific enzymes. D-glucose is the predominant monosaccharide in polysaccharides although D-fructose, D- and L-galactose, D-xylose, and D-arabinose are also common. Some monosaccharide derivatives can also be found as hydrolysis products of natural polysaccharides as D-glucosamine, D-galactosamine, D-glucuronic acid, *N*-acetylneuraminic acid, and *N*-acetylmuramic acid. In these polymers, monosaccharides are linked by glycosidic bonds between the glycosyl residue of the hemiacetal and the hydroxyl residue of another monosaccharide. Polysaccharides differ from each other in their repeated monosaccharidic units, length of their chains, and degree of branching. They can be linear or branched, and with the exception of cyclic polysaccharides known as cycloamyloses or cyclodextrins, their chains have a nonreducing (terminal) end and a reducing end. The general formula of polysaccharides is $C_x(H_2O)$, where x is usually a number between 200 and 2,500 (Izydorczyk et al. 2005; Aluko 2012; Zong et al. 2012).

Polysaccharides can be divided into two classes according to the number of different monosaccharides in their structure: homopolysaccharides containing a single type of monosaccharide and heteropolysaccharides containing two or more different monosaccharidic units. The homopolysaccharides are linear or branched chains, are commonly named by the repeating monosaccharidic unit, and have the same or different glycosidic bonds as presented in Table 1. The glycosidic linkages may have α- or β-configurations in several positions. On the other hand, heteropolysaccharides can have different bond types within each monosaccharidic unit as well as different types and sequences of glycosidic linkages.

According to another classification, polysaccharides can be divided into neutral, acid, or basic. Neutral polysaccharides like amylose, cellulose, and amylopectin are composed only of glucose. Acid or anionic polysaccharides contain derivatives of acid sugars, and their structures have negative charge as pectin and alginates. To date only one cationic polysaccharide is known, the chitosan obtained by modification of the animal polysaccharide chitin. The chitosan is a polymer made of β-(1→4)-2-amino-2-desoxi-D-glucopiranose, which has positive charges at pH between 6 and 7 (Izydorczyk et al. 2005).

2.1 Overview of Biological Activity of Polysaccharides

Over the last years, different research efforts have been done to study the in vitro and in vivo biological activity of different polysaccharides obtained both from natural sources and at industrial level. Among the pharmacological activities reported for polysaccharides, antibiotic, antioxidant (Dahech et al. 2011), antimutagenic, anticoagulant, immunomodulatory (McIntosh et al. 2005;

Table 1 Main natural homopolysaccharides

Type	Polysaccharide	Repeating unit Glycosidic linkage/Monosaccharidic unit
Linear	Amylose	α-(1→4)-Glc
	Cellulose	β-(1→4)-Glc
	Xylan	β-(1→4)-Xyl
	Inulin	β-(2→1)-Fru
	Levan	β-(2→6)-Fru
	Laminarin	β-(1→3)-Glc
	Chitin	β-(1→4)-Glc-*N*-Ac
	β-Glucan	β-(1→4, 1→3)-Glc
	Curdlan	β-(1→3)-Glc
Branched	Amylopectin	α-(1→4, 1→6)-Glc
	Dextran	α-(1→2, 1→3, 1→4, 1→6)-Glc
	Levan	α-(2→1, 2→6)-Fru
	Pullulan	α-(1→6)-maltotriose
	Scleroglucan	β-(1→3, 1→6)-Glc
	Glycogen	α-(1→4, 1→6)-Glc
	Lentinan	β-(1→3, 1→6)-Glc
	Grifolan	β-(1→3, 1→6)-Glc
	Schizophyllan	β-(1→3, 1→6)-Glc

El Enshasy and Hatti-Kaul 2013), anticarcinogenic (Zong et al. 2012), antitumor (Silbir et al. 2014), hypoglycemic, and hypocholesterolemic (Belghith et al. 2012) activities may be highlighted. Different polysaccharides obtained from seaweeds like alginates, carrageenan, and ulvan have shown antibacterial, antiviral, and antifungal activities, among others. For this reason, their use has been evaluated as a dietetic supplement for a variety of fish (Peso-Echarri et al. 2012).

β-Glucans are biopolymers exhibiting a wide range of biological activities. The macromolecular structure of the β-glucans depends on the source and isolation method. Microbial β-glucans are mainly composed of a central linear chain linking D-glucose through β-(1→3) linkages and branches of several sizes originated by β-(1→6) linkages. These branches are presented at different intervals along the central chain. On the other hand, β-glucans derived from cereals are polysaccharides based on glucose with β-(1→3) and β-(1→4) linkages. Different factors like the primary structure, solubility, degree of branching, molecular weight, polymer charge, and structure in aqueous media are related to the biological activity of β-glucans. Some β-glucans obtained from microorganisms (fungi and bacteria) and cereals like oats and barley have exhibited immunomodulatory activity, which depends on the structure, molecular weight, and branching of these polymers. The (1→3)-β-glucans, especially from microbial cells, have shown their ability to stimulate the innate immunity and activate the proinflammatory response, i.e., they exhibit anti-inflammatory properties in vitro and in vivo (Kogan and Kocher 2007; Mantovani et al. 2008; Brown and Williams 2009). In several reports on β-glucans,

their immunomodulatory activity has been related to the induction of the production of tumor necrosis factor alpha (TNF-α), γ-interferon, interleukin 8 (IL-8), and nitric oxide; the activation of macrophages and lymphocytes; and the stimulation of CD8 membrane cells (El Enshasy and Hatti-Kaul 2013).

Some β-glucans having structure with β-(1→3, 1→4) linkages from cereals have presented antimicrobial, antiparasitic, hypocholesterolemic, antithrombotic, and antimutagenic activities. Those β-glucans with β-(1→3, 1→4) obtained from the yeast *Saccharomyces cerevisiae* present antiparasitic, antibacterial, antiviral, antifungal, antimutagenic, and antitumor activities, among others (Zong et al. 2012). Those ones isolated from different fungi such as *Auricularia polytricha*, *Grifola frondosa*, *Candida albicans*, *Poria cocos*, *Agaricus blazei*, *Lentinus edodes*, *Schizophyllum commune*, and *Coriolus versicolor* exhibit antitumor, immunomodulatory, and antimutagenic properties (Mantovani et al. 2008; Lin et al. 2010; Carneiro et al. 2013; Zhang et al. 2013).

The polysaccharides or polysaccharide extracts from bacteria like *Penicillium jiangxiense*, some extracts from plants like *Achyranthes bidentata*, and polysaccharides from plants like *Angelica sinensis* (Oliv.) Diels and *Panax ginseng* C. A. Meyer (ginseng), as well as the pectic polysaccharide obtained from *Angelica gigas* Nakai, have shown anticarcinogenic and antitumor properties and have been traditionally used as medicines in China. In recent years, the interest to develop drugs from polysaccharides against cancer has become evident. Some in vitro studies have shown the potential of these compounds although more in vivo tests are required to confirm the in vitro results (Aluko 2012; Zong et al. 2012; Wasser 2013).

2.2 Natural Occurrence of Bioactive Polysaccharides

2.2.1 Bacteria

Bacteria are often employed as producer of extracellular polysaccharides (exopolysaccharides) at industrial level. Bacterial β-(1→3)-D-glucans are mostly linear glucans, while β-(1→3, 1→6)-D-glucans are branched polymers and β-(1→3, 1→2)-D-glucans have cyclic structures. About 30 species of lactobacilli are exopolysaccharide producers (Badel et al. 2011). The most common are *L. casei*, *L. acidophilus*, *L. brevis*, *L. curvatus*, *L. delbrueckii bulgaricus*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. johnsonii* although their productivities are much lower than species of *Alcaligenes*, *Xanthomonas*, *Sphingomonas*, and *Leuconostoc*, which are the microorganisms most used in industry for the production of curdlan, xanthan, gellan, and dextran (McIntosh et al. 2005; Öner 2013).

The xanthan gum is a component of dietary fiber and is also employed for the formulation of liquid or solid products in the pharmaceutical industry, in the latter case as an agent for controlled release (Tungland and Meyer 2002; Pooja et al. 2014). Gellan gum is composed by repeated tetrasaccharide units of D-glucose, D-glucuronic acid, and L-rhamnose, is obtained from bacterium *Pseudomonas elodea*, and has been proved as a release vehicle of pharmaceuticals, cell

Table 2 Main bacterial bioactive polysaccharides

Name	Composition	Source	Health benefits	Reference
Curdlan	β-(1→3)-D-glucose	*Alcaligenes faecalis*	Immunomodulating and pharmacological responses include anti-tumorigenicity; anti-infective activities against bacterial, fungal, viral, and protozoal agents; anti-inflammatory activity; wound repair; protection against radiation; and anticoagulant activity	McIntosh et al. (2005), Zong et al. (2012)
Hyaluronan	Repeating units of β-(1→4)-linked disaccharides of β-D-*N*-acetyl-glucosamine-β-(1→4)-D-glucuronic acid	*Pseudomonas aeruginosa* *Pasteurella multocida*	Because of its very high immunocompatibility and water binding and retention capacity, hyaluronan is widely used in regenerative medicine and cosmetic applications	Öner (2013)
Fructan: levan type	Repeating fructofuranosyl rings connected by β-(2→6) links	*Zymomonas mobilis*, *Bacillus* spp., *Streptococcus*, *Pseudomonas*, *Xanthomonas*, *and Aerobacter*	Immunomodulator, antitumor and antioxidant agent, hypocholesterolemic, hypolipidemic, hypoglycemic, and a blood plasma substitute	Dahech et al. (2011), Belghith et al. (2012), Silbir et al. (2014)

carrier, material for guided bone regeneration, and wound dressing (Lee et al. 2012).

The curdlan is a polysaccharide produced by fermentation using the bacterium *Alcaligenes faecalis*. The effectiveness of the biological activity of curdlan, as in the case of other β-(1→3)-D-glucans (see Table 2), depends on the chemical structure, molecular weight, and conformation. Several reports on this polysaccharide suggest that the structure influences the type of biological activity, especially in the case of anti-inflammatory and antitumor activities. It has been reported that curdlans with a degree of branching less than 50 are not considered as effective antitumor agents. On the other hand, the carboxymethyl ether and sulfate and phosphate esters of curdlan show higher solubility in water and increased biological activity. Curdlan sulfates present anticoagulant and anti-HIV activities as well as inhibitory effects on the development of malaria parasites in vitro. For this reason, curdlan has been proposed for formulation of cosmetic products and as a protective agent for fish farming (McIntosh et al. 2005; Zong et al. 2012).

The levan-type EPS1 exopolysaccharide was isolated from the bacterium *Paenibacillus polymyxa* EJS-3 and is composed of fructofuranosyl residues with β-(2→6) linkages and branching due to β-(2→1) linkages. This compound showed antiproliferative activity against tumor cells. On the other hand, acetylated, phosphorylated, and benzylated modified EPS1 exhibited and improved antiproliferative activity. Other exopolysaccharide from *Rhizobium* sp. N613 (REPS) that is a β-glucan composed of a main chain of glucose linked by β-(1→4) bonds with β-(1→6) branching can significantly suppress tumor formation and improve the immune response in mice (Zong et al. 2012).

2.2.2 Fungi

The use of medicinal mushrooms for the prevention and treatment of human diseases is very old. Different bioactive compounds can be obtained from fungi such as polysaccharides, polyphenols, fatty acids, colorants, and thickeners, among others (Cheung 2008; Wasser 2013). The polymeric carbohydrates from fungi can be intracellular (intrapolysaccharides) or extracellular. They are typically glycans composed of glucose (glucans) that have different structures, molecular weights, and compositions depending on the organism from which are isolated or on the medium to which are excreted. Thus, when fungi are grown in a liquid medium, several of these compounds are released into the medium, and then they can be recovered by means of different extraction processes. Moreover, a significant amount of intrapolysaccharides makes part of the fungal biomass either within the basidioma's structure or in the mycelium (Montoya et al. 2013).

Most bioactive polysaccharides synthesized by fungi are homoglucans although some compounds can form peptidoglycan or glycoprotein complexes. The structure of the fungal cell wall is depicted in Fig. 1. It is composed of homoglucans like the chitin, β-(1→3)-glucan, β-(1→6)-glucan, α-(1→3)-glucan, heteroglucans, and other compounds as the mannoproteins (Becerra-Jiménez et al. 2011). Fungal β-glucans are typically found in the intermediate layer of the cell wall adjacent to the plasmatic membrane and have the function of maintaining the rigidity and shape of the cells. Some of the most fungal β-glucans known for their pharmacological applications are lentinan, schizophyllan, and krestin (usually abbreviated as PSK or polysaccharide K) (Mantovani et al. 2008). These polymers are β-(1→3, 1→6)-glucans as shown in Table 3. Lentinan obtained from *Lentinus edodes* has a triple-helix structure and a molecular weight between 400 and 800 kDa. Schizophyllan obtained from the fungus *Schizophyllan commune* has also a triple-helix structure and a molecular weight of about 450 kDa. PSK is a proteoglycan composed of 25–38 % protein residues and β-(1→4)-glucan with β-(1→6)-glucopyranosyl side chains, has a molecular weight of 94 kDa, and is obtained from *Coriolus versicolor* (El Enshasy and Hatti-Kaul 2013).

The pharmacological applications of the fungal polysaccharides represent a great interest for both scientific community and industry. For instance, the white-rot fungus *Ganoderma lucidum* (Reishi), known as "Lingzhi" in China, "Reishi" in Japan, and "Youngzh" in Korea, has been widely employed as a tonic to promote the longevity and health in China and other Asian countries for more than 2,000

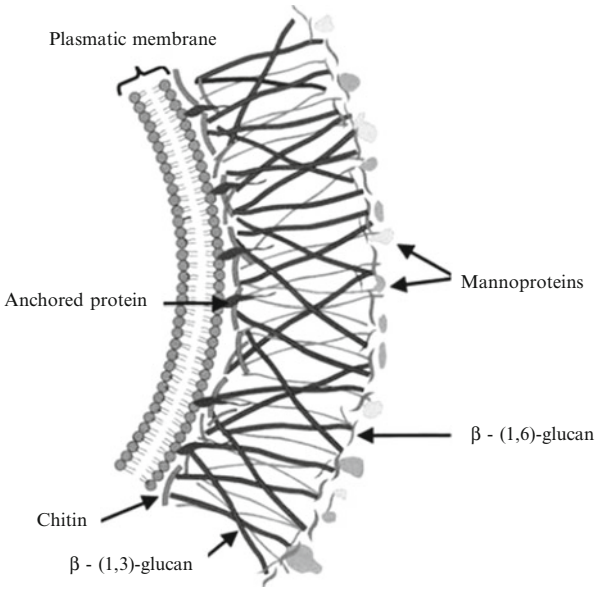

Fig. 1 Structure of the fungal cell wall (Modified from Becerra-Jiménez et al. 2011)

years ago. Bioactive compounds from the different strains of *G. lucidum* can become very diverse depending on the geographic distribution of this fungus, growth conditions, and feedstocks (substrate). According to several reports (Pan et al. 2012, 2013), the polysaccharidic extracts from *G. lucidum* present antioxidant activity and improve the immunity, so they have been suggested for the treatment of gastric cancer. The β-glucan from the macromycetes *Grifola frondosa* (known as Maitake beta-glucan or MBG) has been applied as coadjuvant for cancer treatment like chemotherapy (Lin et al. 2010).

In general, the pharmacological activities of fungal polysaccharides are strongly related to their molecular weight and degree of branching. Recently, Wang et al. (2014) researched five polysaccharide fractions obtained from the fruiting body of *Lentinus edodes*, which showed different structures to those ones reported before. The in vitro and in vivo antitumor tests indicated that the five fractions played a double role. Firstly, they regulate the immune system, and, secondly, they directly kill the cancer cells exhibiting less secondary effect than the chemotherapeutic drugs.

The nutraceutical and chemopreventive properties of the fungus *Agaricus blazei* (*Agaricus brasiliensis*) are related to the presence of β-glucans. In particular, the chemical modification of these β-glucans has been proposed in order to improve their scientific and commercial application, for instance, by increasing their solubility. The sulfation has gained special interest because the β-glucans increase their solubility avoiding granuloma formation. In recent years, several authors have reported the activity of these polysaccharides in healing wounds and burns as well as the antimutagenic, anticarcinogenic, and immunomodulatory activities (Angeli et al. 2009; Sui et al. 2010; Yamanaka et al. 2012).

Table 3 Main fungal bioactive polysaccharides

Bioactive compounds	Source	Health benefits	Reference
Heteroglucans and homoglucans	*Agaricus blazei*, *Ganoderma lucidum*, *Lentinus edodes*, *Grifola frondosa*, *Coriolus versicolor*, *Schizophyllum commune*	Anticancer and antitumor activities	Mantovani et al. (2008), Ziliotto et al. (2009), Ramberg et al. (2010), Hirahara et al. (2012), Wu et al. (2012), Yamanaka et al. (2012), Yue et al. (2012), El Enshasy and Hatti-Kaul (2013), Zhang et al. (2013), Wang et al. (2014)
β-(1→3)-glucan with β-(1→6) branching, β-(1→3)-branched β-(1→2)-mannan, β-(1→3)-glucan, cordyglucan, lentinan, glucan, mannoglucan, galactomannan, grifolan, schizophyllan, acidic polysaccharides, heterogalactan, krestin, heteroglycan, scleroglucan (β-(1→6)-monoglucosyl-branched β-(1→3)-glucan), heteroglucans	*Agaricus blazei*, *Cordyceps sinensis*, *Cryptoporus volvatus*, *Ganoderma lucidum*, *Grifola frondosa*, *Hericium erinaceus*, *Inonotus obliquus*, *Lentinus edodes*, *Morchella esculenta*, *Phellinus linteus*, *Pleurotus ostreatus*, *Polystictus versicolor*, *Schizophyllum commune*, *Sclerotinia sclerotiorum*, *Tremella aurantialba*	Immunomodulators. The immunostimulating effect of β-glucan is probably associated with the activation of cytotoxic macrophages and T helper and natural killer (NK) cells and with the promotion of T-lymphocyte differentiation and activation for the alternative complement pathway	Moradali et al. (2007), Mantovani et al. (2008), (El Enshasy and Hatti-Kaul 2013)
β-glucan and exopolysaccharides	*Agaricus blazei*, *Cordyceps sinensis*, *Lentinus edodes*, *Phellinus baumii*, *Tremella fuciformis*	Antidiabetic effect	Silva et al. (2012)
Prepared derivatives of (1→3)-β-D-glucan	*Saccharomyces cerevisiae*	Antibacterial, antimutagenic, antioxidant, antitumor, and immunostimulating activities	Kogan and Kocher (2007)

(*continued*)

Table 3 (continued)

Bioactive compounds	Source	Health benefits	Reference
Pullulan: α-(1→6)-linked polymer of maltotriose subunits	Synthesized by fermentation of liquefied starch, coconut by-products, beet molasses, and agro-industrial waste with *Aureobasidium pullulans* Chemical reactions	Pullulan has been used for liver and tumor target delivery of drug. Pullulan has the application of targeting drug to liver and cancer cells	Prajapati et al. (2013)
EPS2	Polysaccharide produced by a marine filamentous fungus *Keissleriella* sp. YS 4108	EPS2 exhibited profound free radical-scavenging activities	Laurienzo (2010)
Exopolysaccharides	Marine fungus *Penicillium* sp. F23-2	Three polysaccharides from *Penicillium* sp. F23-2 possessed good antioxidant properties, especially scavenging abilities on superoxide radicals and hydroxyl	Laurienzo (2010)

2.2.3 Algae

An important variety of polysaccharides such as alginates, agar, agarose, and carrageenan may be obtained from marine algae (Laurienzo 2010), some of them with pharmacological applications as can be observed in Table 4. For instance, the alginate consumption in humans has allowed the decrease of cholesterol and glucose in the blood. In addition, the alginates have shown its prebiotic activity, ability to mobilize fatty acids, immunostimulating activity, ability to reduce the blood pressure and enzymatic activity in the intestine, and preventive effect against cancer. Some alginates have been reported as coadjuvant for reparation of the intestinal mucosa. The prebiotic activity of the alginates, carrageenan, and ulvan has enabled the improvement of the intestinal microbiota in fishes as well (Peso-Echarri et al. 2012).

3 Techniques for Cultivation of Microorganisms

Although many polysaccharides can be extracted from other living organisms like plants or even animals, the microorganisms and macromycetes are the most employed source of bioactive polysaccharides. In order to produce these biopolymers at industrial level, fermentation technologies are being implemented.

Table 4 Main algal bioactive polysaccharides

Name	Composition	Source	Health benefits
Alginate	Guluronic and mannuronic acid	Marine brown algae (*Phaeophyceae*) and as capsular polysaccharides in soil bacteria	Alginate dressings for wound healing have been successfully applied for many years to cleanse a wide variety of secreting lesions, and they still remain widely used in many circumstances. Alginates have been shown to be useful also as hemostatic agents for cavity wounds
Agar/agarose	Agar (or agar-agar) is a phycocolloid, which is constructed from complex saccharide molecules (mainly β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose units). Agar and its variant agarose contain also variable amounts of sulfate, pyruvate, and uronate substituents	Agar is extracted from certain species of red algae: *Gelidium*, *Gelidiella*, *Pterocladia*, *Gracilaria*, *Gracilariopsis*, and *Ahnfeltia*	Suspending agent for radiological solutions (barium sulfate), as a bulk laxative as it gives a smooth and nonirritating hydrated bulk in the digestive tract, and as a formative ingredient for tablets and capsules to carry and release drugs
Exopolysaccharides	Soluble polymers in the culture medium (RPS)	*Spirulina platensis*	Antiviral activity

Source: Laurienzo (2010)

In principle, the organisms that contain or synthesize polysaccharides can be cultivated under controlled conditions in special fermentation systems named bioreactors. However, only a few of these organisms are suitable to be grown under such conditions, which can substantially differ from those of the ecosystems where these organisms naturally grow. The microbial organisms (mostly microfungi, yeasts, and microalgae) have the ability to quickly propagate not only in plates and flasks but also in large bioreactors with volumes of thousands of liters. This can be explained by their small sizes (from 0.5 to 10 μm) implying high surface/volume ratios that allow fast exchange of nutritional compounds and metabolites between the cytoplasm and the surrounding medium. The accumulation of very different substances inside the microbial cells enables their transformation into end or intermediate products through enzyme-catalyzed reactions. In this way, the metabolism of microorganisms tends to be very intense compared to higher macroscopic

organisms. In addition, the microorganisms have a wide range of nutritional substances and substrates for their growth. Thus, they can be cultivated using culture media that contain cheap components, e.g., agricultural or agro-industrial residues.

For specific types of polysaccharides, other organisms can be cultivated like the macrofungi (macromycetes), which have a behavior similar to their microscopic counterparts when grown in liquid fermentation media in bioreactors. If the macromycetes are cultivated on the surface of solid materials containing the nutrients required for their development, special biomass structures are formed such as the basidiomata or fruiting bodies. In the particular case of fungi, fungal cells form a filamentous structure named mycelium when liquid media are utilized. If this medium is to be stirred, the diffuse mycelium is converted into spherical cell agglomerations called pellets. When micro- or macrofungi are grown on solid materials, the fungal cells colonize the substrate taking from it the nutrients (in some cases, the solid particles are merely carriers for cells, and the nutrients are taken from the liquid medium that impregnates the solid material). For macromycetes, this growth process is named vegetative stage; after this, the macromycetes form the fruiting bodies that represent the reproductive stage of the growth cycle of the fungi. Evidently, the fructification is not possible when macromycetes are grown in liquid media. The polysaccharidic composition of the fungal biomass obtained from either liquid culture media or solid materials is quite different, and the growth rate of the latter process is usually slower.

As mentioned above, the microorganisms can be cultivated on the surface of solid substrate. This type of aerobic fermentation is called *solid-state fermentation* (SSF). SSF has been commercially implemented mainly in China, Japan, and other Asian countries for the production of different compounds like fermented foods, hydrolytic enzymes, biopesticides, and other value-added products (Pandey et al. 2000). In fact, worldwide production of the most consumed mushroom, *Agaricus bisporus*, is a special type of SSF where the fungal cells colonize previously composted solid substrates. On the other hand, some biological agents used against insects attacking economically important crops are cultivated on cereal grains in several tropical countries. For instance, the entomopathogenic fungus *Beauveria bassiana* is commercially produced in Colombia by SSF of rice grains in order to control the damage caused by the coffee berry borer (*Hypothenemus hampei*). However, SSF has not traditionally received a big attention in Western Europe and North America except for some specific cases in the food industry.

The Western microbiologic industry has been based on the utilization of liquid culture media for the growth of microorganisms. For aerobic fermentations, the microorganisms can develop on the surface of a liquid nutritive medium disposed in trays that, in turn, are located in shelves; purified air is supplied to the cultivation rooms, and the cells take the oxygen directly from the air. This type of cultivation technology is called *surface fermentation* and played an important role in the mid-twentieth century especially in Western Europe. For instance, main part of citric acid was produced by using this technology. However, the surface fermentation was abandoned due to the high risk of infection and elevated labor costs.

The *submerged fermentation* is the most used cultivation technology for the production of cell biomass and value-added products from it (metabolites) in the world. In this case, the microbial cells do not grow on the surface of the liquid medium, but they do in the bulk of the liquid, which is contained in a stainless steel vessel (fermenter). The microorganisms take the nutrients they need from the liquid medium in a solubilized way. If they are aerobic, the air should be supplied to the fermenter by pumping and sparging for the oxygen to transfer from air bubbles to the liquid medium. This technology offers several advantages: low risk of infection, hermeticity and compactness, facility of control and automation, low labor costs, higher reproducibility of the process for different production cycles, and versatility in the usage of a wide range of biological agents (bacteria, molds, yeasts, macromycetes, microalgae, plant cells, animal cells). Nevertheless, the capital costs of the submerged fermentation are higher due to the higher degree of sophistication of the bioreactors (fermenters), and the products tend to be dilute in the liquid medium at the end of fermentation. However, current trends for the development of more effective strains of microorganisms exhibiting elevated titers of the products (e.g., by the development of genetically modified microorganisms) have enabled to neutralize these drawbacks.

3.1 Features of the Submerged Fermentation

Several factors should be defined before the implementation of a submerged fermentation process at industrial scale. Among these factors, selection of the time regime of fermentation, preparation of the culture medium, selection of the process microorganism, aeration, heat transfer, type of bioreactor, process control, cell recovery and downstream operations, and treatment and final disposal of wastes should be highlighted (see Fig. 2). Some of these factors are briefly described below.

3.1.1 Time Regime of Fermentation

Submerged fermentations can be organized depending on how the liquid medium and fermented broth are supplied or removed from the fermenter. During *batch fermentation*, a series of operating procedures are periodically repeated to ensure the growth and development of process microorganisms. These procedures can include the washing and disinfection of the fermenter, fermenter filling-up with the culture medium, sterilization of such medium, inoculation of microbial cells, fermentation, and unloading of the bioreactor content at the end of cultivation process (Cardona et al. 2010). The main drawback of this regime consists in the operating and feedstock costs needed during each fermentation batch to ensure the cell biomass propagation until a concentration high enough to allow appropriate rates of cell growth and product biosynthesis. In addition, the cells are not reutilized that implies not to employ all the potential of cell biomass formed during the process. In general, the production of polysaccharides is accomplished through batch fermentation using bacterial or fungal cells.

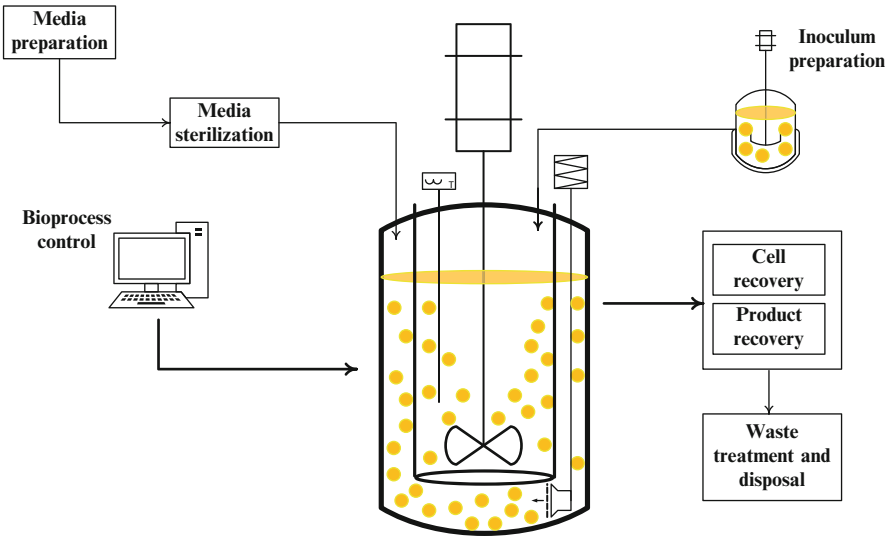

Fig. 2 Schematic diagram of the main aspects affecting the submerged fermentation

Fed-batch fermentation is one of the most employed cultivation regimes when process microorganisms present catabolic repression, i.e., when high substrate concentrations inhibit specific metabolic processes like those related to cell growth rate. For this reason, the microorganisms grow faster at low substrate concentrations. To implement such a process, conventional batch fermentation is performed through employing a less concentrated medium. Once the sugars have been consumed, the bioreactor is fed with portions of fresh medium or by adding a small amount of medium permanently until the end of fermentation. This continuous feeding of the medium can be done in a linear way (with a constant feeding rate) or according to a more complex function defining the rate with which the fresh medium is added to the fermenter, e.g., by an exponential feeding rate (Sánchez and Cardona 2008). Control of flow rate of medium feeding is quite advantageous because the inhibitory effect caused by high concentrations of substrate or product in fermentation broth is neutralized.

Continuous fermentation consists in the cultivation of cells in a bioreactor to which the fresh medium is permanently added and from which an effluent stream of culture broth is permanently removed. The microorganisms are reproduced within the bioreactor at a grow rate that offsets the cells' withdrawal with the effluent achieving the corresponding steady state. To ensure the system homogeneity and reduce concentration gradients in culture broth, continuous stirred-tank reactors (CSTR) are employed. In this way, a constant production of fermented wort can be obtained without the need of stopping the bioreactor operation in order to perform the periodic procedures typical of batch processes like filling-up and unloading. This allows a remarkably increase of volumetric productivity compared to batch or

fed-batch processes. Unfortunately, the microorganisms used to produce bioactive polysaccharides do not exhibit fast growth rates, so the continuous regime is difficult to achieve. In addition, the continuous cultivation during several months can lead to the loss of the ability to produce the target polysaccharides (genetic instability) since the microbial cells tend to form biomass under these conditions and to revert to the native strain in the case of mutants or genetically engineered microorganisms.

3.1.2 Preparation of the Culture Medium

The function of the liquid fermentation medium is to supply the nutritional substances required by the microorganisms to grow and synthesize the desired products. This depends on the metabolism type the process microorganism exhibits under the cultivation process. For instance, if the organism has a fermentative type of metabolism where there is no final electron acceptor (oxidizing agent) added to the medium and the energy source (the compound undergoing oxidation) is organic, there is no need to pump air into the fermenter and therefore the bioreactor design is simpler. The prevalent metabolism type in microorganisms producing polysaccharides is aerobic respiratory metabolism, where an energy source (mainly sugars) is oxidized to CO_2, an electron acceptor (oxygen contained in the air) is added to the medium, a nitrogen source should be present to ensure the synthesis of proteins and nucleic acids, and a series of micro- and oligoelements are also added along with the carbon source (again the sugars) to form new cell biomass. Many bacteria and fungi (micro- and macromycetes) exhibit an aerobic respiratory metabolism. In particular, the fungi utilize as energy/carbon source several plant polysaccharides like starch, cellulose, or pectin thanks to their ability to release hydrolytic enzymes (amylases, cellulases, or pectinases). In the case of the microalgae, the energy source is the sunlight, and the carbon source is the atmospheric carbon dioxide.

Metals play an important role during the metabolic processes leading to the biomass growth and biosynthesis of polysaccharides. In general, they act as cofactor of many enzymes and related proteins such as ligninases, amylases, cytochromes (electron carriers), and cellulases, among others. They also can inhibit many enzymes, so their concentration in the culture medium should be thoroughly determined.

Culture media may be synthetic or complex. In the first case, the composition of the medium is clearly established. Simple sugars are often used as energy source in these media. In the case of complex media, the exact composition is not known since materials like molasses or hydrolyzates are employed. These media are preferred because of their low cost.

3.1.3 Air Supply

The aeration levels should be defined in order to improve the fermentation process. The oxygen contained in the air is needed to oxidize the organic compounds for microorganisms with aerobic respiratory metabolism. These microorganisms exhibit high growth rates, have elevated oxygen consumption rates, and release

significant amounts of heat. As the oxygen has a low solubility in the culture broth (about 6 mg/L), high air flow rates should be used to supply the oxygen needed (between 0.5 and 2.0 vvm or volumes of air per volume of broth in a minute). The aerobic fermenters are limited by the mass transfer of oxygen to the liquid medium. For this reason, many bioreactors are provided with a stirrer with one or more impeller in order to intensify the dissolution of oxygen in the water. In addition, a sparger is mounted below the lower impeller with the same purposes. This gas–liquid system has a tendency to form foam that implies negative effect on the fermentation performance, so antifoam substances or devices are included in the fermenter.

3.1.4 Heat Transfer

All the living organisms transform the energy released by the oxidation of organic or inorganic substances, or collected from the sunlight, into other forms of useful energy, which is employed for the metabolic functions of the cell and for reproduction. These energy transformations are not 100 % efficient. Although the living matter is much more efficient than the artificial human devices (engines, turbogenerators, photovoltaic cells), some portion of energy cannot be utilized and is dissipated in the form of heat. When microbial cells grow and propagate in the culture medium, metabolic heat is produced. If this heat is not removed, the temperature of the system increases, reaching values not optimal for the growth rate or biosynthesis of the target products. High temperatures can even lead to cell death. To avoid this situation, the bioreactors are provided with some devices for heat exchange such as jackets in small fermenters (up to 1,000 L), cooling coils (up to 50–100 m^3), or external heat exchangers with recirculation of liquid medium (above 100 m^3). All these devices have pipelines inside where cooling water flows; the water is not in contact with the liquid culture medium but removes the heat through the metal walls of the pipelines by conduction and convection. The heat transfer in cylindrical tanks has been well studied, so the commercial fermenters for polysaccharide production do not present big difficulties on this matter.

3.1.5 Type of Bioreactors

One bioreactor for fermentation (fermenter) is a complex equipment that should ensure all the optimal conditions for the growth of microbial cells being, at the same time, flexible and versatile in order to be used for different purposes. Most of the bioreactors for submerged fermentation are standard apparatuses that have several internal devices and accessories to intensify the mass transfer of oxygen, ensure the homogenization of the culture broth, remove the metabolic heat, avoid the contamination with non-desired organisms, and maintain the required values of pH, temperature, and oxygen concentration, among other operating parameters. There exist in the market several commercial firms offering standard bioreactors to not only cultivate microorganisms but also macromycetes, plant cells, and animal cells.

Agitation and aeration are very important topics to be considered during the design and construction of bioreactors for submerged fermentation as mentioned above. In addition, the control (automated or not) of the bioreactor is crucial to attain the success in the fermentation industry. The different devices for process

control (probes, transducers, controllers, actuators) along with the software are quite expensive and can add up to 80 % to the main cost of the bioreactor (without control devices). However, some fermentation processes for the production of polysaccharides require a strict pH and temperature control. For this reason, an evaluation of the process viability should be performed in each case to establish if the sophistication level of a given bioreactor justifies its utilization considering the price and market of the target product.

Finally, the target product remains in the culture broth (exopolysaccharide) or inside the cells (intrapolysaccharide). A series of different unit operations are required to separate and purify these polysaccharides without loss of their biological activities. An example of a sequence of those downstream operations to recover a bioactive exopolysaccharide could be as follows: centrifugation, liquid–liquid extraction, adsorption using activated carbon, liquid chromatography, evaporation, drying, and packing. These operations are complex and difficult to design. A more detailed description of this issue is out of the objectives of this chapter.

4 Submerged Fermentation for Production of Fungal Polysaccharides

Submerged fermentation involves the development of microorganisms in a liquid medium enriched with nutrients and with high oxygen concentrations (aerobic conditions). In the case of fungal cells, the hyphal development (especially in basidiomycetes) in submerged cultures results in the uncontrolled development of the mycelium (set of hyphae). The extension of the fungal biomass has significant effects on the mass transfer, growth rate, and product secretion. The fungal mycelium can form pellets causing their proliferation in the whole culture medium and increasing the viscosity, which limits the mass transfer of oxygen. All these drawbacks limit the operation of the bioreactors (Rodriguez-Couto and Toca-Herrera 2007).

The production of extracellular polysaccharides using macromycetes by submerged fermentation is influenced by the process time, temperature, composition of the culture medium, agitation speed, initial pH, and inoculum size, among other factors. The macromycetes can grow under varied environmental conditions, e.g., at a wide range of temperature (Shu et al. 2007; Suárez and Nieto 2013). However, the submerged fermentations are usually carried out at temperature between 26 °C and 36 °C considering that the temperature increase can accelerate the fungus metabolism and diminish the solubility of oxygen in the medium. On the other hand, the aeration also influences the concentration of dissolved oxygen. This can be controlled by fixing the volume of air supplied to the culture medium during the process (Kim et al. 2008; Suárez and Nieto 2013). To accomplish the follow-up and control of all these variables demands time, efforts, and money in order to obtain the experimental data required for the comprehensive evaluation of the interaction of these variables with the production of polysaccharides.

Regarding the culture medium for polysaccharide production from fungi, different reports published in the last decade evidence the importance of the presence of vegetable oils rich in oleic acid as promoters of fungal biomass formation and, consequently, of their constituent polysaccharides (Yang et al. 2000; Hsieh et al. 2008; Hao et al. 2010). When choosing the type of vegetable oil to be used as inductor of polysaccharide production, it is necessary to take into account the fatty acid composition of the oil since several researchers have reported that important amounts of linoleic acid suppress the production of biomass and polysaccharides, while the presence of oleic promotes their production (Park et al. 2002; Hsieh et al. 2006, 2008). Soy and olive oils have been used as promoters of cell growth and polysaccharide production.

More research is needed about the control of submerged fermentation processes using macromycete fungi. For instance, Wu et al. (2006) found that it was necessary to control such variables as substrate composition, pH of the medium, temperature, and other environmental conditions to increase and maintain constant the production of cell biomass and exopolysaccharides by submerged fermentation using the macromycetes *Auricularia auricular*. Previous studies have demonstrated that the pH control plays a very important role during the development of mycelial biomass and polysaccharide production (Fang and Zhong 2002). On the other hand, some reports evidence the effect of pH on the chemical structures and molecular weight of the polysaccharides obtained from fungi. This is particularly important since other studies suggest a clear relationship between the biological properties of the polysaccharides and their molecular weights (Shu et al. 2003, 2004; Hamedi et al. 2012; Zhang et al. 2013). For instance, Shu et al. (2003) concluded that the polysaccharides found in the culture broth where *Agaricus blazei* was grown strongly depended on their molecular weight.

The dispersion of the mycelium in liquid industrial fermentations implies the homogenization and agitation of the branched hyphae. This dispersion produces a broth with a non-Newtonian behavior, and its apparent viscosity increases with the agitation speed. This reduces the transport of nutrients as well as the transfer of oxygen and heat, therefore increasing the operating costs (Prosser and Tough 1991). Thus the agitation plays a very important role regarding the mycelium integrity. If the agitation is very strong, the breakdown of mycelium is produced, and, therefore, pellets formation decreases. At the same time, the biomass formation is affected as well as the target metabolites like the polysaccharides (Cui et al. 1997; Suárez and Nieto 2013).

5 Submerged Fermentation for Production of Bacterial Polysaccharides

The submerged fermentation to obtain polysaccharides from bacteria is used universally. For example, the curdlan exopolysaccharide is obtained by submerged fermentation of *Agrobacterium* sp. or *Alcaligenes faecalis* under nitrogen-limiting conditions. For the production process of this biopolymer, critical factors such as

Table 5 Principal producers and culture conditions for lactobacillus polysaccharide production

Microorganisms	Media	Temperature (°C)	Time (h)	pH	Yield (mg/L)
L. rhamnosus 9595 M	BMM[a]	32–37	72	6	~1,000
L. delb. bulgaricus RR	Whey	38	24–28	5	95–100
L. rhamnosus R	BMM[a]	37	72	6	500
L. delb. bulgaricus	Milk	42	24	/	110
L. delb. bulgaricus	MRS	40	18	/	263
L. rhamnosus GG	Milk	37	20	/	80
L. delb. bulgaricus 291	Skimmed milk	37	22	/	80
L. casei CG11	BMM[a]	25	48	/	130
L. helveticus	Skimmed milk	37	60	5	730
L. delb. bulgaricus	Whey (protein-free)	37	18	6	800
L. rhamnosus 9595	Whey permeate supplemented	37	24	6	2,775
L. paracasei	BMM[a]	32–37	72	6	~80

Source: Badel et al. (2011)
[a]*BMM* basal minimal medium, *MRS* Man Rogosa Sharpe

the carbon source, nitrogen source, phosphate concentration, pH, and agitation rate, among other factors, should be optimized. Moreover, although the conventional nitrogen source for production of curdlan is NH_4Cl, studies with $NaNO_3$, urea, and yeast extract have been reported as well (Jiang 2013).

Badel et al. (2011) studied the main conditions for the production of dextran, levan, inulin, mutan, and reuteran from some lactobacilli species as well as their monosaccharide composition (see Table 5). The cultivation temperature varied between 32 °C and 42 °C, the process time for biopolymer production was between 18 and 72 h, and the pH lied in the range between 5 and 6. The most effective bacteria for their high yields in the production of polysaccharides were *L. rhamnosus* 9595, *L. rhamnosus* 9595 M, *L. delbrueckii bulgaricus*, and *L. helveticus*.

6 Conclusion

Bioactive polysaccharides are value-added products with important applications, especially in the pharmaceutical industry due to their key biological activities: antibiotic, antioxidant, antimutagenic, anticoagulant, immunomodulatory, anticarcinogenic, antitumor, hypoglycemic, and hypocholesterolemic activities. The world market of nutraceuticals and natural pharmaceuticals is steadily increasing. To meet the worldwide demand, especially in the developed countries, it is necessary to not only find new sources and applications of these polysaccharides

but also to improve the technology for the large-scale production of these biopolymers.

The submerged fermentation is the most developed technology in the world to produce a wide range of bio-based products. Fortunately, the organism naturally producing bioactive polysaccharides can be cultivated by fermentation using liquid culture medium. This chapter attempted to provide an overview on the features of the submerged fermentation applied to the production of bioactive polysaccharides from fungi and bacteria. The possibilities to continue the scientific research and technological development on this matter are bright and wide. Undoubtedly, the engineering approach to improve this type of technology in the case of bioactive polysaccharides has the key to reduce production costs and increase their production levels worldwide.

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Cellulose from Lignocellulosic Waste

15

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Contents

Abstract

Bioconversion of renewable lignocellulosic biomass to biofuel and value-added products is globally gaining significant importance. Lignocellulosic wastes are the most promising feedstock considering its great availability and low cost. Biomass conversion process involves mainly two steps: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars and fermentation of the sugars to ethanol and other bioproducts. However, sugars necessary for fermentation are trapped inside the recalcitrant structure of the lignocellulose. Hence, pretreatment of lignocellulosic wastes is always necessary to alter and/or remove the surrounding matrix of lignin and hemicellulose in order to improve

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the hydrolysis of cellulose. These pretreatments cause physical and/or chemical changes in the plant biomass in order to achieve this result. Each pretreatment has a specific effect on the cellulose, hemicellulose, and lignin fraction. Thus, the pretreatment methods and conditions should be chosen according to the process configuration selected for the subsequent hydrolysis steps. In general, pretreatment methods can be classified into four categories, including physical, physicochemical, chemical, and biological pretreatment. This chapter addresses different pretreatment technologies envisaging enzymatic hydrolysis and microbial fermentation for cellulosic ethanol production and other bioproducts. It primarily covers the structure of lignocellulosic wastes; the characteristics of different pretreatment methods; enzymatic hydrolysis; fermentation and bioproducts; and future research challenges and trends.

Keywords

Cellulose • Lignocellulosic wastes • Biorefinery • Pretreatment • Enzymatic hydrolysis • Fermentation • Bioproducts

Abbreviations

BGL	β-Glucosidase
CBH	Cellobiohydrolase
CBP	Consolidated bioprocessing
CF	Co-fermentation
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
DP	Degree of polymerization
EG	Endoglucanase
[emim][OAc]	1-Ethyl-3-methylimidazolium acetate
FAO	Food and Agriculture Organization of the United Nations
GRAS	Generally recognized as safe
IUBMB	International Union of Biochemistry and Molecular Biology
MFC	Microfibrillated cellulose
PDLA	Poly(D-lactic acid)
PLA	Polylactic acid
PLLA	Poly(L-lactic acid)
PW	Primary wall
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification to co-fermentation
SSF	Simultaneous saccharification and fermentation
SSF	Solid-state fermentation
SSSF	Semi-simultaneous saccharification and fermentation
SW	Secondary wall
SW1	Secondary wall inner layer
SW2	Secondary wall middle layer
SW3	Secondary wall outer layer
WHO	World Health Organization

1 Introduction

Conventional petroleum refineries utilize physical and chemical processes to refine crude oil to different fractions that are used for the production of several products as fuels, chemicals, and materials. However, these products have one more thing in common besides the raw material; they are produced because of their economic value. On the other hand, bioprocessing and bioproducts have gained commercial interest because of the perceived "green" advantages of using biomass rather than fossil energy for the production of chemicals and industrial products.

There are several issues influencing the current global interest in the biorefining of biomass feedstocks, more specifically lignocellulosic wastes, rather than fossil reserves, to produce a wide variety of extracts, fuels, and chemicals: the fossil fuels have finite reserves and are nonrenewable, the higher increase of the global warming when fossil fuels are burned to provide energy, and the security of supply of fossil fuels as commodities may not be reliable in the future due to the world regions they originated (Charlton et al. 2009; Anwar et al. 2014). This has led to the concept of biorefinery, which, according to American National Renewable Energy Laboratory, is defined as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass" (www.nrel.gov/biomass/biorefinery.html). Thus, biorefinery concept is analogous to today's petroleum refineries (Yang 2007; Taylor 2008; Cavka 2013).

Availability of resources seems not to be a short-term problem into biorefinery. A multitude of feedstock has been tested for biorefinery applications covering cultivated crops, agricultural wastes, forest resources, urban and industrial wastes, and algae (Kajaste 2014). Recent studies indicate that the demand for biomass to produce required biofuels and platform chemicals of the petrochemical industry can be covered without significant changes to the current agricultural land use. However, there is a clear shift from agricultural products to lignocellulosic feedstocks (plant biomass predominantly comprised of cellulose, hemicellulose, and lignin) for the production of chemicals, using different pretreatments for the achievement of specific fractionation results, depending on the desired solid or liquid by-product to obtain (Bos and Sanders 2013; Forster-Carneiro et al. 2013; García et al. 2014a).

Currently the most promising and abundant cellulosic feedstocks derived from plant residues in the USA, South America, Asia, and Europe are corn stover, sugarcane bagasse, rice, and wheat straws, respectively (Limayem and Ricke 2012; Phitsuwan et al. 2013), as they are abundant, low-cost, nonfood materials.

However, the expertise required to exploit biomass as a viable source of base commodities is also diverse and requires a multidisciplinary approach. Taking into account the emerging research trends, the concept of fractionating biomass into its core constituents, an important step in the development of biorefining technologies, has the potential to benefit a wide range of bioprocessing industries due to the ease and improved efficiency associated with working with less variable material feedstocks (FitzPatrick et al. 2010).

2 Structure of Lignocellulosic Wastes

Lignocellulosic wastes are the most abundant source of unutilized biomass. They are composed mainly of cellulose (30–50 %), hemicellulose (15–35 %), and lignin (10–20 %) (Mielenz 2001; Gírio et al. 2010). Celluloses and hemicelluloses correspond to approximately 70 % of the entire biomass and are tightly linked to the lignin through covalent and hydrogenic bonds that make the structure highly robust and resistant to any kind of hydrolysis (Knauf and Moniruzzaman 2004; Limayem and Ricke 2012). This highly recalcitrant nature is related to the presence of lignin, the degree of crystallinity of cellulose, the degree of polymerization of the polysaccharides, the available surface area, and the moisture content (Van Dyk and Pletschke 2012). In addition, other components can be found to a lesser extent, such as pectin, proteins, extractives, and ash (Jorgensen et al. 2007; Gírio et al. 2010).

These compounds are present in the cell wall and in the middle lamella of plant cell walls. In general, the plant cell wall is composed of distinct layers and can be subdivided as primary (PW) and secondary (SW) walls (Fig. 1). The distribution of

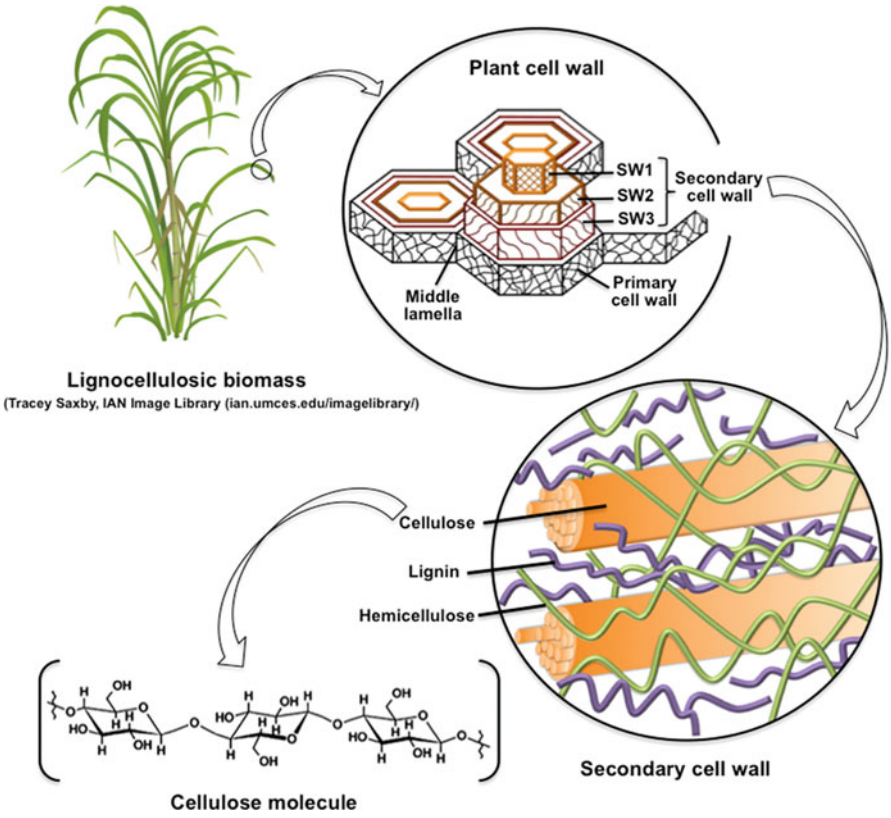

Fig. 1 Structure of the plant cell wall (Adapted from Brett and Waldron 1996; Boudet et al. 2003)

cellulose, hemicellulose, and lignin varies considerably among these layers. The PW is generally very thin and has a low cellulose content (~10 %), some lignin, but lots of pectin and hemicellulose. The SW is composed of three layers, namely, SW1, SW2, and SW3, where SW2 (which comprises the major part of the cell wall) is usually thicker than the others and contains the major portion of cellulose (about 70 %). The middle lamella, which binds the adjacent cells, is almost entirely composed of lignin (Pandey 2009; Menon and Rao 2012; Aitken 2012; Gibson 2012).

However, the composition of various lignocellulose materials varies substantially (Table 1) depending on the source and even with age, stage of growth, climatic conditions, and others (Perez et al. 2002). In sequence, the structure of the main constituents of lignocellulosic wastes will be described.

2.1 Cellulose

The most predominant component found in all plant cell walls is cellulose. It is a linear homopolymer of D-glucose monomers linked by β-1,4-glycosidic bonds that can contain over 10,000 glucose units (Cheng and Timilsina 2011). The cellulose chains are cross-linked by strong hydrogen bonds to form cellulose microfibrils. These microfibrils exhibit a crystalline region, which is very recalcitrant to degradation and a small part of amorphous cellulose, which is easier to degrade (Van Dyk and Pletschke 2012). The orientation of cellulose microfibrils in the secondary cell wall has a strong effect on the structural properties of various plant types (Abdul Khalil et al. 2012).

The cellulose microfibrils are present in the secondary cell wall usually embedded in an amorphous matrix of hemicelluloses and lignin (Cziple and Marques 2008; Huber et al. 2012). These microfibrils in the matrix are often associated in the form of bundles or macrofibrils (Menon and Rao 2012), and lignin and hemicellulose fill the spaces between cellulose microfibrils in primary and secondary cell walls, as well as the middle lamellae (Eriksson and Bermek 2009).

The high molecular weight and ordered tertiary structure makes natural cellulose insoluble in water. Although starchy materials require temperatures of only 60–70 °C to be converted from crystalline to amorphous form, cellulose requires 320 °C and a pressure of 25 MPa to change from a rigid crystalline structure to an amorphous structure in water (Deguchi et al. 2006; Limayem and Ricke 2012). Cotton, flax, and chemical pulp represent the purest sources of cellulose (up to 90 % cellulose), while wood contains approximately 50 % cellulose (Aitken 2012; Limayem and Ricke 2012).

2.2 Hemicellulose

Hemicellulose is another polysaccharide found in abundance in plant cell walls. It is a complex branched heteropolymer of sugars and sugar derivatives which form a

Table 1 Composition of some lignocellulosic wastes

Lignocellulosic waste	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Almond shell	26.8	32.5	27.4	Nabarlatz et al. 2007
Bamboo	50.4	24.2	23.4	Alves et al. 2010
Banana waste	13.2	14.8	14.0	John et al. 2006
Barley husk	21.4	36.6	19.2	Parajó et al. 2004
Barley straw	30.8	25.3	16.7	Nabarlatz et al. 2007
Corncob	34.3	37.2	17.7	Garrote et al. 2002
	34.4	40.8	18.8	Parajó et al. 2004
	38.5	36.8	18.7	Nabarlatz et al. 2007
	34.3	40.6	18.8	Garrote et al. 2007
	35.4	35.2	14.8	Michelin et al. 2012
Cotton stalk	58.5	14.4	21.5	Jiménez et al. 2007
Eucalyptus	46.6	25.8	22.9	Parajó et al. 2004
	46.7	20.7	29.2	Alves et al. 2010
Faba bean	28.4	20.4	14.4	Petersson et al. 2007
Hybrid poplar	43.8	14.9	29.1	Kim et al. 2009
Maple	40.9	19.4	29.1	Kim et al. 2011
Oilseed rape	27.3	21.9	14.2	Petersson et al. 2007
Olive stone	24.0	27.8	31.3	Nabarlatz et al. 2007
Olive tree biomass	25.0	18.3	16.6	Cara et al. 2008
Sorghum straw	35.1	24.0	25.4	Vázquez et al. 2007
Sugarcane bagasse	44.7	36.9	11.0	Song and Wei 2010
	43.1	32.3	23.2	Alves et al. 2010
Sunflower stalk	42.1	29.7	13.4	Jiménez et al. 1990
Rice husk	36.7	20.0	21.3	Parajó et al. 2004
	36.7	20.0	21.3	Garrote et al. 2007
	29.7	19.6	24.6	Nabarlatz et al. 2007
	33.6	37.2	19.3	Kim et al. 2008
Vine shoot	41.1	26.0	20.3	Jiménez et al. 2007
Wheat straw	39.7	36.5	17.3	Jiménez et al. 1990
	48.6	27.7	8.2	Saha et al. 2005
	31.5	25.2	15.9	Nabarlatz et al. 2007
	37.4	33.8	19.4	Ruiz et al. 2011a
Winter rye	40.8	26.1	16.1	Petersson et al. 2007

highly branched network. It consists of about 100–200 sugar units typically made up of different sugars including hexoses (D-glucose, D-galactose, and D-mannose), pentoses (D-xylose and L-arabinose), and/or sugar acids (uronic acids), namely, D-glucuronic, D-galacturonic, and 4-O-methyl-D-glucuronic acids (Cheng and Timilsina 2011; Limayem and Ricke 2012). Other sugars such as α-L-rhamnose and α-L-fucose may also be present in small amounts, and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Gírio et al. 2010).

Xylans are the main hemicellulose components of secondary cell walls, its backbone chain is primarily composed of D-xylose residues (nearly 90 %) linked by β-1,4-glycosidic bonds (Gírio et al. 2010; Limayem and Ricke 2012). Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain such as acetyl, arabinosyl, and glucuronosyl residues (Michelin et al. 2013). Branch frequencies vary depending on the nature and the source of feedstocks. The hemicelluloses of softwood are typically glucomannans, while hardwood hemicellulose is more frequently composed of xylans (McMillan 1994).

The primary role of the hemicelluloses has been proposed to act as a bonding agent between lignin and cellulose. Its covalent linkage to lignin and its noncovalent interaction with cellulose may be important in maintaining the integrity of the cellulose in situ and helping to protect the fibers against degradation by cellulases (Uffen 1997; Michelin et al. 2013).

2.3 Lignin

Lignin is another highly polymerized molecule but is quite amorphous relative to cellulose and hemicellulose (Aitken 2012). It is a complex hydrophobic cross-linked aromatic biopolymer with a molecular weight of 10,000 Da (Mielenz 2001; Limayem and Ricke 2012), composed of three major phenolic components, namely, *p*-coumaryl, coniferyl, and sinapyl alcohol (Menon and Rao 2012).

Its ratio varies between different plants, wood tissues, and cell wall layers (Menon and Rao 2012). Older and more woody plants contain higher levels of lignin deposited in cell walls to provide rigidity and strength, conferring impermeability to cell walls and forming an effective physic–chemical barrier against microbial attack (Raven et al. 1999; Kumar et al. 2009; Van Dyk and Pletschke 2012) and oxidative stress. Therefore, it is the most recalcitrant component of lignocellulosic material to degradation (Himmel et al. 2007; Sánchez 2009; Michelin et al. 2013).

Lignin covalently bonds to hemicellulose and is responsible for much of the mechanical strength of wood (Aitken 2012). Forest woody biomass is primarily composed of cellulose and lignin polymers. Softwood barks have the highest level of lignin (30–60 %) followed by the hardwood barks (30–55 %), while grasses and agricultural residues contain the lowest level of lignin (10–30 % and 3–15 %, respectively) (Demirbas 2005).

3 Biorefinery Processing of Cellulose

As mentioned above, the biorefinery concept is comparable to the classical petroleum refinery and includes the conversion of biomass into fuels and chemicals with high value-added (Fig. 2). The implementation of this demands the identification of the most promising bioproducts, chemicals, and bioenergy, considering environmental and economic aspects (Cherubini and Ulgiati 2010; Ruiz et al. 2013a).

Nowadays, the application of a biorefinery process based on cellulose has increased, for example, in the pulp and paper industry (Jahan et al. 2013). Moshkelani et al. (2013) mentioned that the incorporation of a biorefinery unit into an operating kraft pulping process has significant technological, economic, and social advantages over the construction of a grassroot biorefinery. Recently, Fornell et al. (2013) performed a techno-economic analysis of a kraft pulp mill-based biorefinery producing both ethanol and dimethyl ether and concluded that the biorefinery can be self-supported in terms of fuel if the process is well integrated. Pettersson and Harvey (2012) reported black liquor gasification as an alternative technology for energy and chemical recovery in kraft pulp mills by comparing the

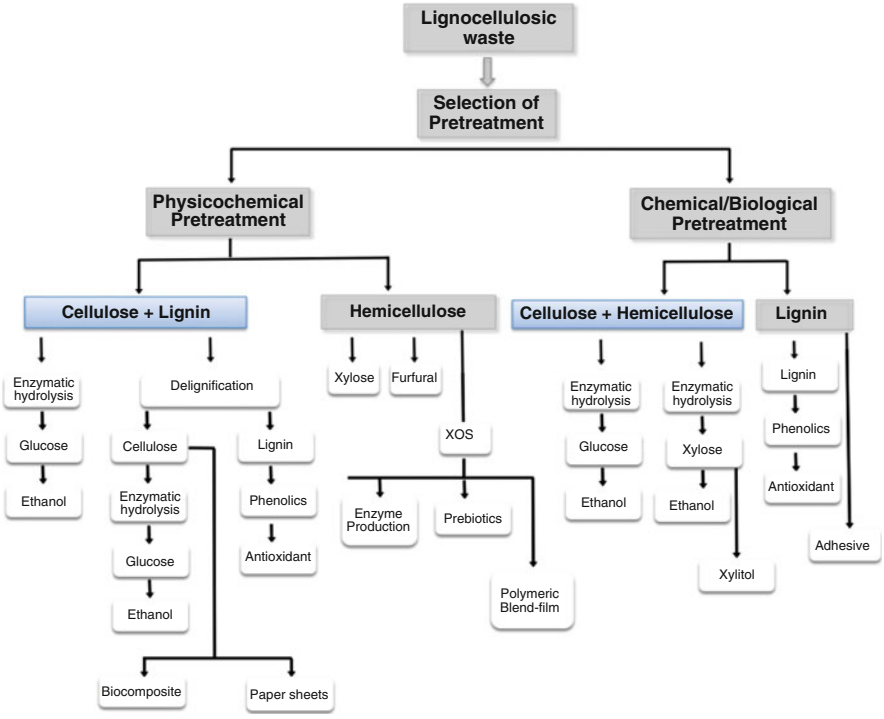

Fig. 2 Flowchart of conceptual biorefinery options for lignocellulosic wastes (Adapted and modified from Ruiz et al. 2013a)

black liquor gasification with downstream production of dimethyl ether or electricity and with recovery boiler-based pulping biorefinery for different types of mills. In a recent work, Lundberg et al. (2014) investigated the conversion of an existing Swedish kraft pulp mill to the production of dissolving pulp, with export of electricity, lignin, and hemicellulose stream suitable for upgrading, and they concluded that the profitability of this kraft pulp mill biorefinery is very dependent on the particular mill and the specific investment needs. Moreover, there are few companies that operate under the concept of biorefinery. An overview of almost all the biorefinery demonstration and pilot plants has been published in www.iea-bioenergy.task42-biorefineries.com, and new plants are continuously being built around the world, the USA being one of the main operators of biorefineries and producer of biofuels and high value-added compounds.

An important step in biorefineries of second generation is the pretreatment process. Ruiz et al. (2013a, b) reported that the pretreatment stage plays an important role in the biorefinery concept, since the pretreatment allows the fractionation of the main components of the biomass, producing substrates for conversion into biofuels and high value-added chemicals (Fig. 3).

3.1 Pretreatment Technologies

Due to the robust structure of lignocellulosic wastes, a pretreatment is required to alter its structure and chemical composition and promote fractionation (Fig. 3). According to Romaní et al. (2013a), the criteria for an effective pretreatment include: (1) avoiding size reduction, (2) preserving hemicellulose or lignin fraction, (3) limiting formation of inhibitors or sugar degradation, (4) minimizing energy input, and (5) being cost-effective. Yang and Wyman (2008) mentioned that the choice of pretreatment technology is not trivial and must take into account sugar production and solid concentrations for each pretreatment in conjunction with their compatibility with the overall process, feedstock, enzymes, and organisms to be applied and to meet the biorefinery concept. In the following sections, some different pretreatments used in second-generation processing of lignocellulosic wastes will be addressed.

3.1.1 Physical Pretreatment

Some of the important variables in the pretreatment process are residence time, temperature, and particle size. Normally, when larger chips are used, heat transfer problems lead to overcooking of the exterior (with associated formation of inhibitors) and incomplete hydrolysis of the interior. This problem can be overcome by reducing particle size before the application of the pretreatment. This size reduction process not only changes the particle size and shape, but also increases bulk density, improves flow properties, and increases porosity and surface area. This higher surface area increases the number of contact points for chemical reaction (Ruiz et al. 2011a). Also, reduction of the crystallinity of the cellulose is one of the important objectives of physical pretreatment. Chipping, grinding, and milling

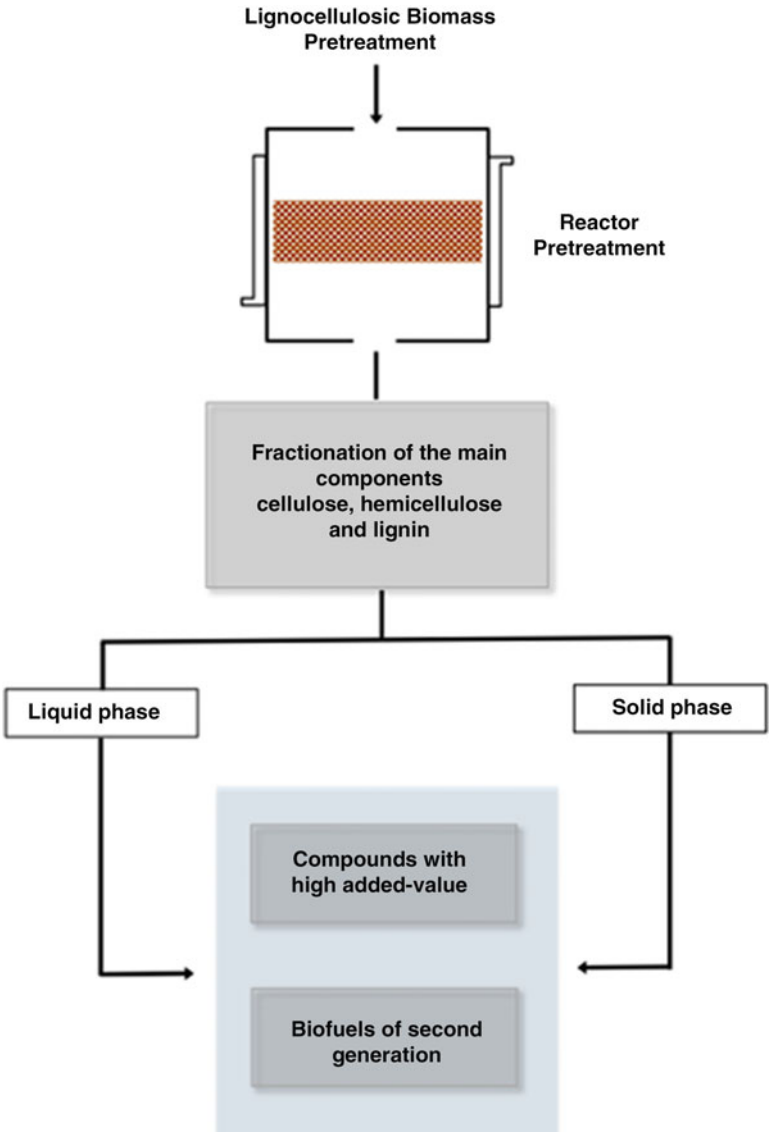

Fig. 3 Scheme for biomass fractionation

are among the different mechanical size reduction methods that have been used to enhance the digestibility of lignocellulosic wastes (Agbor et al. 2011). However, the power and energy requirement of this pretreatment is relatively high depending on the final particle size and the lignocellulosic waste characteristics. Delgenes et al. (2002) cited by Agbor et al. (2011) reported that the milling pretreatment increases biogas, biohydrogen, and bioethanol yields.

Extrusion pretreatment is a promising physical pretreatment method for lignocellulosic waste conversion to bioethanol production (Alvira et al. 2010). According to Lamsal and Brijwani (2010), the extrusion pretreatment can provide a unique continuous reactor environment for a combination of thermomechanical and chemical pretreatment of lignocellulosic wastes at higher yields. The materials are subjected to heating, mixing, and shearing, resulting in physical and chemical modifications during the process (Alvira et al. 2010). Lamsal et al. (2010) compared the particle size reduction and thermochemical extrusion pretreatment for the structural modification of wheat bran and soybean hull as raw material. They concluded that the use of chemical pretreatment in combination with extrusion did not result in improvement in hydrolysis of the lignocellulosic materials. Washing of thermomechanically treated samples was found to be very critical in maximizing the reducing sugar yields. Karunanithy et al. (2012) investigated the effect of biomass moisture content and extruder parameters such screw speed and barrel temperature on sugar recovery. They concluded that these variables were significant to the cellulose, hemicellulose, and total sugar recovery.

3.1.2 Physicochemical Pretreatment

Autohydrolysis Pretreatment

Autohydrolysis process is an environmentally friendly process in which the lignocellulosic wastes are pretreated with compressed hot water; it is based on the selective depolymerization of hemicellulose, which is catalyzed by hydronium ions generated in situ by water autoionization and by acetic acid from acetyl groups. Moreover, autohydrolysis pretreatment causes re-localization of lignin on the surface of lignocellulosic biomass. This process avoids difficult steps in chemical handling and recovery (e.g., sulfuric or hydrochloric acid) compared with dilute sulfuric acid or base pretreatment (Ruiz et al. 2012a, 2013a). Autohydrolysis process has been considered a cost-effective pretreatment, and in general, the major advantages that this process offers are as follows: (1) the process does not require the addition and recovery of chemicals different from water, (2) limited equipment corrosion problems, and (3) simple and economical operation. For that reason, autohydrolysis can be considered an environmentally friendly fractionation process.

According to Ruiz et al. (2013a), after autohydrolysis pretreatment, the cellulose shows degradation at temperatures >230 °C. It has been reported that the cellulose started to degrade in hexoses and oligosaccharides above 230 °C, and almost all cellulose was decomposed at 295 °C (Sakaki et al. 2002). An application of the solid residue (cellulose + lignin) after autohydrolysis pretreatment is used as raw material for pulp and paper marking (Ruiz et al. 2011b). Alfaro et al. (2010) reported that the cellulose pulp autohydrolysis reduces Kappa number and viscosity and decreases paper strength. Vila et al. (2011) studied the susceptibility of autohydrolysis solids to kraft processing pulp, being obtained cellulose pulps with low Kappa numbers and highly susceptible to alkaline oxygen bleaching.

Microwave Pretreatment

Microwaves as an alternative heating source have been successfully applied for extraction of numerous biologically active compounds from a wide variety of natural resources, because it is characterized as a selective, efficient, and environmental friendly process. This technique consists in a rapid delivery of energy to the total volume and subsequent rapid heating of the material structure accelerating the solubilization of compounds. Polar solvents have permanent dipole moment and can absorb microwave radiation, and water as a polar compound can absorb the microwave energy and transfer it to the sample, having as advantage over the organic solvents being a secure and ecologic reagent (Rodriguez-Jasso et al. 2011).

Budarin et al. (2010) reported the interaction between cellulose with microwave irradiation with high quality fuels produced from biomass. The substrate pretreated with microwave process has also been used for cellulose production. Zhao et al. (2010) reported that when some substrates were pretreated with microwave irradiation, the reducing sugar content and carboxymethylcellulase production were increased, and most cellulase was produced by the substrates pretreated at 450-W microwave for 3 min. Moreover, the microwave pretreatment has been used for enhancing enzymatic hydrolysis of cellulose (Peng et al. 2013).

Steam Explosion Pretreatment

This process is also considered as a hydrothermal process and has almost the same fundament of autohydrolysis. However, in this process, the high pressure steaming is followed by a rapid decompression. Steam explosion of lignocellulosic wastes has been largely studied in the last 15–20 years. This physicochemical method modifies lignocellulose material to allow the fractionation of the three polymer streams: hemicellulose into the liquor, and lignin and cellulose remaining in the insoluble fraction. Steam pretreatment of the lignocellulosic materials at temperatures of 160–220 °C generates acetic acid (from hemicellulose), which catalyzes the hydrolysis of hemicellulose resulting in its solubilization. When the process reaction is performed under high pressures followed by explosive decompression, the resulting residue is rendered highly degradable by enzymes and microorganisms.

Deepa et al. (2011) extracted cellulose nanofibers from banana fibers by steam explosion pretreatment; these nanofibers had better thermal properties over the untreated fibers. Romaní et al. (2013b) used steam exploded pretreated solids (*Eucalyptus globulus* as raw material) subjected to simultaneous saccharification and fermentation at high solids loading and reached an ethanol concentration of 51 g/L with a 91 % yield. Oliveira et al. (2013) reported the use of an industrial-scale steam explosion pretreatment of sugarcane straw for the production of bioethanol and high value-added chemicals.

Ultrasound Pretreatment

The ultrasound pretreatment is a new emerging technology that has potential as an alternative pretreatment technology. According to Yunus et al. (2010), the ultrasound pretreatment does not hydrolyze the biomass to soluble sugars. The function is to generate a pretreated substrate that is more easily hydrolyzed via increasing the

accessible surface area and affecting the crystallinity. This process has been recently reviewed by Bussemaker and Zhang (2013) for biorefinery and biofuel applications, and they commented that the ultrasonic waves create pressure differences within a solution for the enhancement of physical (mechano-acoustic) and chemical (sonochemical) processes; this ultrasonic phenomenon is generated by either piezoelectric or magnetostrictive transducers, where the transducer is attached to a vessel filled with a sonication solution and the mechanical vibrations of the piezoelectric material creates a pressure wave through the solution. This effect of ultrasound on lignocellulosic wastes has been employed to improve the extractability of hemicelluloses, cellulose, and lignin or to get clean cellulosic fiber from used paper; however, only a few attempts to improve the susceptibility of lignocellulosic materials according to biorefinery concept have been made (Bussemaker et al. 2013).

Yunus et al. (2010) studied the effect of ultrasonic pretreatment on oil palm empty fruit bunch fiber prior to acid hydrolysis, and they concluded that the exposition of this material to ultrasonication power has a marked effect on the efficiency of low temperature acid hydrolysis. García et al. (2011) reported the ultrasound pretreatment of lignocellulosic waste and showed that the application of this pretreatment improved the effectiveness of the classic pretreatments, obtaining higher yield and selectivity of the products. Nikolic et al. (2011) reported the utilization of microwave and ultrasound pretreatments in the production of bioethanol using corn as raw material, having concluded that ultrasonic and microwave pretreatments effectively increased the glucose concentration obtained after liquefaction and consequently improved the ethanol yield and productivity during the SSF process.

3.1.3 Chemical Pretreatment

Several chemical pretreatments have been studied in order to remove the hemicellulosic fraction, to cleave the bindings between the lignin and the polysaccharides, and to distort the arrangement of the cellulose crystallinity, improving the accessibility of the cellulose component to the action of hydrolytic enzymes so that an efficient hydrolysis of carbohydrates to fermentable sugars occurs (Sun and Cheng 2002; Mosier et al. 2005). Moreover, the combination of sequential chemical pretreatments is an interesting alternative for the lignocellulosic wastes being in accordance with the biorefinery concept (Ruiz et al. 2011b).

Alkaline Pretreatment

The alkaline pretreatment is one of the most studied and makes use of several reagents as sodium hydroxide, calcium hydroxide, potassium hydroxide, aqueous ammonia, and ammonia hydroxide, sometimes in mixture with hydrogen peroxide (Chen et al. 2013; Gonçalves et al. 2014). According to Chen et al. (2013) and García et al. (2014a), alkali reagent is believed to cleave hydrolyzable linkages in lignin and glycosidic bonds of polysaccharides causing a reduction in the degree of polymerization, increasing the internal surface area and decreasing crystallinity of the cellulose and disruption of the lignin structure which increases the reactivity

of the remaining polysaccharides as delignification occurs (Pedersen and Meyer 2010). Also, the alkali pretreatment is effective for agricultural residues and herbaceous crops, due to the smaller amount of lignin present in these types of materials (Galbe and Zacchi 2012). The alkaline pretreatment has as advantage the low energetic demand due to the severity of the alkaline media, where high reaction temperatures are usually not required (below 140 °C) and as disadvantage the alkali price and the difficulty of its recuperation that still involves prohibitive costs (Sun and Cheng 2002; Hamelink et al. 2005).

In a recent work, Gonçalves et al. (2014) studied the production of bioethanol using a sequential alkali pretreatment with hydrogen peroxide and sodium hydroxide, obtaining a high susceptibility of pretreated materials to enzymatic action. Selig et al. (2009) pretreated corn stover with alkaline peroxide at pH 11.5 resulting in reduction of lignin content in the pretreated solids and improvement of sugar production by cellulases. Prinsen et al. (2013) studied the modification of the lignin structure of a eucalyptus feedstock during alkaline delignification by kraft, soda–anthraquinone, and soda-O_2, indicating that soda-O_2 process produced higher lignin degradation and provides results as a pretreatment for the deconstruction of eucalyptus feedstock for subsequent use in lignocellulose biorefineries.

Acid Pretreatment

The use of acid pretreatment to catalyze the hydrolysis of lignocellulosic wastes in their sugar constituents is well known and effective. Acid pretreatment has been considered as a suitable technology for industrial-scale bioethanol production from glucose and hemicellulosic sugars (Ruiz et al. 2013c). Different types of acids have been used in these pretreatments such as: phosphoric, sulfuric, and organic acid (oxalic, citric, tartaric and acetic) (Qin et al. 2012; Avci et al. 2013; Ruiz et al. 2013c).

Castro et al. (2014) optimized the phosphoric acid pretreatment of *Eucalyptus benthamii* wood chips and produced bioethanol from this pretreated material with an ethanol yield of 240 g ethanol/kg of raw material. García et al. (2014b) produced bioethanol from the shells of *Jatropha curcas* using dilute sulfuric acid pretreatment, the cellulose conversion being above 80 %.

Organosolv Pretreatment

As alternative to conventional chemical pulping, processes utilizing aqueous organic solvents, known as organosolv, have been studied in the last 30 years. This pretreatment involves the use of organic solvents or their aqueous solutions for extracting lignin, based on the fact that lignin can be solubilized in certain solvents in a wide range of temperatures (100–250 °C) (González Alriols et al. 2009; Agbor et al. 2011). During the organosolv process, the lignin structure is broken into smaller parts and dissolved from the raw material and separated in the form of a liquor rich in phenolic compounds that represents the process effluent. This lignin can be isolated and has the advantage of being a relatively pure product with excellent properties that may be used as a precursor in the production of various commercial products according to the biorefinery concept (Ruiz et al. 2011b).

The organosolv pretreatment has been investigated due to the effective results for hemicelluloses/lignin depolymerization, increasing the cellulosic fraction digestibility (Geng et al. 2012). However, most of the used organic solvents need to be recovered for economic and environmental reasons (Alvira et al. 2010; Galbe and Zacchi 2012). The organosolv process has been developed as part of a commercial lignocellulose biorefinery technology known as the Lignol process. Lignol is a pilot plant that obtained several high value products in a cost-effective process where solvents were recovered and recycled at the end of the process (Pan et al. 2005).

Torre et al. (2013) reported that the cellulose pulp with the organosolv process is especially attractive because it can be used in boiler combustion chambers. Li et al. (2012) studied the fractionation of organosolv lignin with organic solvents and reported that this fractionation provides a way to prepare lignin with homogeneous structure and good functional properties for several potential applications. Kautto et al. (2013) reported that the organosolv pulping can be used as a pretreatment step in bioethanol production allowing, in complement to the production of bioethanol, the production of a pure lignin and other coproducts according to biorefinery concept.

Ozonolysis Pretreatment

Ozonolysis pretreatment includes using ozone gas, a powerful oxidant and soluble in water, in order to breakdown lignin and hemicelluloses and increase cellulose biodegradability; soluble compounds of smaller molecular weight such as acetic and formic acid (Balat 2011) may also be released. The lack of degradation by-products and operation at ambient conditions constitute the advantages of this pretreatment (Garcia-Cubero et al. 2009), while the disadvantages reside in the cost of ozone (Sun and Cheng 2002).

Travaini et al. (2013) pretreated sugarcane bagasse in a fixed bed reactor with ozone and studied the effect of ozone concentration and sample moisture, and they concluded that ozonolysis process is a promising pretreatment to obtain a high glucose conversion from cellulose.

Ionic Liquid Pretreatment

Ionic liquids are organic salts that exist as liquids at low temperatures, with tunable physicochemical properties, low vapor pressures, good thermal stability, and different combination of anions and cations to their synthesis (Fort et al. 2007; Lee et al. 2008). Recent studies of particular interest have indicated that both cellulose and lignin can be dissolved in a variety of ionic liquids and, perhaps more important, easily regenerated from these solutions. Thus, studies have shown ionic liquids with potential to be used as an environmentally benign pretreatment of lignocellulosic wastes (Fort et al. 2007; Zhu 2008; FitzPatrick et al. 2010).

Yuan et al. (2013) mentioned that anionic liquid-based biorefining strategy could integrate biofuel production into a biorefinery scheme in which the major components of poplar wood can be converted into value-added products. Shafiei et al. (2013) produced bioethanol from spruce wood chips using ionic liquid as pretreatment; they reported that ethanol yield was between 66.8 % and 81.5 %.

Labbé et al. (2012) investigated three ionic liquids as potential media to act on biomass and make the cellulose component more accessible to hydrolytic enzymes. The ionic liquid [emim][OAc] was the most efficient in the fractionation of lignocellulosic waste. In general terms, the treatment with ionic liquids is a useful technology for the development of the biorefinery concept (Stark 2011).

3.1.4 Biological Pretreatment

The biological or microbial pretreatment involves the use of microorganisms or enzymes. In addition, in biological pretreatment, particle size, moisture content, resident time, and temperature, besides the microbial agents used, could affect lignin degradation and enzymatic hydrolysis yield (Patel et al. 2007; Wan and Li 2010).

Unlike most of the chemical and physicochemical pretreatment methods, biological pretreatment offers as advantages low energy consumption and no chemical requirement, in addition to mild operational conditions and likely ease of integration into a consolidated bioprocessing setup (Sun and Cheng 2002; Yang and Wyman 2008; FitzPatrick et al. 2010). The disadvantages include long-time process, large space requirement, and the need for continuous monitoring of microorganism growth (Wyman et al. 2005; Taherzadeh and Karimi 2008).

Research on fungal pretreatment is mainly focused on evaluating fungi that selectively degrade lignin and hemicellulose, while utilizing little cellulose (Chen et al. 1995; Singh et al. 2008; Shi et al. 2009; Wan and Li 2010). Cellulose is more recalcitrant to fungal attack than other components. On the other hand, microbial consortium pretreatment is conducted by microbes screened from natural environment's typically rotten lignocellulosic biomass. In contrast to fungal pretreatment, which is usually conducted under sterilized conditions, in most cases, sterilization of lignocellulosic feedstocks is not necessary when using a microbial consortium for pretreatment, which is an advantage over fungal pretreatment. In enzymatic pretreatment, the most commonly used enzymes are cellulases and hemicellulases with the disadvantages of the cost of enzymes to be high and therefore its application is limited.

3.1.5 Integrated Pretreatment

Other pretreatment processes, using integrated methods, have been a target of some researches in order to improve the efficiency of fractionating, decrease the formation of inhibitors, and shorten process time (Mood et al. 2013; Zheng et al. 2014).

Thus, in literature, there are several studies with combination of alkaline and dilute acid pretreatments resulting at more effective delignification and less carbohydrate degradation, in comparison with alkali and acid pretreatment solely (Lu et al. 2009), and combinations of dilute acid and microwave pretreatments resulting in highest biomass fragmentation and swelling as well as complete hemicellulose degradation (Chen et al. 2011). Combination of biological and steam explosion pretreatment reduced the pretreatment time significantly (Taniguchi et al. 2010), while the combination of biological and dilute acid pretreatments led to enhanced enzymatic hydrolysis (Na et al. 2010). The combination

of dilute acid and steam explosion pretreatment revealed a high xylose yield, a low level of inhibitors, and an enhanced saccharification efficiency (Sun and Cheng 2002). The use of microwave-based heating, instead of the conventional heating, in alkali pretreatment, removed more lignin and hemicelluloses in shorter pretreatment time (Yuanxin et al. 2005, 2006), while the synergic effect of the combination of ultrasonic pretreatment, instead of the conventional heating pretreatment, and different ionic liquids also enhanced the saccharification ratio (Ninomiya et al. 2010).

3.2 Enzymatic Hydrolysis

After the pretreatment, the cellulose is more susceptible to enzymatic attack (Cheng and Timilsina 2011). Therefore, the enzymatic hydrolysis is the second step in the biorefinery processing of cellulose from lignocellulosic wastes. It involves cleaving the cellulose polymers to soluble monomeric sugars using a class of enzymes known as cellulases.

The cellulases are highly specific, and the enzymatic hydrolysis of cellulose is usually carried out under mild conditions of pressure, temperature, and pH (Binod et al. 2010). Most cellulases show an optimum activity at temperatures and pH in the range of 45–55 °C and 4–5, respectively (Duff and Murray 1996; Galbe and Zacchi 2002; Talebnia et al. 2010; Cheng and Timilsina 2011). Enzymes tolerant to high temperature and low pH are preferred for the enzymatic hydrolysis due to most current pretreatments making use of acid and heat. Besides, thermostable enzymes have several advantages including higher specific activity and higher stability, which improves the overall enzymatic performance.

Ultimately, improvement in catalytic efficiencies of enzymes reduces the cost of process by enabling lower enzyme dosages (Dashtban et al. 2009). A cellulase dosage of 10–30 FPU/g cellulose is often used in laboratory studies because it results in an efficient hydrolysis with high glucose yield in a reasonable time (48–72 h). However, enzymes loading may vary depending on the pretreatment, type, and concentration of raw materials (Talebnia et al. 2010). Although the cost of enzyme production is still high, a reduction in the costs may be obtained in the case less noble materials are used (Castro and Pereira Jr 2010).

Fungi and bacteria can produce cellulases for the hydrolysis of lignocellulosic wastes, and these enzymes have been mainly produced by species of *Trichoderma*, *Aspergillus*, *Schizophyllum*, and *Penicillium*. Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora*, and *Streptomyces* can produce cellulases. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens*, produce cellulases with high specific activity, they do not produce high enzyme titers (Sun and Cheng 2002).

Therefore, most research for commercial cellulase production has been focused on fungi (Talebnia et al. 2010). Besides, due to the promising thermostability and

acidic tolerance of thermophilic fungal enzymes, they have good potential to be used for hydrolysis of lignocellulosic wastes at industrial scale (Dashtban et al. 2009).

The use of a mixture of cellulases from different microorganisms or a mixture of cellulases and other enzymes has been extensively studied since this can raise the rate of enzymatic hydrolysis of lignocellulosic biomass. The combination of enzymes such as cellulase, xylanases, and pectinases exhibits a significant increase in the extent of cellulose conversion (Sun and Cheng 2002; Binod et al. 2010). Tabka et al. (2006) observed that the addition of accessory enzymes, such as xylanases, feruloyl esterase, and laccase, on pretreated wheat straw could act in synergistically, improving the enzymatic hydrolysis of cellulose on pretreated material.

Currently, the use of enzymes from a cellulolytic complex in the hydrolysis of biomass is one of the more emerging applications. The lignocellulosic feedstocks contain from 20 % to 60 % of cellulose, which may be fully converted to glucose by enzymatic action. In subsequent steps, the monosaccharide can be used as a building block for obtaining a vast range of products, which range from biofuels to polymers. These technologies fall under the definition of cellulosic biorefineries, which aim to the integrated and integral use of agro-industrial wastes generated in a given production chain with value addition (Castro and Pereira Jr 2010).

The three enzymes involved in hydrolysis of cellulose to glucose by synergistic action include:

3.2.1 Endoglucanase (EG)

EG (Endo-1,4-β-D-glucanohydrolase; EC 3.2.1.4) cleaves the β-1,4-glucosidic linkages in the interior of cellulose molecule to produce cellooligosaccharides with free chain ends. It is responsible for starting cellulose hydrolysis by attacking randomly regions of low crystallinity in the cellulose fiber and making it more accessible for cellobiohydrolases (Talebnia et al. 2010; Cheng and Timilsina 2011). The EG is the cellulolytic enzyme responsible for the rapid solubilization of cellulose due to their fragmentation in oligosaccharides. It is also referred as carboxymethylcellulase (CMCase) because of the use of carboxymethylcellulose (CMC) as substrate to measure its activity (Michelin et al. 2013).

Studies have shown that many fungi produce multiple EGs. For example, *T. reesei* produces at least five EGs (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A and EGV/Cel45A), whereas three EGs were isolated from white-rot fungus *Phanerochaete chrysosporium* (EG28, EG34 and EG44) (Dashtban et al. 2009).

3.2.2 Cellobiohydrolase (CBH)

CBH (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) is an exoglucanase that preferentially hydrolyzes β-1,4-glycosidic bonds of the cellulose, releasing cellobiose units from chain ends (Michelin et al. 2013). Cellobiose, the end product of CBHs, acts as an inhibitor, which can limit the ability of the enzymes to degrade

cellulose (Dashtban et al. 2009). Microcrystalline cellulose (Avicel) has been used as substrate to measure its activity (Michelin et al. 2013).

Although IUBMB (International Union of Biochemistry and Molecular Biology) defines the CBH as a catalyst of the hydrolysis of only the nonreducing ends of the cellulosic fiber and oligosaccharides with degree of polymerization (DP) higher than three in cellobiose, studies have shown that some CBHs can act from the reducing ends of the cellulosic chains, which increases the synergy between opposite-acting enzymes (Lynd et al. 2002; Michelin et al. 2013). For example, *T. reesei* has shown to have two CBHs acting from nonreducing (CBHII/Cel6A) and reducing (CBHI/Cel7A) ends (Zhang and Lynd 2004; Dashtban et al. 2009).

3.2.3 β-Glucosidase (BGL)

BGL (1,4-β-D-glucosidase glucanohydrolase; EC 3.2.1.21) cleaves cellobiose and other cellodextrins with a DP up to six releasing glucose units. The hydrolysis rates decrease markedly as the substrate DPs increase (Zhang et al. 2006). BGL is also inhibited by its end product (glucose).

BGLs are very amenable to a wide range of simple sensitive assay methods, based on colored or fluorescent products released from some synthetic substrates, such as *p*-nitrophenyl β-D-1,4-glucopyranoside. Also, BGL activities can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases (Ghose 1987; Zhang and Lynd 2004; Zhang et al. 2006).

BGLs have been isolated from many different fungal species including *Ascomycetes* such as *T. reesei* and *Basidiomycetes* such as white-rot and brown-rot fungi. In *T. reesei*, two β-glucosidases (BGL I/Cel3A & BGL II/Cel1A) have been isolated from culture supernatant, but the enzymes were found to be primarily bound to the cell wall. However, BGL production in *T. reesei* is very low compared to other fungi such as *A. niger* (Dashtban et al. 2009).

3.3 Fermentation and Bioproducts

3.3.1 Fermentation

Fermentation can be defined as a process performed mainly by microorganisms such as bacteria, yeast, and fungi in order to obtain a bioproduct from a suitable nutrient source. This process can be performed by solid-state or submerged cultures. Solid-state fermentation (SSF) is a complex heterogeneous three-phase (gas–liquid–solid) process defined as the growth of microorganisms, often fungi, on the surface of a porous and moist solid substrate particle in which enough moisture is present to maintain microbial growth and metabolism; submerged fermentation has been defined as fermentation in a liquid medium (Pandey 2003; Ruiz et al. 2012b).

Currently, the use of lignocellulosic wastes has been employed as nutrient source in fermentative process, and special attention has been given to cellulose present in these materials. Due to the complexity of the cellulose, several forms to lead the fermentative process were developed in order to obtain a high efficiency.

For example, for the conversion of the sugars from cellulose in molecules of interest, such as ethanol, a step of enzymatic hydrolysis of the cellulose prior to submerged fermentation is necessary, and both steps can be performed separately (SHF – separate hydrolysis and fermentation), simultaneously (SSF – simultaneous saccharification and fermentation), and semi-simultaneously (SSSF– semi-simultaneous saccharification and fermentation) (Dashtban et al. 2009; Castro and Pereira Jr 2010; Gonçalves et al. 2014).

In SHF, the hydrolysis of cellulose occurs in a separate step and after that, the sugars released are fermented to ethanol. The advantage of this method is that both steps can be carried out in each optimum condition (e.g., optimum temperature for cellulase hydrolysis is around 50 °C and for fermentation 30 °C). Besides, in this process, the cells can be recycled, since there is no raw material in suspension during fermentation. The main drawback is the accumulation of intermediate sugars (cellobiose and glucose) during the hydrolysis, which can cause inhibition of the cellulase enzymes and a reduction in the final conversion to glucose. This makes the process inefficient, and the costly addition of β-glucosidase is needed in order to promote a reduction of the inhibition of endo- and exoglucanase by its hydrolysis products (particularly cellobiose) and increase the final conversion of substrate to glucose that will be used for fermentation (Philippidis et al. 1993; Kádár et al. 2004).

In SSF, the enzymes are less susceptible to inhibition by hydrolysis products because the released glucose is fermented simultaneously. Therefore, addition of high amounts of β-glucosidase is not necessary, which reduces the costs of the process (Dashtban et al. 2009). The maintenance of low glucose concentration in the medium also promotes the continuous hydrolysis reaction and reduces the risk of contamination of the system. Besides, this process contributes to lower cost of investment at the plant since the two steps are performed in the same reactor (Castro and Pereira Jr 2010). The main drawback of SSF is the need to lead the process in suboptimal conditions of temperature and pH. The development of recombinant yeast strains (i.e., improved thermotolerance) is expected to enhance the performance of SSF (Galbe and Zacchi 2002). The semi-simultaneous saccharification and fermentation (SSSF) is a good alternative that includes a short presaccharification period before the SSF process (Gonçalves et al. 2014).

Considering the overall conversion of lignocellulosic wastes into ethanol, another alternative has been described. In this process, known as co-fermentation (CF), the fermentation of pentoses (mainly xylose from hemicellulosic fraction of the lignocellulosic wastes) and hexoses (glucose from cellulose) occurs in one reactor. The conduction of co-fermentation with concomitant hydrolysis of cellulose and/or hemicellulose fractions is called SSCF (simultaneous saccharification to co-fermentation) (Castro and Pereira Jr 2010).

Further process integration can be achieved by a process known as consolidated bioprocessing (CBP) which aims to minimize all bioconversion steps into one step using one or more microorganisms (enzyme production, hydrolysis of cellulose and hemicellulose, and fermentation of glucose and xylose happen in one reactor) and, thus, reduce product inhibition and operation costs (Limayem and Ricke 2012).

These processes are integrated within the concept of "biorefinery"-industrial installations designed to produce a wide range of bioproducts from conversion of biomass (Guo et al. 2010). A number of high value bioproducts can be obtained from lignocellulosic wastes such as bioethanol, organic acids, biohydrogen, enzymes, packaging materials, biocomposites, and others. In the sequence, some bioproducts that can be obtained from lignocellulosic wastes will be described.

3.3.2 Bioproducts

Bioethanol

In the past few years, much research has been done to find a viable alternative for ethanol production from lignocellulosic wastes (known as bioethanol, or cellulosic ethanol or second-generation ethanol) in view of fast depletion of fossil fuels and food shortages.

Ethanol is either used as fuel (pure or as an additive to gasoline) or a chemical feedstock and can be obtained from: (1) sucrose-containing feedstocks (e.g., sugar cane, sweet sorghum and sugar beet), (2) starchy materials (e.g., corn, wheat, and barley), and (3) lignocellulosic biomass (e.g., sugar cane bagasse, corncob, wood and straw) (Balat et al. 2008). The ethanol production from lignocellulose wastes has several advantages, such as: is a renewable energy source, abundant, of low cost, and noncompetitive with food crops. The use of ethanol fuel can significantly reduce the use of petroleum and reduce greenhouse gas emission. Currently, ethanol production from lignocellulosic wastes is one of the most studied and promising alternatives for reuse of these feedstocks, due to the large incentive that has been given to use of biofuels in replacement of gasoline (Mussato and Teixeira 2010).

Brazil and the USA produce ethanol from the fermentation of sucrose from sugarcane juice and starch from corn, respectively, generating wastes as the sugarcane bagasse and straw and corncob and corn straw. Therefore, a variety of widely available sugar feedstocks can be used (Sánchez 2009).

The production of fuel ethanol from lignocellulosic wastes includes an initial pretreatment (e.g., steam explosion or diluted acid) to render cellulose more accessible to the subsequent step of enzymatic hydrolysis (with cellulases), which breaks down cellulose to fermentable sugars, and finally the fermentation of the sugars to ethanol by yeast *Saccharomyces cerevisiae*. This conventional strain presents optimum temperature at 30 °C and tolerates a high osmotic pressure, low pH levels, and inhibitory products (Limayem and Ricke 2012).

Hsu et al. (2011) investigated the ethanol production by *S. cerevisiae* BCRC 21812 from reducing sugar released by hydrolysis of corncob material with cellulases (CMCase, Avicelase, and β-glucosidase) from *Streptomyces* sp. strain. Sukumaran et al. (2009) studied the saccharification of three different feedstocks, i.e., sugarcane bagasse, rice straw, and water hyacinth biomass (using cellulase and β-glucosidase) for ethanol production by *Saccharomyces cerevisiae*. The highest yield of reducing sugars was obtained from rice straw followed by sugarcane bagasse.

Intensive efforts have been done in the last years to (1) develop efficient technologies for the pretreatment of lignocellulosic wastes, since several factors have been described to affect the hydrolysis of cellulose, such as porosity (accessible surface area) of the lignocellulosic waste, crystallinity of cellulose fiber, and lignin and hemicellulose contents (McMillan 1994); (2) develop enzymes for enhanced cellulose/hemicellulose saccharification; and (3) develop suitable technologies for the fermentation of both hexose and pentose sugars (Soccol et al. 2010).

Biohydrogen

Hydrogen is considered as a clean fuel, forming water (instead of greenhouse gases) as the only combustion product. It has high energy content and can be used directly as fuel for transportation or, after purification, to produce electricity (Guo et al. 2010). Therefore, the development of renewable and cost-effective process for its production will contribute to increase the energy production and to reduce greenhouse effect (Kapdan and Kaegi 2006). Other applications of hydrogen include the use as chemical reactant in the production of fertilizers, for refining diesel and for the industrial synthesis of ammonia (Guo et al. 2010).

The use of the hydrogen as energy resource has been restricted in large part due to the high production costs, technical storage requirements, and distribution methods (Dunn 2002). Nowadays, the most part (around 88 %) of the hydrogen production derives from fossil fuels (natural gas, heavy oils or coal) (Nath and Das 2003), and up to 4 % of hydrogen production derives from water electrolysis. However, all such processes consume high energy (Guo et al. 2010).

Recently, biohydrogen gas production from lignocellulosic biomass, mainly agricultural wastes, has received more and more attention. It seems economically viable and technically feasible to produce biohydrogen from lignocellulose biomass by an integrated process involving pretreatment steps for the raw material and enzymatic hydrolysis for the yield of fermentable reducing sugars. After, the biohydrogen production can be performed by anaerobic fermentation process (Chen et al. 2008; Lo et al. 2008b, 2009b).

According to Magnusson et al. (2008), although there are several different methods of hydrogen production, biohydrogen production by dark fermentation, compared to alternative methods such as biophotolysis of water or photofermentation, is advantageous due to its higher rate of production. Besides, nonbiological methods of hydrogen production such as electrolysis and steam reformation of methane require extensive amounts of energy and also are sources of polluting emissions, such as CO_2, CO, NOx, and SOx.

The generation of biohydrogen from lignocellulosic wastes by using dark fermentation usually requires a step of pretreatment of substrate, which increases the production cost. Some investigators demonstrated a two-stage process (i.e., hydrolysis and hydrogen production) where cellulose hydrolysis can be performed by using mixed or pure microbial culture, and after, the hydrolysates (rich in reducing sugars) are used for sequential biohydrogen production by an efficient hydrogen producer. In this way, the hydrogen yield could be increased, and thus, the process

becomes more advantageous in practical applications due to a higher economical feasibility and less energy consumption (Lo et al. 2008a, 2009a).

Although pure cultures have been intensively investigated over the past years, involving species such as *Bacillus coagulans* (Kotay and Das 2007), *Thermoanaerobacterium* spp. (O-Thong et al. 2008), and *Clostridium butyricum* (Chen et al. 2005), few studies refer to the characterization of mixed cultures. In relation to mixed cultures producing biohydrogen, a wide range of species has been studied. In relation to mesophilic microorganisms, species from the genera *Clostridium* (*C. pasteurianum, C. saccharobutylicum, C. butyricum*), *Enterobacter* (*E. aerogenes*), and *Bacillus* can be cited, and in relation to thermophilic or extremophilic microorganisms, species include the genera *Thermoanaerobacterium* (*T. thermosaccharolyticum*), *Caldicellulosiruptor* (*C. saccharolyticus*), *Clostridium* (*C. thermocellum*), and *Bacillus* (*B. thermozeamaize*) (Guo et al. 2010).

Clostridium thermocellum is a thermophilic, acetogenic, anaerobic bacterium that degrades cellulose directly synthesizing a mixed product, such as acetate, hydrogen, and carbon dioxide, as well as lactate and ethanol under different growth conditions (Ng et al. 1977; Lynd et al. 1989). This bacterium expresses a suite of cellulolytic enzymes that degrades the cellulose to glucose and cellulodextrans. Of all known cellulose degrading microorganisms, *C. thermocellum* displays the highest rate of cellulose degradation (Lynd et al. 1989). Because of this characteristic and propensity to synthesize hydrogen, carbon dioxide, and acetate, *C. thermocellum* offers the potential for directly producing biohydrogen from cellulosic wastes. The high optimum growth temperature of *C. thermocellum* (60 °C) also reduces the chance of contamination by precluding the growth of predominant mesophilic microorganisms and allows for *C. thermocellum*-enriched cultures to be maintained. Besides, as the solubility of gases decreases with higher temperatures, the higher growth temperature also facilitates the efficient removal of product gases such as hydrogen and carbon dioxide.

Chen et al. (2005) reported *Clostridium butyricum* CGS5 as an efficient microorganism in converting sugars (e.g., glucose, xylose, sucrose) into hydrogen via dark fermentation. Unfortunately, *C. butyricum* CGS5, like most hydrogen-producing strains, cannot directly utilize cellulose or hemicellulose as carbon source for hydrogen production, and therefore, pretreatment and hydrolysis steps of the cellulosic feedstock were required to enable efficient cellulosic biohydrogen production.

Organic Acids

Some organic acids including citric and lactic acids can be produced by fermentation using hydrolysates rich in glucose obtained from cellulose of lignocellulosic wastes.

Currently, citric acid is produced mainly by fermentation (a small part is yet extracted from citrus fruits in Mexico and South America) submerged (SmF), in surface and in solid-state (SSF), using starch- or sucrose-based media (Jianlong 2000; Vandenberghe et al. 2000). The microorganism used is the filamentous

fungus A. niger, which can accumulate citric acid in media rich in carbohydrate but deficient in phosphate and trace elements like Fe^{+2} and Mn^{+2}.

Kumar et al. (2003) used pineapple, mixed fruit, and maosmi wastes as substrates to produce citric acid by solid-state fermentation using *Aspergillus niger* DS 1. Dhillon et al. (2011) also used different agro-industrial wastes, such as apple pomace, brewers' spent grain, citrus waste, and sphagnum peat moss to evaluate their suitability for the production of citric acid through solid-state and submerged fermentation by *A. niger* NRRL 567 and NRRL 2001.

Citric acid is used in several industrial sectors such as the food, beverage, and pharmaceutical industries. It is mainly used as an additive (antioxidant and acidulant) in the production of soft drinks, desserts, jellies, candies, and wines. It is also used as flavor enhancer and plasticizer. In pharmaceutical industry, it is used as anticoagulant (blood transfusion) and in the production of effervescent products. It is also used to adjust the pH of astringent lotions, as sequestering agent, and hair fixatives.

The food industry consumes about 70 % of the total production of citric acid (Rohr et al. 1983), due to some characteristics such as its pleasant acidic taste and its high solubility in water. Besides, it is worldwide accepted as "GRAS" (generally recognized as safe), approved by the joint FAO/WHO Expert Committee on Food Additives. The pharmaceutical industry consumes 12 %, and the rest 18 % has market for other applications (Penna 2001).

Lactic acid is another important organic acid that has attracted great attention because of its wide applications in food, chemical, and pharmaceutical industries. It is widely used as acidulant and preservative in the food industry, being used as taste-enhancing additive in soft drinks, jellies, syrups, and fruit juices. In the pharmaceutical industry, it is used for the adjustment of pH of pharmaceutical preparations and topical wart preparations. Other applications include blood coagulant and dietary calcium source. Lactic acid has been used in the manufacture of cellophane, resins, and some herbicides and pesticides. Another important application of lactic acid is in textile and tanning industries (Penna 2001).

Lactic acid has two enantiomers: D-(−) and L-(+) lactic acid. The production of optically pure D- or L-lactic acid can be obtained by fermentation when the appropriate microorganism is used (Abdel-Rahman et al. 2013).

Lactic acid is produced by fermentation and by chemical synthesis. Fermentative production offers some advantages, such as the use of cheap and renewable substrates, low production temperature, and low energy consumption. Currently, most of lactic acid produced worldwide is obtained from fermentative pathway (Abdel-Rahman et al. 2011). A racemic mixture of DL-lactic acid is usually produced by the chemical pathway.

Lactic acid can be produced from renewable materials by various microorganism species, including bacteria, fungi, yeast, microalgae, and cyanobacteria. Strains of *Lactobacillus* sp. have been common among the bacterial cultures (John et al. 2007a) and that of *Rhizopus* sp. among the fungal cultures (Tay and Yang 2002; Koutinas et al. 2007).

A major concern in lactic acid production by fermentation is to reduce the process costs with respect to substrate costs and production efficiency. Inexpensive and renewable materials can be used as substrates, including by-products of agricultural industries, food industries, and natural unutilized biomass such as starchy and lignocellulosic wastes (Nguyen et al. 2013).

Recently, lactic acid production has been studied with increased interest because of its application in the synthesis of biodegradable and biocompatible polylactic acid (PLA) polymers (Koutinas et al. 2007). L-(+) lactic acid can be polymerized to form polylactic acid (PLA). This polymer can be used in the manufacture of new biodegradable plastics. In comparison with petrochemical plastics, PLA production is considered a relatively immature technology at the industrial scale. This is mainly due to the high production cost of lactic acid – the feedstock for PLA (Abdel-Rahman et al. 2013).

Production of lactic acid from fermentation aiming the production of polylactic acid has increased, due to decreased in petrochemical resources and the problems of environmental pollution caused by the petrochemical industry. The pure polymers of poly(L-lactic acid) (PLLA) and poly(D-lactic acid) (PDLA) are relatively heat sensitive, while stereo complexes of polylactic acid produced by blending PLLA and PDLA have a melting point approximately 50 °C higher than their respective pure polymers and are more biodegradable. The ratio of L- and D-lactic acid influences the properties and the degradability of the resulting polylactic acid (Datta and Henry 2006; John et al. 2007b).

Cellulases

Cellulases, enzymes involved in cellulose hydrolysis, represent currently the third largest sale in the world market for enzymes (Singhania et al. 2010), due to their wide applications in several industry sectors, such as in extraction and clarification of fruit juices; as animal feed additive to enhance the absorption of nutrients, improving the feed value and performance of animals (Bhat 2000; Kuhad et al. 2011); in the textile industry for cotton softening and denim finishing; in the detergent industry to improve the appearance and color brightness, besides softening the garment and removing dirt particles trapped within the microfibril network (Bhat 2000); and in paper recycling (Singhania et al. 2010). However, cellulases may become the largest volume industrial enzyme, if ethanol fuel production from lignocellulosic biomass becomes a reality, since in this process, a step of enzymatic hydrolysis using cellulases is necessary.

Cellulases are relatively costly enzymes, and a significant reduction in production cost will be important for their commercial use in biorefineries. Strategies that will make the use of cellulases in biorefinery as a more economical process include the increase of volumetric productivity for commercial enzyme production, the production of enzymes using cheaper substrates, the production of enzyme preparations with greater stability for specific processes, and the production of cellulases with higher specific activity on solid substrates (Zhang et al. 2006).

Agro-industrial wastes such as wheat straw, corncobs, and sugarcane bagasse (pretreated or not) have been widely included in the nutrient media composition as

the main strategy in microbial cellulase biosynthesis and other enzymes that degrade biomass, due to their high cellulose content, wide availability, and low cost (Michelin et al. 2013).

Currently, Genencor International and Novozymes Biotech companies are the two largest producers of cellulases. Both companies have played a significant role in bringing down the cost of cellulase through active research and are continuing to bring down the cost by adopting novel technologies (Singhania et al. 2010). They reported the development of technology that has reduced the cellulase cost for the process of bioethanol production from US$5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol (Moreira 2005; Zhang et al. 2006).

A wide variety of microorganisms including fungi and bacteria have been reported to degrade cellulose by synthesizing enzymes of the cellulolytic complex. Filamentous fungi are especially interesting since they secrete these enzymes into the medium, with levels superior to those found in yeasts and bacteria. These microorganisms can degrade lignocellulosic wastes better than other microbes as they closely resemble their natural habitat.

One of the most extensively studied cellulase producers is the *Trichoderma reesei* fungus, which is capable of hydrolyzing native cellulose. It produces two cellobiohydrolases (CBHI and CBHII) and two endoglucanases (EG1 and EG2), in a rough proportion of 60:20:10:10, which together can make up to 90 % of the enzyme cocktail, while seven β-glucosidases – BGL I–VII – secreted by this fungus typically make up less than 1 % (Lynd et al. 2002; Aro et al. 2005; Herpoël-Gimbert et al. 2008). Other fungi that produce cellulases include *Aspergillus*, *Humicola*, and *Penicillium* (Singhania 2009).

Most commercial cellulases (including β-glucosidase) are produced by *Trichoderma species* and *Aspergillus species* (Zhang et al. 2006). Recently, Genencor Company has launched Accellerase®1500, a cellulase complex intended specifically for lignocellulosic biomass processing industries, which is produced with a genetically modified strain of *T. reesei*. This enzyme preparation is claimed to contain higher levels of β-glucosidase activity than all other commercial cellulases available today, to ensure almost complete conversion of cellobiose to glucose (Singhania et al. 2010).

The large market potential and the important role that cellulases play in the bioenergy and bio-based products industries provide a great motivation to develop better cellulase preparations for cellulose hydrolysis of lignocellulosic wastes.

Biocomposites

Currently, there is a wide variety of wood and non-wood resources suitable for the production of cellulose nanostructures. The use of non-wood resources such as agricultural/agro-industrial wastes is particularly interestingly due to their renewable nature (Alemdar and Sain, 2008; Lavoine et al. 2012). Besides, the shortage of natural resources such as wood and the concerns over the environmental impact of other widely used materials have encouraged researches with alternative materials (Ghaderi et al. 2014). Other advantage of non-wood materials is that the cellulose

microfibrils from wall of agricultural wastes are easier to break down than wood wall, and the fibrillation of this pulp demands less energy.

Microfibrillated cellulose (MFC) is being increasingly studied from a number of different cellulosic sources (Lavoine et al. 2012). Due to its nanometer scale, its high surface energy and its ability to form a nanoporous network, MFC has been studied for use in nanocomposites as a mechanical reinforcement (Siqueira et al. 2010) and as a dispersion stabilizer (Andresen et al. 2006).

All-cellulose nanocomposites are classified as biocomposite, a class of materials that has been recognized and studied during the past decade (Nishino et al. 2004). The matrix and reinforcement phases in these biocomposite materials are noncrystalline and undissolved cellulose, respectively (Soykeabkaew et al. 2009). These nanocomposites have promising properties, as they are a completely bio-based material and fully biodegradable. Besides, they also have remarkably high mechanical performance and transparency (Nishino et al. 2004; Gindl and Keckes 2005). These characteristics give composite materials the potential for many applications.

The use of MFC suspensions as coating slurries has several applications, such as in printing industry and, more recently, in the food packaging sector. One further possibility is the use of MFC in drug release applications (Lavoine et al. 2014). In the literature, there are diverse agricultural/agro-industrial sources, such as wheat straw and soy hulls (Alemdar and Sain 2008) and bagasse (Bhattacharya et al. 2008), that are being used to produce MFC.

4 Future Challenges and Trends of Cellulose

Recent advances in understanding the biochemical complex in cell wall structure and their biochemical characteristics offer new avenues for the development of new biological-based processes for biomass conversion to different bioproducts at industrial scale. In that sense, a huge potential exists in upgrading fuel and energy producing pathways into biorefineries in order to improve its financial performance and long-term sustainability. However, high production costs remain the bottleneck for large-scale development of this pathway. Another important issue is how extensively can biomass be utilized without causing significant and irreversible harmful environmental and social impacts.

One of the main problems during the pretreatment and hydrolysis of biomass is the variability in the content of lignin and hemicellulose. This variability depends on factors as the type of plant from which the biomass is obtained, crop age, method of harvesting, and others. This makes that no one of the pretreatment methods can be applied in a generic way for many different feedstocks (Claassen et al. 1999). The future trends for improving the pretreatment of lignocellulosic feedstocks also include the production of genetically modified plant materials with higher carbohydrate content or modified plant structure to facilitate pretreatment (Sanchez and Cardona 2008).

Therefore, the success and efficiency of the applied pretreatment must be accomplished by the proper selection of treatment conditions for raw materials that present different recalcitrance. A single pretreatment method does not provide efficient results due to its limited specificity. For this, the proper combination of pretreatment steps (firstly, for hemicelluloses solubilization and, secondly, for effective delignification) should be applied in order, not only to improve the effectiveness of the following biorefinery processing stages but also to avoid high contaminated hemicellulosic or lignin containing streams. These fractionation by-products could be properly exploited avoiding further purification steps, which are usually more expensive and require greater energy and amount of chemicals (García et al. 2014a). Thus, a number of lignocellulosic by-products and residues can be the focus of a biorefinery. The integration of the different processes outlined will certainly have a positive effect on the scientific, technological, economic, environmental, and social areas, not only for lignocellulosic materials but also for a wide range of coproducts (biofuels, chemicals, and others compounds).

Other challenges also need to be addressed including optimization of the fermentation technology in order to produce a range of bioproducts by selective fermentation of different chemicals, characterization of new enzymes or new enzymatic systems in order to lead to low-cost conversion of lignocellulosic biomass into biofuels and biochemicals, as well as logistics considerations and careful choice of raw material for the biorefinery.

Still in the context of future challenges and trends of cellulose, it is clear that different countries and regions have potential access to the dry matter as lignocellulosic wastes, for use in a commercial scale biorefinery. However, there are clearly a number of challenges that need to be addressed to the success of these processes based on renewable technologies that are expected to improve the environment and enhance the quality of life in rural areas through the diversification of the rural economy. Overall, these biorefineries will contribute to the establishment of a link between economic and social development and environmental protection.

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Sugarcane Straw and Its Cellulosic Fraction as Raw Materials for Obtainment of Textile Fibers and Other Bioproducts

16

Sirlene M. Costa, André Aguiar, Sandra M. Luz, Adalberto Pessoa, and Silgia A. Costa

Contents

Abstract

This chapter describes the different ways of performing sugarcane straw exploitation. This is an important agro-residue in Brazil that has a wide range of feasible applications. For these specific usages, it is essential to have an effective knowledge on the sugarcane straw fiber related to its chemical composition (lignin and carbohydrate contents) and on cellulose extraction processes. We address the recent studies on biological processes, such as alternative

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pretreatment in combination with chemical processes and bleaching methods for residual lignin removal. This chapter also addresses some recent examples on the production of ethanol, bio-oil, enzymes, composites, and pulps from sugarcane straw. In addition, we discuss the development of lyocell textile fibers. The scientific and technological advances in the exploitation of sugarcane straw have a strong appeal, and the use of an agro-residue in this application is an important issue.

Keywords
Sugarcane straw • Cellulose • Textile fibers • Lyocell • Ethanol • Enzyme

Abbreviations

AFEX	Ammonia fiber expansion
AQ	Anthraquinone
NMMO	*N*-methylmorpholine-*N*-oxide
SEM	Scanning electron microscopy

1 Introduction

The Brazilian industry currently has 380 sugarcane-ethanol plants that process approximately 600 million tons of sugarcane per year for the extraction of its sucrose-rich juice. Brazil is the greatest sugarcane producer in the world, with an estimated production of 587 million tons in 2013–2014 (UNICA 2013). This sizeable production leads to an accumulation of by-products, mainly sugarcane straw and bagasse. The sugarcane straw is composed of all the material removed before the cane is crushed in the harvesting and consists of plant tops and leaves (Gómez et al. 2010; Santos et al. 2012).

Significant biomass residues – either sugarcane bagasse or straw – are generated and become potential raw materials for the industry. One ton of sugarcane plant produces about 140 kg of straw and 140 kg of bagasse (in terms of dry mass), generating approximately 84 million tons of each sugarcane residue per year. The energy content of these residues can support its use for bioethanol production. Usually, the sugarcane bagasse is burned in the mills for steam and electricity generation, making the ethanol and sugar industry energetically sustainable (Ferreira-Leitão et al. 2010a; Santos et al. 2012).

In Brazil, about 50 % of the sugarcane straw is left in the field to prevent erosion and maintain moisture and soil agronomic properties. These properties are related to its carbon/nitrogen ratio and potassium, phosphorus, calcium, magnesium, and sulfur content. It also serves as weed control and preserves soil microbiota (Gómez et al. 2010; Ferreira-Leitão et al. 2010a). Moreover, as the traditional practice of burning sugarcane leaves prior to manual harvesting is being terminated (Brazilian Federal Law 2661/98 and São Paulo State Law 11241/02), the mechanized harvest will gradually take its place and higher quantities of strategically placed sugarcane straw will be available for industrial application (Gómez et al. 2010). Thus, it is

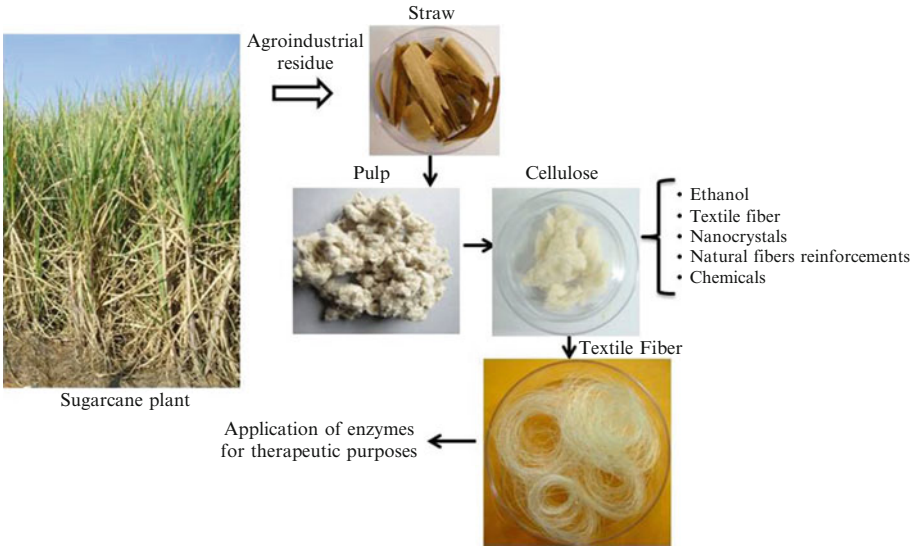

Fig. 1 Sugarcane straw and some potential applications based on its chemical composition

critical to evaluate the best applications and technologies for the use of this biomass. Figure 1 suggests some potential applications for sugarcane straw based on its chemical composition, which will be discussed in this manuscript.

2 Composition and Morphology of Sugarcane Straw

A large variation in chemical component content is observed on the sugarcane straw. Generally, it is composed of 32.4–44.4 % cellulose, 24.2–30.8 % hemicelluloses, and 12.0–36.1 % lignin. Ashes (2.4–7.8 %) and extractives (2.5–10.6 %) are also found in lesser proportions (Table 1). Dry leaves have markedly low lignin content and high ashes content in comparison with other sugarcane residues. The chemical composition varies depending on the material-collecting site, climatic conditions, stage of plant development, and variety (Gómez et al. 2010; Santos et al. 2012).

Cellulose is the most abundant component in sugarcane straw, as observed in Table 1. Cellulose is a linear homopolymer composed of glucose units linked by β-(1→4)-glycosidic bonds, forming cellobiose that is repeated several times in its chain. The interaction between the hydroxyl groups via hydrogen bonds in the chains limits the access of agents to the functional groups in the cellulose chain, which hinders both its solubilization and reactivity. Hemicellulose is a heteropolysaccharide composed of hexoses (D-glucose, D-galactose, and D-mannose), pentoses (D-xylose, L-arabinose), and acid units (acetic, D-glucuronic, and 4-*O*-methyl-D-glucuronic acid). Hemicellulose differs substantially from cellulose, as it is

Table 1 Chemical composition of sugarcane residues (straw) reported in the literature

Material reported	Components (% m/m, dry basis)					References
	Glucan	Hemicelluloses	Lignin	Extractives	Ash	
Straw	39.4 ± 0.3	26.2 ± 0.1	21.5 ± 0.3	–	–	Saad et al. (2008)
Trash (dry leaves)	44.4 ± 2.7	–	12.0 ± 0.8	–	–	Singh et al. (2008)
Leaves	33.3	24.2	36.1	–	–	Ferreira-Leitão et al. (2010b)
Dry leaves	44.5 ± 0.5	30.4 ± 0.3	12.3 ± 0.2	3.7 ± 0.1	7.5 ± 0.3	Gómez et al. (2010)
Fresh leaves	40.5 ± 0.8	30.8 ± 0.8	22.8 ± 0.2	2.5 ± 0.1	2.1 ± 0.2	
Straw	40.1 ± 0.4	30.7 ± 0.2	22.9 ± 0.2	3.0 ± 0.3	2.2 ± 0.2	
Straw	33.6 ± 0.9	28.9 ± 0.6	–	–	5.7 ± 0.1	Silva et al. (2010)
	32.4 ± 0.6	25.3 ± 0.9	–	–	6.0 ± 0.1	
Leaves	35.3 ± 0.6	26.4 ± 0.6	19.6 ± 0.7	7.5 ± 0.6	7.8 ± 2.1	Krishnan et al. (2010)
Straw	38.1 ± 0.2	29.2 ± 0.3	24.7 ± 0.2	4.7 ± 0.2	3.4 ± 0.1	Miléo et al. (2011)
Straw	33.5 ± 0.2	27.1 ± 0.3	25.8 ± 0.5	–	2.5 ± 0.2	Costa et al. (2013)
Fresh leaves	39.8 ± 0.3	28.6 ± 0.2	22.5 ± 0.1	6.2 ± 0.3	2.4 ± 0.3	Oliveira et al. (2013)
Straw	33.3	27.4	26.1	10.6	2.6	Luz and Gonçalves (2001)

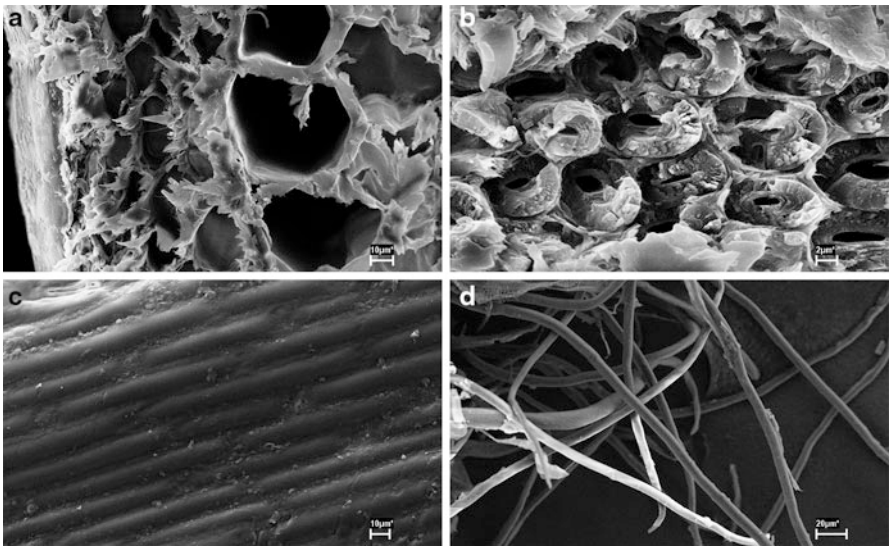

Fig. 2 SEM micrographs from sugarcane straw exhibiting its cellular morphology. (**a**) Internal part of sugarcane straw leaf showing the dandified rings from the extremity of cells (600× magnification). (**b**) Internal part of sugarcane straw leaf showing the vegetable cell wall (2,750× magnification). (**c**) External part of sugarcane straw leaf showing the protection wall. (**d**) Fibrillar structure of sugarcane straw pulps

amorphous and has branches and lower molecular mass, which makes it easier to be hydrolyzed than cellulose. Xylan is the predominant hemicellulose in sugarcane biomass (Ferreira-Leitão et al. 2010b). Lignin is a noncarbohydrate complex aromatic macromolecule formed by radical polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols. The structure of lignin is heterogeneous and globular, with amorphous regions. Silicon is the predominant compound in sugarcane straw ashes. The sugarcane straw can contain up to 5 % m/m of sand and debris as impurities. The presence of impurities is due to the type of transport and harvesting of sugarcane (Gómez et al. 2010).

In the plant cell wall, lignin and hemicelluloses involve the cellulose elementary fibrils, providing protection against chemical and/or biological degradation (Santos et al. 2012; Aguiar and Ferraz 2011; Canilha et al. 2012). The lignin content and its distribution are responsible for the recalcitrance of lignocellulosic biomass to its chemical or enzymatic conversion (Santos et al. 2012; Canilha et al. 2012).

Sugarcane straw fibers have a common cellular morphology when compared to other lignocellulosic materials, as we can see in Fig. 2. The cell wall morphology shows a specific aspect for the internal (Fig. 2a, b) and external part of the straw leaf (Fig. 2c) and the cellulose from sugarcane straw by alkaline process (Fig. 2d).

Figure 2a presents the details of the sugarcane straw leaf cell, showing the vessel form, and in Fig. 2b, we can observe the walls of various adjacent plant cells. On the other hand, in Fig. 2c, we notice that there is a kind of globular form that seems to

involve the internal part. Visually, this external part is smoother than the internal part of the leaf. After the alkaline process, which will be presented in this work, a cellulose extraction results in fibrillar structure, as we can see in Fig. 2d.

3 Sugarcane Straw Fractionation for Cellulose Extraction

Pulping processes modify the lignocellulosic materials by disrupting the plant cell wall structure, removing, solubilizing, and/or fragmenting the lignin. The types of processes depend on the lignocellulosic material and on the purpose of the use of their fractions, which may be mechanical, physical, biological, and/or chemical (Focher et al. 1991; Wood and Saddler 1988; Kokta and Ahmed 1992).

The separation of lignocellulosic materials by a chemical process is the most widely used method in pulp and paper industries. The studied pulping processes for sugarcane straw in the literature are acetosolv (Gonçalves et al. 2008; Saad et al. 2008), ethanol/water (Moriya et al. 2007), and soda/anthraquinone (AQ) (Luz and Gonçalves 2001; Costa et al. 2013).

In the studies by Gonçalves et al. (2008) on acetosolv pulping of sugarcane straw, they used 93 % acetic acid (m/m) and 0.3 % hydrochloric acid (m/m) as catalyst and a straw/solvent ratio of 1:16 (m/v) with a 2-h cooking time at 115 ± 5 °C. In another work, a kinetic study of acetosolv pulping of sugarcane straw was performed, varying time (0–5 h) and acetic acid concentration (73–93 %). The catalyst concentration (hydrochloric acid) was defined at 0.3 % (m/m), and pulping temperature was 115 ± 5 °C. The pulp yields were 45–50 % after a 1-h treatment (Saad et al. 2008).

The ethanol/water pulping of sugarcane straw was carried out by Moriya et al. (2007). The influence of independent variables (temperature and time) on sugarcane straw cooking with solvent mixtures was studied, in order to determine the operating conditions for obtainment of pulps with high cellulose contents. An experimental 2^2 design was applied to temperatures of 185 and 215 °C and time of 1 and 2.5 h, with constant ethanol/water mixture concentration and straw/solvent ratio. The system was scaled up at 200 °C cooking temperature for 2 h with 50 % ethanol/water concentration and 1:10 (m/v) straw/solvent ratio to obtain a pulp with 3.14 cP viscosity and 58.09 kappa number. The pulp yields ranged from 45.18 % to 55.61 % (Moriya et al. 2007). At 215 °C, the pulp presented 33 % lignin content, and this amount is higher than in the pulps obtained at 185 °C and 200 °C. In organosolv pulping process, lignin reprecipitation occurs on cellulosic fibers (Xu et al. 2007).

In another method, the cellulose from sugarcane straw can be obtained by soda/AQ pulping. The pulping conditions adopted by Luz and Gonçalves (2001) were 16 % of Na_2O, 0.15 % AQ, and straw/liquor ratio of 1:12 (m/v). The pulp yield was about 35 %, presenting 83 % of cellulose content. The values for sugarcane straw pulp viscosity were low, about 5.7 cP (Luz and Gonçalves 2001). In alkaline pulping, the lignin reactions are based on two mechanisms: condensation and hydrolysis (Vallaquiran 1987). The former is undesirable, as it hinders the removal

of lignin and makes its solubilization in the pulping liquor very difficult. During hydrolysis, the alkali acts on the lignin, breaking the macromolecule into units of low molecular mass, which are soluble in the liquor. However, due to the aggressiveness of alkaline pulping reactions, the cellulose is also much degraded, decreasing the pulp yield. In order to minimize the carbohydrate degradation, AQ is utilized during treatment (Blain 1993).

Biopulping is also an alternative for the fractionation of lignocellulosic materials. This bioprocess is defined as a solid-state fermentation process carried out by white-rot fungi acting on lignocellulosic materials. Biopulping aims at minimizing some problems associated with conventional methods of chemical (pollution, low yield, and high cost) and mechanical pulping (high energy consumption, low mechanical strength pulp) (Akhtar et al.1998; Messner et al. 1998; Ferraz et al. 2008).

Biopulping of sugarcane straw has been carried out using the white-rot fungus *Ceriporiopsis subvermispora*. The results showed that this fungus is selective to lignin degradation, reducing cooking times and/or chemical loads in the subsequent pulping process (Saad et al. 2008). Manganese peroxidase was the main oxidative enzyme produced by *C. subvermispora* on sugarcane straw. Laccase and lignin peroxidase activities were not detected with the biodegradation time. Xylanase activities predominated on biodegradation time, reaching its maximum value on the 30th day. Cellulase levels were very low and did not attain the appropriate concentrations to allow determinations of filter paper activity based on the standard assay (Ghose 1987). After pretreatment with *C. subvermispora*, sugarcane straw was subjected to the acetosolv pulping process. Along with the pulping time, the viscosity for biopulps decreased (5.8–4.3 cP) after 5 h. However, for control pulps, the viscosity remained constant (~6.0 cP). The resulting pulp strength is low, but it can be used for the production of dissolving pulps or chemicals (carboxymethylcellulose, cellulose acetate, fuels) according to authors (Saad et al. 2008; Gonçalves et al. 2005). The control and biopulp yield were similar, reaching 45–50 % after 1 h. Within a 1-h reaction, the cellulose content in the biopulp was 65 %, and within 4 h, it reached 70 %. For the control pulp, the cellulose content was 60 % within a 1-h reaction, increasing to 65 % within 5-h pulping (Saad et al. 2008). The pulping kinetics showed that, for biopulps, the final lignin content was about 7.5 %, with a reduction of 40 % in the pulping time to reach 12.5 % pulp lignin. The factorial design showed that the biological pretreatment had a positive effect on the acetic acid reduction of 21.5 % during pulping process (Saad et al. 2008).

The pulping processes are not enough to extract all the lignin from the feedstock, why a stage for the bleaching of pulp is required. The treatment with sodium chlorite followed by extraction with sodium hydroxide releases chlorine, which reacts with lignin to oxidize and degrade it, thus producing substances soluble in sodium hydroxide (Machado 2000). After bleaching in a chlorite medium, the viscosity of the soda/AQ pulps decreased at 39.5 % (Luz and Gonçalves 2001). As the action of chlorite is not selective to lignin, the cellulose can be also degraded. Furthermore, during bleaching, the fibers were intensely stirred, causing the cellulose macromolecules to break and, in turn, decrease the viscosity of sugarcane straw pulps (Luz and Gonçalves 2001).

Legislative and environmental pressures have been forcing the pulp and paper mills to modify their bleaching technologies, mainly because of the chlorine used in the traditional sequences (Wang et al. 1992; Gonçalves et al. 2008). One of these modifications is the use of xylanases, which could be used together with conventional chemical sequences (Wong et al. 1997; Gonçalves et al. 2008). This enzyme degrades xylan, which constitutes a physical barrier for the action of bleaching agents on lignin. This facilitates the bleaching stage and reduces the consumption of chlorine (Viikari 1991). However, the cost of enzyme production and its low yield are major challenges in the use of these biocatalysts for industrial application. Strategies aimed at overcoming these challenges have mainly focused on hyper-producing microbial strains and using cheaper substrates (Kapoor et al. 2008; Ncube et al. 2012). Another strategy to reduce the enzyme cost is to reuse them several times in the process (Greeg and Saddler 1996; Gonçalves et al. 2008). The applicability of xylanases in sugarcane straw pulps and the possibility of recovering this enzyme were evaluated by Gonçalves et al. (2008). A commercial xylanase (Pulpzyme HC) was used for pulp treatment and was recovered and reused several times in the new pulp treatment without adding a new enzyme load. Furthermore, a combined process with fungal pretreatment of sugarcane straw (biopulping) and the acetosolv pulping was evaluated. The amount of recovered enzyme in the first treatment was approximately 7 % from the initial enzyme load. After the second treatment, no enzyme recovery was possible. Several factors interfered on xylanase recovery, such as composition, treatment medium (buffered or aqueous), and the methodology employed in the enzyme recovery (Gonçalves et al. 2008).

In addition, the chemical bleaching to remove residual lignin from the sugarcane straw pulp may be performed with hydrogen peroxide (Costa et al. 2013). With this chemical, Costa et al. (2013) evaluated a series that included three bleaching agents: sodium hydroxide (3 % m/m based dry pulp), diethylenetriaminepentaacetic acid (0.4 % m/m), and hydrogen peroxide (50 % m/m). After the bleaching stage, sugarcane straw pulps presented a reduction of 6.9 % to 1 % in the lignin content. This work used hydrogen peroxide (50 %), because cellulosic pulps should have a high level of chemical purity to obtainment of textile fibers (Costa et al. 2013).

The pulping and bleaching processes promote the isolation of fibers (cellulose-rich) from plant biomass. Therefore, the obtainment of pulps with high level of chemical purity is desired for the production of textile fibers, as the effectiveness of these two processes is an important aspect.

4 Textile Fibers

Textile fiber is a generic term for any type of natural, synthetic, or artificial material that forms the basic elements of a textile product (yarns, fabrics, knits, nonwovens, etc.), which is characterized by having a length of at least a hundred times greater than its diameter (Maluf and Kolbe 2003). In addition to the length and width or diameter of fibers, other characteristic properties – such as tensile strength, absorption, elongation, elasticity, and resistance to abrasion – are important in the

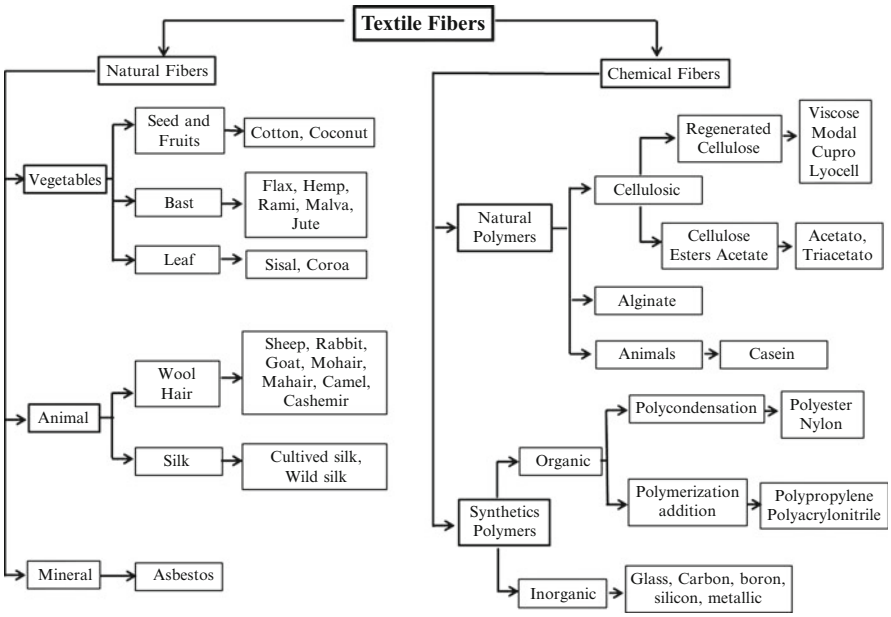

Fig. 3 Classification for textile fibers (Adapted Salem 2010)

development of a textile product. The characteristics of the fibers also play an important role in determining the machines that will be used in processing, the nature of treatments, and the products that are used (APIM 2001). Given the large amount of existing and currently in use fibers in the production of textile fibers, it is convenient to classify them (Fig. 3).

A natural or synthetic polymer should present certain essential properties or characteristics that are important to the forming process of fibers. The primary properties are known to include a high aspect ratio length, toughness and adequate strength, flexibility or malleability, cohesion "quality for spinning," reliability, and uniformity on the fibers (Ribeiro 1984; Neto Aguiar 1996). Secondary properties aim at increasing the comfort and improving maintenance: physical shape, density or specific gravity, luster, "regain," stretching, elastic recovery, resilience, thermal behavior, resistance to microorganism, chemical resistance, and resistance to environment and light. In addition, in order to qualify as a practical and economically feasible fiber, the material should have a competitive price, consistent supply, and be quantitative in terms of demand (Neto Aguiar 1996; Maluf and Kolbe 2003).

The minimal length-width ratio should be approximately 100, although the majority of fibers have a higher ratio. Fibers smaller than 1.27 cm are rarely used in yarn manufacturing. Cotton fibers, for example, have a length that varies between 1.27 and 5.08 cm. In addition, in order to be a satisfactory textile fiber, a raw material requires some properties such as toughness and resistance to equipment process. It also needs to provide adequate durability for the final product (Neto Aguiar 1996; Maluf and Kolbe 2003).

The fibers should be soft and flexible so that yarns and fabrics can be bent and have the capacity to move with the body, allowing freedom of movement. Reliability and cohesion are important to fiber properties. The fiber cohesion determines certain characteristics such as fineness, appearance, texture, volume, durability, and ease of maintenance. A yarn can consist of a single (monofilament yarn) or many fibers (multifilament yarn). Staple fibers (natural and manufactured) can be spun into yarns by twisting (staple yarns). Staple yarns have more surface texture and softness. A yarn can be made by blending several different fibers for unique properties (Neto Aguiar 1996).

When the properties or desired features are absent or only present at very low levels, selected finishes can be applied in order to introduce these features or increase those present at low levels. Therefore, several finishes may reduce or modify the undesirable characteristics of fibers due to their secondary properties (Araújo and Castro 1984; Maluf and Kolbe 2003).

Nowadays, due to the great evolution in manufacturing textile fibers that has occurred, new synthetic and natural fibers have been discovered. Particular attention is given to those obtained from polymers, concerning chemical man-made fibers from natural polymers.

Nature is the greatest source of extraction of raw materials for the manufacturing of industrial products; however, the high product demand means that the biosphere is increasingly threatened. In order to minimize the impacts, wastes from other industrial sectors, such as the agribusiness sector, have been increasingly sought after. In Brazil, among these residues, sugarcane bagasse and straw are generated on a large scale. Several processes have been reported to utilize sugarcane bagasse as raw material. These include electricity generation, pulp and paper production, and products based on fermentation. The various products from sugarcane bagasse include chemicals and metabolites such as alcohol and alkaloids, mushrooms, protein-enriched animal feed (single-cell protein), enzymes, etc. (Parameswaran 2009). One of the significant applications of the sugarcane bagasse is the production of protein-enriched cattle feed and enzymes. These applications can also be extended to the use of sugarcane straw. The textile industry is another sector that has great potential for the use of both bagasse and straw for the production of regenerated fibers, therefore reducing the use of raw material of high commercial value and contributing to the reduction of this waste in the environment (Parameswaran 2009).

4.1 Chemical Fibers from Natural Polymers: Artificial Fibers

Man-made textile fibers are those that do not occur in nature, although they may be composed of naturally occurring materials. Using the adjective “artificial” to designate some man-made fibers is not quite correct, because, in principle, they are all artificial, particularly if released before synthetic fibers. Artificial fibers are those produced from natural polymers (macromolecules) with emphasis on cellulose. The cellulosic fibers are made from cellulose that may be derived from wood

or to a lesser extent the "linters," which are short fibers covering cotton seeds. Within the cellulosic fibers, we can also consider two groups: (a) regenerated cellulose fibers, designated as such because the fibers, after its passage into spinnerets transformations, are ultimately made from cellulose. Its raw materials include viscose, modal, cupro, and lyocell. (b) Fibers derived from cellulose, so called because its passage into spinneret transformations generates acetate and triacetate (Neves 1987; APIM 2001).

The first one is the most common and a commercial man-made cellulosic fiber and is produced by viscose process. The viscose fiber is produced by the viscose process, which involves the production of cellulose xanthate solution. Cellulose xanthate is an alkali-soluble salt formed by the reaction of cellulose and carbon disulfide in the presence of strong alkali (Broughton and Brady 1995; Kennedy and Knill 2006). Extrusion of the viscose solution into a coagulating bath, containing sulfuric acid and other salts, causes the regeneration of cellulose (Cole 1992). During the viscose production process, chemical changes occur and these sulfur products arise: sulfur trioxide and hydrogen sulfide (SH_2), which saturate the bath and are released to the atmosphere. To effectively remove toxic gases into the atmosphere spinning viscose, machines have, at the top, a powerful extractor fan which takes the gases to a treatment plant (Neto Aguiar 1996). As legislation imposes ever-tighter restrictions, this is inevitably more and more costly and in some instances can limit the economic viability of the process (Cole 1992; Broughton and Brady 1995).

In recent years, there has been a great scientific and technological interest in the development of new methods of spinning cellulosic fibers, particularly in one that satisfies technical, economical, modern, and environmentally friendly demands (Navarret 2002).

4.1.1 Lyocell Fibers

Lyocell is a type of cellulose manufactured from dissolving pulp, also known as bleached wood pulp. A company known as Courtaulds Fibers, UK, was the first to develop and manufacture it in the 1980s. In 1990, Courtaulds announced a major investment in a production plant for its new cellulose-based man-made fiber, the Tencel (Cole 1992; Rosenau et al. 2001). Courtaulds developed a fiber spinning process based on the use of an amine oxide, *N*-methylmorpholine *N*-oxide (NMMO), to affect the dissolution (Cole 1992). Wood pulp and amine oxide, as a solution in water, are mixed and then passed to a continuous dissolving unit to yield a clear, viscous solution. This could be extruded onto a dilute aqueous solution of the amine oxide, which precipitated the cellulose as fiber. They are washed and dried and finally baled as staple or tow products as required by the market. The spin-bath and wash liquors are passed to solvent recovery systems (Borbély 2008). Literature data show that NMMO is highly recovered (up to 99.7 %), recycled, and reused in the process (Rosenau et al. 2001, 2003; Lim et al. 2003; Fink et al. 2001). Figure 4 shows Courtaulds' semicommercial production system.

The main disadvantage of the lyocell fiber is its relatively low surface energy, which makes it difficult for dyes to bind to it. This is not to say it is impossible to

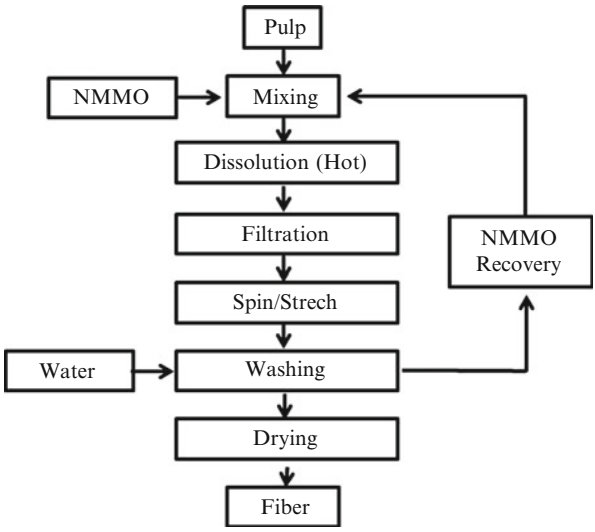

Fig. 4 Direct dissolution: Courtaulds' lyocell (Borbély 2008)

dye, but the required dyes are more expensive for this proposal. It also has a tendency to fibrillate during processing and wearing. Fibrillation is what causes the effect known as "pilling," where small balls of fiber form on the surface. The finishing processes of lyocell articles are considered different from the conventional processes of other fibers (Hall et al. 1995; APIM 2001; Borbély 2008).

Cellulase enzymes improve the appearance of lyocell fabrics and produce a more homogeneous, shorter fibrillation in a second process with mechanical agitation. This fibrillation, also called secondary fibrillation, provides a "peach skin feeling" to lyocell garments. Cellulase enzymes are well-known finishing agents that are successfully applied to remove fuzz fibers and pills from lyocell fabrics, as well as from all cellulosic-based fabrics (Morgado et al. 2000).

Renewable resources will become increasingly important as the planet's stocks of fossilized reserves are depleted and as governments realize that biomass can provide a truly sustainable, cost-effective source of energy and materials (Woodings 1995). Global trends in scientific and technological advancement in the area of new materials demonstrate the importance of using industrial and agro-industrial waste, for example, sugarcane bagasse and straw for the production processes of textile fibers. The use of this residue can minimize the environmental problems related to accumulation and reduce the use of noble raw materials (Costa et al. 2013).

4.1.2 Lyocell from Sugarcane Straw

In the studies by Costa et al. (2013), sugarcane straw cellulose was used for the production of lyocell. Sugarcane straw pulps were obtained after alkaline pulping using soda/AQ (yield of 30 %). For the removal of residual lignin, pulps were submitted to chemical bleaching. Afterward, bleached pulps were used to obtain fibers with NMMO. Fibers were obtained using 10 % (m/m) of cellulose, 80 % (m/m)

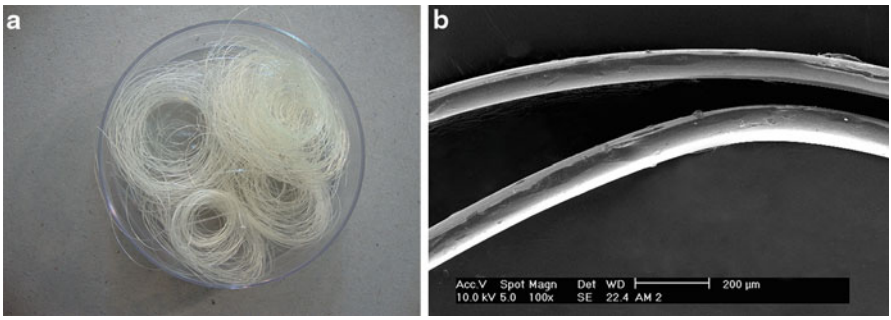

Fig. 5 Aspect of textile fiber from sugarcane straw (**a**) photo and SEM (**b**)

of NMMO, and 10 % (m/m) of water. The obtained gel was extruded with water at room temperature. The fibers showed water uptake capacity of around 73 %, and the mass loss profile was about 26 % in 30 days. Scanning electron microscopy (SEM) was used to evaluate the quality of fibers, as it allows evaluating the lack or the presence of scale, thickness, or irregularity forms along fibers (Fig. 5).

The cellulose fibers obtained from sugarcane straw, still in testing phase at laboratory scale for assessment of other important properties such as tenacity, were evaluated to confirm if they are compatible with the commercial lyocell produced from cellulose from wood pulp (Costa et al. 2013). Studies on enzyme immobilization on fibers, such as lysozyme and bromelain, for the obtainment of fibers with therapeutic properties for medical applications are under investigation. Immobilized enzymes are already being used in medical applications for clinical diagnosis and also for intra- and extracorporeal enzyme therapy. Applications in clinical analysis are mainly related to biosensors, which have been used to detect the presence of various organic compounds for many years. Glucose oxidase and catalase have been used to measure blood glucose concentration, cholesterol oxidase, and cholesterol esterase to determine cholesterol levels. In addition, enzymes can be immobilized on different prosthetic devices or used for extracorporeal therapies (e.g., artificial heart, artificial lung, artificial kidney, equipment for hemodialysis, and specific blood purification) as surface modifiers in order to increase the biocompatibility of these devices and to prevent blood clotting (Costa et al. 2004). Various methods have been developed for the immobilization of biocatalysts, which are being used extensively today. A wide range of support materials has also been employed for enzyme immobilization. The support type can be classified according to their chemical composition, as organic or inorganic supports, and the former can be further classified into natural or synthetic matrices. Immobilization techniques can be divided into different categories: physical, chemical, enzymatic, and genetic engineering methods (Costa et al. 2004).

Lysozymes are hydrolytic enzymes, characterized by their ability to cleave the β-(1,4) glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer (Callewaert and Michiels 2010).

This enzyme is currently used as an antimicrobial agent in many types of foods, acting like "chemical condoms" or controlling microbial processes in the production of cheese, beer, and wine and antibacterial films in food packaging processes. A commercially available lysozyme is obtained by using a combination of conventional methods such as crystallization, precipitation, centrifugation, or adsorption (Wan et al. 2006). The human skin is known to produce several antimicrobial agents that form a chemical protection, such as lysozyme. Many of these agents act together to kill pathogenic microorganisms such as Gram-positive bacteria, Gram-negative bacteria, fungi, and viruses (Niyonsaba and Ogawa 2005; Zdybicka-Barabas et al. 2012).

The fibers obtained from the work carried out by Costa et al. (2013) are being tested for the immobilization of the enzyme bromelain in addition to lysozyme.

The medicinal qualities of pineapple are recognized in many traditions in South America, China, and Southeast Asia. These qualities are attributed to bromelain, a 95 % mixture of proteases. Medicinal qualities of bromelain include anti-inflammatory, antithrombotic, antiedematous, antimetastatic, fibrinolytic, and anti-cancer functions (Chobotova et al. 2010; Neetu et al. 2008; Fitzhugh et al. 2008; Kalra et al. 2008; Gupta et al. 2007). Because of its efficacy, safety, and lack of side effects after oral administration, bromelain has earned growing acceptance and compliance among patients as a phytotherapy drug. Another wide range of therapeutic benefits has been attributed to bromelain, such as inhibition of platelet aggregation, angina pectoris, bronchitis, sinusitis, surgical traumas, thrombophlebitis, pyelonephritis, and increased absorption of drugs, particularly antibiotics. Existing evidence derived from clinical observations, as well as from mouse- and cell-based models, suggests that bromelain acts systemically, affecting multiple cellular and molecular targets. In recent years, studies have shown that bromelain has the capacity to modulate key pathways that support malignancy. It is now possible to suggest that the anticancer activity of bromelain consists in the direct impact on cancer cells and their microenvironment, as well as in the modulation of the immune, inflammatory, and hemostatic systems (Chobotova et al. 2010).

The results of a study by Majid and Mashhadani (2014) showed that oral bromelain is an effective therapy to improve quality of life after the surgical removal of impacted lower third molars, with an effect on the postoperative sequelae comparable to that of preemptive diclofenac sodium. In conclusion, perioperative bromelain in a daily oral dose of 4×250 mg started 1 day before surgery and continued for 4 days showed a significant analgesic and antiedemic effect, a significant improvement in quality of life, and a limited reduction of trismus in the early postoperative period for patients who had undergone lower third molar surgery. Biochemical experiments indicate that these pharmacological properties depend only in part on the proteolytic activity, while suggesting the presence of non-bromelain protein factors (Wen et al. 2006).

5 Ethanol and Other Bioproducts from Sugarcane Straw

The application of sugarcane straw as industrial raw material for ethanol production requires its fractionation to increase the accessibility to carbohydrates. The first stage in this process is pretreatment, in which a considerable part of hemicelluloses is solubilized and cellulose is activated toward enzymatic hydrolysis. Different kinds of chemical, physical, biological, and physicochemical pretreatment methods have been proposed to disrupt the cellulose-hemicelluloses-lignin complex. Because of the intimate association between the three polymeric components of biomass, the release of carbohydrates is among the most important and urgent priorities in the research and development of new products. For new pretreatment methods, it is advisable to use cheap and easily recoverable chemicals and low-cost equipment. The use of environmentally friendly and low-energy-intensive approaches is highly desired. Reviews on the types of pretreatments applied to sugarcane biomass followed by enzymatic hydrolysis are reported in the literature (Santos et al. 2012; Canilha et al. 2012).

Ethanol produced from sugarcane residues is one of the most suitable alternatives for partial replacements of fossil fuels, because it provides energy that is renewable and less carbon intensive than gasoline. Bioethanol reduces air pollution and contributes to mitigate climate change by reducing greenhouse gas emissions. As feedstock cost is crucial, agro-industrial residues, which are less expensive than conventional agricultural feedstock, could play a central role in the production of ethanol and chemicals (Santos et al. 2012; Canilha et al. 2012).

Considering that 80 L of first-generation ethanol (from sucrose juice) are produced per ton of sugarcane and that a surplus of 36.5 L could be produced from the conversion of sugarcane residues (bagasse and straw), ethanol production could increase to almost 50 % without increasing the planted area, a topic of great environmental importance (Ferreira-Leitão et al. 2010a).

There has been an increase on the number of studies on the use of sugarcane bagasse for the production of second-generation ethanol (cellulosic ethanol). However, there are few works using sugarcane straw for this purpose. Krishnan et al. (2010) evaluated AFEX pretreatment to improve the accessibility of carbohydrates during enzymatic hydrolysis of sugarcane bagasse and leaves. The maximum glucan conversion of AFEX-pretreated bagasse and leaves by cellulases was 85 %. Supplementation with xylanases during enzymatic hydrolysis improved the xylan conversion up to 95–98 %. AFEX-treated sugarcane leaf residue was found to have a greater enzymatic digestibility compared to AFEX-treated bagasse. Co-fermentation of glucose and xylose from leaves produced 34–36 g L^{-1} of ethanol with 92 % theoretical yield (Krishnan et al. 2010).

In other work, Silva et al. (2010) verified the effectiveness of milling pretreatments on sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation. Glucose and xylose hydrolysis yields at optimum conditions using ball-milling pretreatment for straw were 77.6 % and 56.8 %, respectively.

Maximum glucose and xylose yields using wet disk milling were 68.0 % and 44.9 % for sugarcane straw, respectively. Ball milling decreased the cellulose crystallinity, while the defibrillation effect observed for wet disk milling samples seems to have favored enzymatic hydrolysis. Ethanol yields from total fermentable sugars using a glucose-fermenting strain reached 91.8 % for straw hydrolysates and 78 % when using a glucose-/xylose-fermenting strain (Silva et al. 2010).

Ferreira-Leitão et al. (2010b) used carbon dioxide as an impregnating agent (in comparison with sulfur dioxide) in steam pretreatment of sugarcane bagasse and leaves for ethanol production. The highest glucose and xylose yields for sugarcane leaves (97.2 % and 62.8 % respectively) were obtained after pretreatment at 220 °C for 5 min. On the other hand, Oliveira et al. (2013) evaluated the effect of temperature on steam explosion pretreatment for 15 min for sugarcane dry leaves followed by sodium hydroxide delignification. The pretreatments led to remarkable hemicellulose solubilization, with the maximum (92.7 %) for pretreatment performed at the highest temperature (200 °C). Alkaline treatment of the pretreated materials led to lignin solubilization above 80 %. All pretreatment conditions led to high enzymatic conversion of cellulose, with the maximum (80.0 %) achieved at 200 °C. The best effect of delignification was observed on the material pretreated at 180 °C, increasing its enzymatic conversion from 58.8 % to 85.1 %.

The components of sugarcane straw can also be utilized as substrates for the production of enzymes by solid-state fermentation. Singh et al. (2008) evaluated diverse fungi and bacteria to produce cellulases and xylanases during the biodegradation of sugarcane dry leaves. The total cellulose and lignin content of the substrate were significantly reduced by the microbial action. Maza et al. (2014) evaluated the production of oxidative and hydrolytic enzymes on this same agro-industrial residue. Enzymes as laccases, peroxidases, xylanases, endoglucanases, and β-glycosidases were produced in vitro by autochthonous fungi in sugarcane plantations. Hydrolytic enzymes as cellulases and xylanases are actually important to biomass enzymatic conversion, whereas laccases and peroxidases are effective for bioremediation of organopollutants (Durán and Esposito 2000; Aguiar and Ferraz 2011; Canilha et al. 2012).

Sugarcane straw can be used for bio-oil production by pyrolysis process. Bio-oil is a very complex mixture containing many organic compounds formed by the thermal degradation of plant biomass. Using two-dimensional gas chromatography coupled with time-of-flight mass spectrometry, Moraes et al. (2012) identified 123 compounds in sugarcane straw bio-oil, mainly oxygenated compounds (acids, ethers, aldehydes, ketones, and phenols) with a predominance of furfural and hexenoic acid. These compounds can be important feedstocks for the chemical industry and for energy purposes. During biomass pyrolysis, the generated solid residue, known as biochar, can present adsorptive properties. Melo et al. (2013) evaluated chromium and zinc sorption capacity by sugarcane straw-derived biochar. The authors observed higher sorption capacity in contaminated soils for biochars from highest pyrolysis temperature, which is attributed to their highest superficial area and porosity (Melo et al. 2013).

Composite materials from natural fibers have been intensely studied in recent years, and the sugarcane straw also can be used for this application. Luz et al. (2010) and Miléo et al. (2010) evaluated the use of sugarcane straw cellulose as reinforcement of polypropylene and polyurethane, respectively. These composite materials presented good mechanical and thermal properties, regardless of the matrix. Associated with that, the other important application for sugarcane straw can be the nanocrystals. The nanocrystals from cellulose can be extracted after hydrolysis, as we observed in studies with sugarcane bagasse and other agro-residues (Teixeira et al. 2011; Johar et al. 2012).

6 Conclusions and Future Perspectives

This work described potential applications for sugarcane straw. The appeal of the search for new ways to avail sugarcane straw is strong, and the economic factor has a greater impact on analyzing their processing and utilization. The economic use of sugarcane straw will depend on further research on the cost-effectiveness of collecting, loading, and transportation technologies, in addition to the cost of using this material, turning it into a higher commercial value product. In this work, we cited some products that can be obtained from sugarcane straw (ethanol, bio-oil, enzymes, enzyme support, composites, cellulose pulp, and commercial fibers). It remains to be evaluated which process can be economically viable for the obtainment of products using sugarcane straw as raw material.

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Endogenous Hydrolyzing Enzymes: Isolation, Characterization, and Applications in Biological Processes

17

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Contents

Abstract

Enzymes are the key substances responsible for a variety of biotechnological processes involved in the production of useful bioproducts. Malt and microbial species (bacteria, fungi, etc.) are the main sources of endogenous hydrolyzing enzymes (EHEs). EHEs are primarily involved in the digestion of complex substrates into simpler units and the resulting formation of biological products. Based on origin and substrate specificity, EHEs are categorized into cell wall-, starch-, protein-, lipid-, nucleic acid-, polyphenol-, and thiol-hydrolyzing enzymes. The substrate specificities and reaction mechanisms of individual EHEs and groups of EHEs have been verified through isolated and purified enzymes. A number of methods have been reported for high-yield, economically feasible isolation of enzymes. The endogenous enzymes contained in microbial cells are separated from cells, cellular fragments, and organelles through several cell lysis and separation methods. Analysis of the mechanism of action has revealed that most enzymes systematically undergo biological processes through

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a cascade of enzyme-specific reactions. The applications of these EHEs are involved in almost every aspect of human and animal life and are important in food, animal feed, textile, paper and pulp, fuel (energy), pharmaceutical, and chemical industries. In this chapter, we describe the origins, classes, isolation techniques, mechanisms, and applications of various EHEs with examples from updated literature.

Keywords
Endogenous hydrolyzing enzyme • Malt • Microbial species • Cell lysis • Mechanism of action

1 Introduction

Most of biochemical reactions occur spontaneously, while others depend on specific catalysts in order to reduce the magnitude of the energy difference essential for successful conversion of substrate into product (Illanes 2008). Enzymes are highly specialized catalysts that lead to increases in reaction rates and the specificity of metabolic reactions, from simple to highly complex biological reactions. Additionally, each biological reaction may be catalyzed by a specific enzyme, enabling reactions to function at rates required to sustain life. In theory, enzymes should not be consumed or altered during conversion of the substrate into product. However, in practice, the sustainability and functionality of any enzyme during reaction catalysis is strongly dependent upon the conditions adopted for substrate conversion because enzymes are primarily proteins (Shuler and Kargi 2001).

The enzymes catalyzing a biological reaction can be broadly divided into two types (exogenous hydrolyzing enzymes and endogenous hydrolyzing enzymes [EHEs]), based on their source. Generally, exogenous enzymes are expensive and ultimately increase the final cost of a bioproduct. Moreover, optimization of operational conditions, such as selection of appropriate temperature, pH, and substrate and enzyme concentrations, is a tedious job. In contrast, EHEs are attractive candidates for industrial-scale production of various bioproducts. All biological processes and metabolic pathways are driven and regulated by a group of enzymes catalyzing a cascade of reactions. These enzymes can act on the substrate both in vivo and in vitro and mediate reactions that ultimately result in improved product yields, up to severalfold. However, in the absence of a biological catalyst, the reaction rate is retarded, and biological reactions are ultimately blocked. EHEs are also advantageous in that they are encoded by the genome of a particular cell, which controls the expression level and activity of the EHE, resulting in enhanced biological activity within the same cell. Generally, EHEs are expressed within the cell when required; however, their expression can be regulated (enhanced or suppressed) depending on the cell's needs and on environmental conditions. Khattak et al. (2012) reported the presence of several EHEs from waste fermentation broth; these EHEs included starch-hydrolyzing enzymes,

cellulose-hydrolyzing enzymes, cell wall-hydrolyzing enzymes, protein-hydrolyzing enzymes, lipid-hydrolyzing enzymes, and several other important enzymes of industrial importance (Khattak et al. 2012).

Cell extracts containing EHEs can be isolated through cell disruption and further processed and utilized for various useful applications, such as development of synthetic pathways, cell-free enzyme systems, and commercialization. Cell disruption can be carried out by various methods, including chemical, enzymatic, alkaline lysis, and mechanical insults, such as ultrasonication, bead beating, and high-pressure homogenization. The obtained cellular extracts are further evaluated for extracellular and intracellular enzymes from cells and cellular fragments, respectively, through various approaches, such as filtration, centrifugation, flocculation, and flotation. Because the obtained extract possesses a lower concentration of enzymes, extracts can be further concentrated through various approaches, such as thermal methods, precipitation using different reagents, and ultrafiltration. For enhanced activity and storage to prior utilization for various purposes, enzymes can be further purified through crystallization, electrophoresis, and various chromatographic techniques.

The ease of use, cost-effectiveness, distinctive properties, and efficacy of EHEs make them attractive candidates for utilization in various industries, including breweries, food processing, and several medical and pharmaceutical industries. This chapter briefly describes the history of EHEs and then introduces various types of EHEs based on the type of substrate; discusses different techniques employed for the isolation, separation, and purification of EHEs from various sources; and describes the mechanisms of action of various EHEs.

2 History of EHEs

Hundreds of years have passed since humankind started to use enzymes for the production of beverages and baking products, as first described by ancient Egyptians. Indeed, ancient Egyptians used biological catalysts to produce beer, wine, bread (leavened bread), and cheeses, and later civilization used biological catalysts to make yogurt and other fermented milk products, pickles, vinegar, butter, and sauerkraut (Steinkraus 2002). In late eighteenth century, de Réaumur (1752) and Spallanzani reported that gastric juices secreted by the stomach are responsible for the digestion of meats (de Réaumur 1752; Modlin and Sachs 2004). In later years, a number of similar phenomena were observed, including endogenous secretions in plant extracts and saliva, which participate in the breakdown of starch into simple sugar. In 1814, glutinous (proteinaceous) secretions were reported to be responsible for the hydrolysis of starch into sugar (Segel 1993). Although the substances involved in digestion of meat, starch, and amygdalin have been identified, these early studies did not define these endogenous secretions and their mechanisms of action (Williams 1904).

After the first clear identification of endogenous enzymes made by Payen and Persoz in 1833 (Payen and Persoz 1833), it was unclear until the nineteenth century

whether the molecules responsible for the catalysis of biological processes belonged to living species or were chemical substances. The substance they obtained (referred as "diastase") was employed for the hydrolysis of starch. A second well-known enzyme obtained from an animal source in 1836 was pepsin, described by Schwann as the water-soluble factor found in gastric juice that was responsible for albumin digestion (Modlin and Sachs 2004). The next year (1837), Berzelius elucidated the details of the catalyst, which, by its mere presence, could "exert its influence and arouse affinities and relativities in the complex body thereby causing a rearrangement of the constituent of the complex body." By studying the mechanism of action of both pepsin and diastase, along with phenomenon observed during yeast fermentation, Berzelius concluded that in all three cases, only a single chemical agent (called the catalyst) is responsible for conversion of one substance into other (Segel 1993). In 1838, Charles Cagniard de Latour investigated the nature of yeast and its involvement in alcohol fermentation. His findings supported the hypothesis that fermentation was caused by a living organism (Berche et al. 2009). After additional careful studies, Louis Pasteur reported the presence of some vital force, referred to as a "fermenter," which was responsible for fermentation, thus providing a strong foundation for the vitalistic protoplasm theory (Dubos 1951).

Like most notable chemists of the time, Stahl and Liebig initially accepted the existence of a "vital force" but subsequently introduced the concept of the inorganic world, in contrast to the "vital force," in the 1850s (Goujon 2001). In their era, a clear distinction was made between organized and unorganized ferment claim to be involved in the biological process. In 1858, Moritz Traube, a student of Liebig, clearly defined this entity as a specific protein-like compound, which catalyzes all cellular activity, including fermentation, respiration, and putrefaction; later, he proposed the theory of enzyme/substrate interaction that occurs inside the cell (Rollin et al. 2013; Renneberg 2008). In 1878, Kühne introduced, for the first time, the term "enzyme," meaning "in yeast," for both the organized and unorganized ferment (Whitehurst and Oort 2009; Stead 1987). The suffix "*ase*," most commonly used as the suffix in enzyme names, was proposed by Duclaux and became the standard for enzyme nomenclature in 1883; this suffix generally indicates the substrate on which the enzyme acts (Traut 2008). The concept of the lock-and-key model was proposed by Emil Fischer in 1894 to account for the high degree of specificity of an enzyme toward a particular substrate (Segel 1993).

Moritz Traube isolated an endogenous enzyme in its active form from potatoes and proved that an enzyme from a plant extract was able to catalyze a reaction in vitro. In 1897, the concept of a cell-free enzyme system, proposed by two brothers, Hans and Edouard Buchner, provided a solid and logical background to refute Pasture's assertion of "vital force" and Liebig's "organic world" (Segel 1993). The same year, Gabriel Betrand proposed the role of heat-stable, dialyzable material, which he called "*coenzymes*," in cell-free glucose fermentation (Semenza and Turner 2005). In 1902, Henri derived a mathematical equation for the effects of substrate concentration on the enzymatic reaction rate, and Sorenson described the effects of pH on enzyme activity in 1909 (Kuby 1990). The industrial

use of endogenous enzymes began in 1913, when German chemist Otto Röhm evaluated the specific activity of pancreatic trypsin for the removal of proteinaceous stains from cloth (Aehle 2007). The same year, Michaelis and Menten reestablished the equation derived by Henri and further evaluated this equation after confirming careful control of pH. The Henri-Michaelis-Menten equation is based on the chemical equilibrium of enzyme kinetics (Kuby 1990). In 1925, Briggs and Haldane explained the steady-state concept of enzyme kinetics and modified the Michaelis-Menten equation with the more general valid assumptions of the steady state (Palmer and Bonner 2007). Today, both approaches are still applied to enzyme kinetics. In 1926, Summer obtained the enzyme urease in crystalline form and announced it to be a simple protein (Sumner 1948). However, the crystalline forms of several other enzymes were not established until the 1930s, when Northrop and his fellows crystallized pepsin, trypsin, and chymotrypsin as pure crystals (Segel 1993). In the 1940s and 1950s, hundreds of new enzymes were discovered, purified, and crystallized (Segel 1993). In 1955, the complete sequence of amino acids of insulin was reported by Sanger (Sanger 1960). In 1960, ribonuclease was sequenced, and the first chemical synthesis of an enzyme (ribonuclease) was achieved in 1969 (Segel 1993). In 1957, Kendrew discovered the three-dimensional geometry of myoglobin through X-ray diffraction analysis (Kendrew 1963). In 1963, the primary structures of two enzymes, pancreatic ribonuclease and egg white lysozyme, were reported by Smyth et al. and Canfield as containing 120 amino acids, further supporting the protein-like nature of these enzymes (Smyth et al. 1963; Canfield 1963). In 1965, the isolation of *Taq* polymerase from *Thermus aquaticus* by Thomas D. Brock set the foundation of a new era in molecular biology and biotechnology (Rollin et al. 2013). Heat-shock proteins (HSPs), identified by Tissieres et al. in 1974, are group of polypeptides expressed under stress conditions that play an important role in the stability of endogenous enzymes under stress conditions (Tissieres et al. 1974).

Currently, the most common definition of an enzyme is "a protein with catalytic activity based on the specific activation of its substrate." However, with the discovery of other catalytic biomolecules, such as catalytic RNA, the identification of ribozymes in 1982 began a new debate regarding the definition of the enzyme (Yonaha and Soda 2007). The efficiency of an enzymatic reaction was increased to a certain extent by using different advanced techniques, including immobilization, in the early 1980s (Rollin et al. 2013). In contrast to cellular extracts, a reconstituted cell-free enzyme system was employed by Welch and Scopes in 1985 for the production of bioethanol from a glucose solution. Compared to the Buchner system, the Welch and Scopes reconstituted cell-free system was evaluated as an effective system for bioethanol production, characterized by near-100 % efficiency, which was quite an achievement (Welch and Scopes 1985).

The concept of the reconstituted cell-free system, as proposed by Welch and Scopes, was the beginning of studying the activities of enzymes in a sequential manner, similar to the methods for studying particular metabolic pathways. Later, this concept was further extended to include the production of certain others biocommodities, including biofuels, biochemicals, and food (You and Zhang 2013).

Endogenous enzyme-based biotransformation systems have become an interesting tool for the production of certain other high-value biocommodities. Using a combination of selective endogenous enzymes as a single biosystem for the biotransformation of substrate into product has advantages over whole-cell systems; for example, this method is highly targeted and generally much more efficient. The best example is the cell-free enzyme system developed by Khattak et al. (2014); this system consists of both glycolytic and fermentation enzymes and can be used for bioethanol production at elevated temperatures at which conventional fermentation is impossible (Khattak et al. 2014).

3 Types of EHEs

Each cell contains genetic information encoding thousands of different endogenous enzymes, and their expression and activity in specific cell compartments usually determine which of the many possible biological reactions take place within the cell. Enzyme expression is either induced within the cell or supplied exogenously under definite circumstances. It is critical to understand the characteristics of the enzyme and its specificity to the available substrate before application in a specific biological process. Known EHEs are classified based on substrate specificity into the different categories shown in Table 1.

3.1 Starch-Hydrolyzing Enzymes

Starch, the most common storage carbohydrate in plant, is made up of D-glucopyranose linked together via α-(1→4) and α-(1→6) glycosidic bonds. Structurally, starch consists of 20–25 % amylase, a linear and helical molecule, and 75–80 % amylopectin, a branched molecule. Amylopectin provides the starch with a greater tendency to resist enzymatic hydrolysis because the residues involved in α-(1→6) glycosidic branch points constitute 4–6 % of the glucose present. Although most of the hydrolytic enzymes are specific for α-(1→4) glycosidic linkages, α-(1→6) glycosidic bonds must also be cleaved for complete hydrolysis of amylopectin to glucose. The most recently developed processes carry out the hydrolysis of starch in three steps, involving gelatinization, liquefaction, and saccharification. In gelatinization, nanogram-sized starch granules are dissolved in water or some other liquid to form a suspension. This is followed by liquefaction, which involves partial hydrolysis of starch through enzymes. Finally, glucose and maltose are formed through saccharification by enzymes.

Malt contains several starch-hydrolyzing enzymes, such as α-amylase, β-amylase, limit dextrinase, and β-glucosidase (MacGregor 1987; Bamforth 2009). Alcohol precipitates of malt extracts (known as diastase), which liquefy starch into simple sugars (Payen and Persoz 1833), have two catalytic units (Maercker 1878) classified as α-amylase (or dextrinogen) and β-amylase (or saccharogen) (Ohlsson 1926). α-Amylases from different sources, such as

Table 1 Major classes of hydrolyzing enzymes involved in the hydrolysis of longer chain into their respective constitutive subunits

Substrate	Class	Enzyme	Reference
Cell wall	1	Endoglucanase	Khattak et al. 2012
		Xylanase	Khattak et al. 2012
		Arabinofuranosidase	Khattak et al. 2012
		Feruloyl esterase	Khattak et al. 2012
		Acetyl xylan esterase	Khattak et al. 2012
		Carboxypeptidase	Khattak et al. 2012
		Exo-β-glucanase	Khattak et al. 2012
		β-Glucosidases	Khattak et al. 2012
		Glucanase	Khattak et al. 2012
Starch	2	α-Amylase	Khattak et al. 2012
		β-Amylase	Khattak et al. 2012
		Limit dextrinase	Khattak et al. 2012
		α-Glucosidase	Khattak et al. 2012
Cellulose	3	Cellobiohydrolases	Aro et al. 2005
		Endoglucanase	Aro et al. 2005
		β-Glucosidases	Aro et al. 2005
Protein	4	Endopeptidase	Khattak et al. 2012
		Carboxypeptidase	Khattak et al. 2012
		Proteases	Khattak et al. 2012
		Lipid transfer proteins	Khattak et al. 2012
Lipids	5	Lipase	Khattak et al. 2012
		Lipoxygenase	Khattak et al. 2012
		Hydroperoxide lyase	Khattak et al. 2012
		Hydroperoxide isomerase	Khattak et al. 2012
		Hydrase	Khattak et al. 2012
Nucleic acid	6	Exoribonucleases	Mishra 1995
		Exodeoxyribonucleases	Mishra 1995
		Endoribonucleases	Mishra 1995
		Endodeoxyribinucleases	Mishra 1995
		Restriction endonucleases	Mishra 1995
		Damage-specific deoxyribonucleases	Mishra 1995
		Topoisomerases	Mishra 1995
		Recombinases	Mishra 1995
Phytin	7	Phytase	Khattak et al. 2012
Polyphenol	8	Peroxidase	Khattak et al. 2012
Thiols	9	Thiol oxidase	Khattak et al. 2012

Bacillus amyloliquefaciens, *B. licheniformis*, *A. oryzae*, *A. niger*, and *B. subtilis*, act as endo-acting enzymes that randomly cleave α-(1→4) glycosidic linkages to yield α-dextrins and maltose. These molecules play an important role in the metabolism of starches and are usually produced in the beginning of the malting process, after which they are moved to and stored in the mature endosperm of seeds

(Svensson 1994). β-Amylase from malted barley is a typical exo-acting enzyme that cleaves the α-(1→4) glycosidic linkage from nonreducing ends, yielding limit dextrins, β-glucose, and β-maltose (Hoseney 1994; Svensson 1994). Glucosidases also function as exo-acting enzymes and cleave α-(1→4) glycosidic linkages in starch. Compared to β-amylase, glucosidase has the ability to bypass the side chain at α-(1→6) bonds. Glucoamylase from *A. niger* cleaves α-(1→4) and α-(1→6) glycosidic linkages from nonreducing ends to yield β-glucose. Pullulanase from *B. acidopullulyticus* cleaves α-(1→6) glycosidic linkages to yield straight-chain maltodextrins. Limit dextrinase cleaves amylopectin α-(1→6) linkages of branched dextrins to complete the hydrolysis of starch into its monomeric sugars at the end of the starch-hydrolyzing reaction (Bowles 1996).

3.2 Cellulose-Hydrolyzing Enzymes

Cellulose is an organic compound that constitutes the major portion of plant cell walls and vegetable fibers, such as cotton, ramie, and wood. It is a highly stable, water-insoluble polysaccharide consisting of chains of glucose monomers. The molecular formula of cellulose is $(C_6H_{10}O_5)_n$, producing an unbranched homopolysaccharide composed of α-D-glucopyranose units linked by (1→4) glycosidic bonds (Purves 1954). It is the most abundant biopolymer on earth and is produced at a rate of about 1×10^{11} tons per year. The enzymes responsible for cellulose hydrolysis are referred as cellulases.

Cellulases are *O*-glucoside hydrolases (GHs) that hydrolyze the β-1,4 linkages of cellulose. These enzymes are predominantly found among prokaryotes and fungi (Hildén and Johansson 2004). Several fungal species are known to produce cellulases with known catalytic abilities (Cunningham and López 1994). Cellulase genes have also been identified in the marine yeast *Aureobasidium pullulans* and *Bursaphelenchus xylophilus* (Chi et al. 2009). GHs are classified into cellulase families based on amino acid sequence similarity. Out of the 122 families of proteins identified to date, 14 correspond to cellulases. Most cellulases form complexes with other GHs; these complexes contain a catalytic module, a highly *O*-glycosylated linker, and a cellulose-binding module (CBM). The latter domain facilitates cellulose hydrolysis by holding the catalytic module in close proximity to its substrate. Cellulases are classified, depending on their enzymatic activity, in three major groups: exoglucanases, endoglucanases, and β-glucosidases. Exoglucanases catalyze the successive hydrolysis of residues from the reducing and nonreducing ends of the cellulose polysaccharide, releasing cellobiose molecules as the main product of the reaction (Aro et al. 2005). These enzymes account for 40–70 % of the total components of the cellulase system and are able to hydrolyze crystalline cellulose. Exoglucanases are monomeric proteins with molecular weights ranging from 50 to 65 kDa, although smaller variants (41.5 kDa) have been identified in some fungi. Exoglucanases have low levels of glycosylation (from 0 % to 12 %), their optimum pH is 4.0–5.0, and their optimum temperature varies from 37 °C to 60 °C, depending on the specific enzyme-substrate

combination. Endoglucanases randomly cleave internal linkages in amorphous cellulose filaments, generating randomly sized oligosaccharides and creating new chain ends that can in turn be attacked by exoglucanases (Aro et al. 2005). These are the enzymes that initiate the cellulolytic process, randomly cleaving internal linkages at amorphous regions of the cellulose fiber and creating new reducing and nonreducing ends that are susceptible to the action of cellobiohydrolases. Endoglucanases are monomeric enzymes with molecular weights ranging from 22 to 45 kDa. Endoglucanases are not glycosylated; however, they sometimes may be conjugated with relatively low amounts of carbohydrates (from 1 % to 12 %). These enzymes function best at an optimal pH of 4.0–5.0 and temperature of 50–70 °C. β-Glucosidases hydrolyze cellobiose, releasing two molecules of glucose, and thereby provide a carbon source that is easy to metabolize. These enzymes have molecular weights ranging from 35 to 640 kDa; they can be monomeric, reaching molecular weights of approximately 100 kDa, or exist as homo-oligomers, such as in the yeast *Rhodotorula minuta*. β-Glucosidases are generally glycosylated and have an optimal pH of 3.5–5.5 and temperature of 45–75 °C. The activity of a cellulase enzyme system is much higher than the sum of the activities of its individual subunits, a phenomenon known as synergism. Cellulase systems are not just simply a conglomerate of enzymes with components from all three cellulase types, but act coordinately to efficiently hydrolyze cellulose fibers (Lynd et al. 2002).

3.3 Cell Wall-Hydrolyzing Enzymes

The plant cell wall is a highly organized network of lignocellulosic material containing cellulose and cross-linked glycans. The cell wall constitutes the outermost boundary in most cell types. It is localized outside the cell membrane and provides the inner cell material with structural support and protection. The cell wall also acts as filtering mechanism and prevents the cell from expanding. Chemically, the cell wall of starch endosperm is composed of (1→3, 1→4)-β-D-glucans (75 %), arabinoxylane (20 %), cellulose (2 %), glucomannan (2 %), and traces of acetic and ferulic acids. Aleurone cells are composed of arabinoxylane (71 %), (1→3, 1→4)-β-D-glucans (26 %), and 3 % cellulose and glucomannan (Fincher 1992; Lazaridou et al. 2008). A number of cell wall-hydrolyzing enzymes are present in malt, including endoglucanase, exoglucanase, arabinofuranosidase, esterase (Bamforth et al. 2009), carboxypeptidase (Bamforth et al. 1979; Sørensen et al. 1989), xylanase (Chithra and Muralikrishna 2008), and β-glucosidase (MacGregor 1987). The cell wall-hydrolyzing enzymes are glycosyl hydrolases, which comprise one of the two main classes of carbohydrate-active enzymes (Jamar et al. 2011).

The (1→3, 1→4)-β-D-glucans are also referred as β-glucan, a linear polysaccharide composed of an unbranched chain of D-glucose residues linked together by β-(1→4) and β-(1→3) bonding with a ratio of 3.2:1–6.6:1 (Jamar et al. 2011). Based on their solubility in water, these compounds are classified as either soluble

or insoluble (Swanston and Ellis 2002). The molecular weight of β-glucan varies from 800 to 1,220 kDa (Jin et al. 2004). β-Glucans are hydrolyzed directly by β-glucan exohydrolases into glucose or indirectly by a number of enzymes involved in removal of the outer layer of the cell wall followed by hydrolysis into glucose. Enzymes participating in the breakdown of β-glucans include (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidase (Jin et al. 2004).

Xylan is a major structural component of plant cell walls and is the second most abundant renewable polysaccharide in nature (after cellulose) (Collins et al. 2005). Xylan interacts with lignin and cellulose via covalent and noncovalent linkages; these interactions are important for both protecting the cellulose microfibrils against biodegradation and maintaining the structural integrity of cell walls. Due to its heterogeneity and complex nature, the complete breakdown of xylan requires the action of a variety of hydrolytic enzymes (Biely 1985; Coughlan and Hazlewood 1993). Degradation of the xylose backbone depends on xylanases, which cleave bonds within the polymer, and β-xylosidases, which release xylose units from xylobiose and xylooligomers. Four enzymes, endo-β-(1→4)-xylanase, exoxylanase, β-xylosidase, and α-arabinofuranosidase, are involved in the hydrolysis of arabinoxylan (Hrmova et al. 1997). Although exoxylanase cleaves the outer β-(1→4) xylosidic linkages, endo-β-(1→4)-xylanase attacks inner β-(1→4) xylosidic linkages in arabinoxylan polymers, separating the arabinofuranosyl residues (Egi et al. 2004). β-Xylosidase catalyzes the hydrolysis of β-(1→4) xylosidic bonding within xylo-oligosaccharides, while arabinofuranosidase cleaves the α-(1→2) and α-(1→3) linkage formed between arabinofuranose units.

3.4 Protein-Hydrolyzing Enzymes

Proteins are large biological molecules consisting of one or more chains of amino acid residues. Proteins perform several principle functions, such as catalyzing metabolic reactions, facilitating the replication of DNA, mediating stimulus responses, participating in transport, and maintaining the structures of different organelles in the cell, including the nucleus, nuclear membrane, and cell membrane. Chemically, the building blocks of proteins (i.e., amino acids) are linked together through peptide bonds. These bonds are hydrolyzed by a group of enzymes designated as peptidases, which account for about 2 % of genes within the genomes of various organisms. Peptidases are important for many biological processes, including digestion of food proteins, recycling of intracellular proteins, the blood coagulation cascade, antigen presentation, and activation of a variety of proteins, such as enzymes, peptide hormones, and neurotransmitters.

Certain proteolytic enzymes synthesized during germination are involved in the production of free amino nitrogen (FAN) and di- and tri-amino acids through their synergistic action. These enzymes can provide a greater exposed surface area for starch-hydrolyzing enzymes (Briggs 1998). All enzymes share the same substrate (polypeptides), but have different catalytic sites. The product is a modified protein

that ultimately results in seed softening and increased friability being required for the effective growth of seedlings (Wentz et al. 2004). Some of the enzymes involved in protein modification already exist in mature barley grain, while some are synthesized de novo in aleurone cells during germination (Briggs 1992). Endopeptidases and exopeptidases are two principal groups of malt proteolytic enzymes. Endopeptidases cleave the bonds between two amino acids in a protein molecule at random, producing relatively smaller peptide chains, while exopeptidases attack these smaller peptide chains and cleave the links between terminal amino acids. Therefore, exopeptidase activity ultimately leads to protein molecules being hydrolyzed into FAN or di-/tri-amino acids. Endopeptidases function at a lower optimum temperature; thus, most of these enzymes are degraded during the malting process. However, exopeptidases can withstand high temperatures and complete the protein hydrolysis process. Exopeptidases consist of two major enzymes: carboxypeptidase and aminopeptidase, both of which have different active sites. Specifically, carboxypeptidase has the ability to hydrolyze protein molecules from the carboxyl end, while aminopeptidase functions from the amino end (Lalor and Goode 2010).

3.5 Lipid-Hydrolyzing Enzymes

Lipids are a group of naturally occurring, small, hydrophobic, or amphiphilic molecules, including fats, waxes, sterols, fat-soluble vitamins, monoglycerides, triglycerides, phospholipids, and others. The amphiphilic nature of some lipids allows them to form structures, such as vesicles, liposomes, or membranes, in an aqueous environment. The principle biological functions of lipids include acting as a natural food reservoir (natural glycosides), storing energy, providing a source of hydrocarbons (waxes), functioning as a cofactor in intermediate metabolism (quinines) and structural component of cells membranes (polar glycerides), and participating in signaling (Subramaniam et al. 2011). Grain lipids consist of 65–78 % natural lipids, 7–13 % glycolipids, and 15–26 % phospholipids (Briggs 1978). Lipids in cereal grains are associated with starch granules on their surface or within the starch structure (Morrison 1988). The lipids inside starch granules alter the gelatinization properties of starch granules (Briggs et al. 1981). The hydrolysis of numerous biopolymers is governed by respective hydrolyzing enzymes in the initial stage of germination during malting. Commonly used lipid-hydrolyzing enzymes include lipases (Baxter 1982), lipoxygenases (Antrobus et al. 1997), hydroperoxide lyases, and hydroperoxide isomerases (Bamforth 2009; Bamforth et al. 2009).

Lipases are hydrolyzing enzymes that catalyze esters of long-chain aliphatic fatty acids to produce free fatty acids and glycerol. Their natural substrates are insoluble lipid compounds prone to aggregation in aqueous solution. In eukaryotes, lipases may be confined within an organelle (i.e., the lysosome) or can be found in the spaces outside cells, playing roles in the metabolism, absorption, and transport of lipids. In lower eukaryotes and bacteria, lipases can be localized either intracellularly or can be secreted, functioning to degrade lipid substrates present in the

environment. Moreover, in some pathogenic organisms (*Candida albicans*, *Staphylococcus* and *Pseudomonas* species, and *Helicobacter pylori*), lipases can even act as virulence factors. Their ability to hydrolyze insoluble fatty acyl esters makes lipases different from all other esterases. Cereals contain storage fats or oils called lipid bodies, which are hydrolyzed into free fatty acids by lipase. The hydrolysis of lipid bodies is directly related to the concentration of lipase in the aleurones and scutellums of grains, including both preexisting and newly synthesized lipases, in response to physical and chemical changes (Huang 1992). Free fatty acids produced by triglyceride hydrolysis are readily utilized as metabolic energy in the synthesis of glucose when grains containing soluble sugars are depleted or as precursors for phospholipid synthesis, which is involved in the growth of the cell membrane. In malt, the enzyme lipoxygenase can hydrolyze the dioxygenation of polyunsaturated fatty acids (linoleic and linolenic acids) into hydroperoxy acids. This hydrolyzed product is used as a precursor for the production of the stale testing aldehydes used in the wine brewing industry. Barley genetic material encodes two types of lipoxygenases secreted in embryonic tissues, LOX-1 and LOX-2. LOX-1 is already present in raw barley, and its expression increases with germination. In contrast, LOX-2 is only expressed during germination of barley grain (de-Almeida et al. 2005). Lipoxygenase is heat sensitive and loses most of its activity (96–98 %) during the kilning process. Even with the dramatic reduction in hydrolyzing activity after the kilning process, almost 30 % of malt lipids are hydrolyzed by lipoxygenase during mashing. This enzyme also plays a crucial role in senescence, wound healing, infection, and resistance against pests. Hydroperoxide lyase (HPL), which is very common in plants, is known for catalysis of the hydrolysis reaction of fatty acid hydroperoxides into oxo-acids and aldehydes. HPL is a member of the cytochrome P450 family, which plays a major role in phyto-oxylipin synthesis resulting from interactions among plant herbivores. HPLs can be divided into two groups based on substrate activity: 13-HPL (CYP74B) and 9-/13-HPL (CYP74C). Because it can change the constituents of volatile aldehydes, HPL plays an important role in determination of the characteristics of food products. The hydroxyperoxide isomerase catalyzes the conversion of hydroperoxylinoleic acid into α- or β-ketols. The localization of HPL expression changes with germination, and the enzyme is only expressed in the embryo in its dormant form during early development.

3.6 Nucleic Acid-Hydrolyzing Enzymes

Nucleic acids are the polymeric biological macromolecules essential for all known forms of life. Nucleic acids, such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are made up of basic repeating units called nucleotides. Each nucleotide contains three essential components: a nitrogenous base, a phosphate group, and a five-carbon sugar molecule. Each cell of a living organism contains nucleic acids in the nucleus and in some other organelles, such as mitochondria and chloroplasts (in plants only); these nucleic acids function to encode, transmit, and

express genetic information. The building blocks of nucleic acids (i.e., nucleotides) are synthesized enzymatically (5′–3′ direction) and chemically (3′–5′ direction). During synthesis, the nucleotides are sequentially added to the growing oligonucleotide chain. Different types of oligonucleotides, such as antisense oligonucleotides, small-interfering RNA, primers (for DNA sequencing and amplification), and probes (for detecting the complementary DNA or RNA via molecular hybridization technique), have several applications in a variety of biological fields and act as tools for the targeted introduction of mutations and restriction sites and for the synthesis of artificial genes or gene fragments.

Several nucleic acid-hydrolyzing enzymes are used in genetic engineering approaches; these include restriction endonucleases, nucleases, alkaline phosphatases, and RNase P. Among these, restriction enzymes or, more specifically, restriction endonucleases (REases) are the principle nucleic acid-hydrolyzing enzymes. These produce discrete DNA fragments upon cleavage, which serves as a useful feature for analyzing and recombining the DNA molecules from different origins. These characteristics have led to the development of various genetic engineering techniques and the search for additional enzymes with similar functions. REases recognize a unique short sequence (four to eight base pairs) in the DNA fragment and produce a nick at the target site (type II), vicinity (type I), or away from the site of attachment (type III), depending upon the type of enzyme. This process produces fragments with sticky ends or blunt ends. Several applications of restriction enzymes have been reported, including manipulation of DNA, insertion of genes into plasmids, distinguishing between gene alleles, digestion of genomic DNA for gene analysis, etc. Nucleases are enzymes that cleave DNA or RNA (called DNases and RNases, respectively). DNases are further classified into exonucleases, which act on the ends or terminal regions of DNA, and endonucleases, which act at nonspecific regions in the center of DNA. Exonucleases require at least two 5′ and 3′ ends and thus cannot act on circular DNA, such as plasmids. However, endonucleases can act on both linear and circular DNA molecules. Exonucleases release single nucleotides, while endonucleases produce short DNA segments. Phosphatases remove the phosphate from DNA, RNA, and proteins. Most of these enzymes act in basic buffers and are known as alkaline phosphatases. Three common types of alkaline phosphatases used in molecular cloning experiments are bacterial alkaline phosphatases (BAPs), calf intestine alkaline phosphatases (CIAPs), and shrimp alkaline phosphatases. Alkaline phosphatases are metalloenzymes containing Zn^{2+}. RNase P, which specifically cleaves at the 5′ end of RNA, is a complex ribozyme containing a 20-kDa protein and a 377-nucleotide RNA molecule possessing at least part of the enzymatic activity of the complex.

3.7 Other Hydrolyzing Enzymes

Besides the above-described enzymes, several other enzymes also catalyze hydrolytic reactions; these include glucose oxidase, galactose oxidase, cholesterol

oxidases, catalases, phytase, peroxidase, and thiol oxidase. Glucose oxidase is a flavoprotein that catalyzes the oxidation of β-D-glucose by O_2 to δ-gluconolactone and H_2O_2. Galactose oxidase is a type II (non-blue) copper protein with a molecular mass of 69 kDa that catalyzes the oxidation of a wide range of primary alcohols and polysaccharides (e.g., D-galactose) to the corresponding aldehydes. Cholesterol oxidases are bifunctional flavoenzymes that catalyze two reactions at a single active site. The first is the oxidation of cholesterol to cholest-5-ene-3-one and the isomerization of the labile cholest-5-ene-3-one intermediate to a cholest-4-ene-3-one product. Catalases or hydroperoxidases catalyze the degradation of H_2O_2 to H_2O and O_2. Phytase is involved in the hydrolysis of phytate into phytic acid, inorganic phosphate, and myoinositol during the malting process and thus decreases its total contents in the final product. Two types of phytases, 3- and 6-phytase, are known for their dephosphorylation of phytate. 3-Phytase is found in microbial species, while 6-phytase is primarily found in seeds of higher plants, including barley. Thiol oxidase belongs to the family of oxidoreductases that specifically act on the sulfur group of donors, with oxygen as the acceptor.

4 Isolation of EHEs

As mentioned earlier, enzyme-catalyzed reactions require different types of enzymes either supplied externally or induced within the biological system. Different types of EHEs have been isolated from different sources at various states of purity before being fully characterized. However, partially purified EHEs isolated from different sources tend to be analyzed more completely for elucidation of their inhibition characteristics, substrate specificity, and interaction with activators molecules during the initial characterization. The conclusions from all these preliminary studies will guide us in understanding the mechanisms through which these proteins are controlled under different conditions. Therefore, it is important to understand the basic features of techniques most commonly used for the isolation of EHEs from different sources.

4.1 Occurrence/Sources of EHEs

Malt, comprised of germinated cereal grains that have been dried in a process known as "malting," is considered an enriched source of EHEs (Khattak et al. 2012). During the malting process, cereal grains (mostly often wheat or barley) induce signals for the expression of different types of EHEs, including cell wall-hydrolyzing enzymes, starch-hydrolyzing enzymes, proteases, lipases, and phytases (Fig. 1) that mediate the internal physiological changes of seeds. Soaking of seed results in scutellum-induced gibberellin, which functions as a signal for the expression of starch-hydrolyzing enzymes; this is thought to be the first change in seeds that leads to the production of monosaccharides, including glucose and fructose, as well as disaccharides essential for the initial growth and

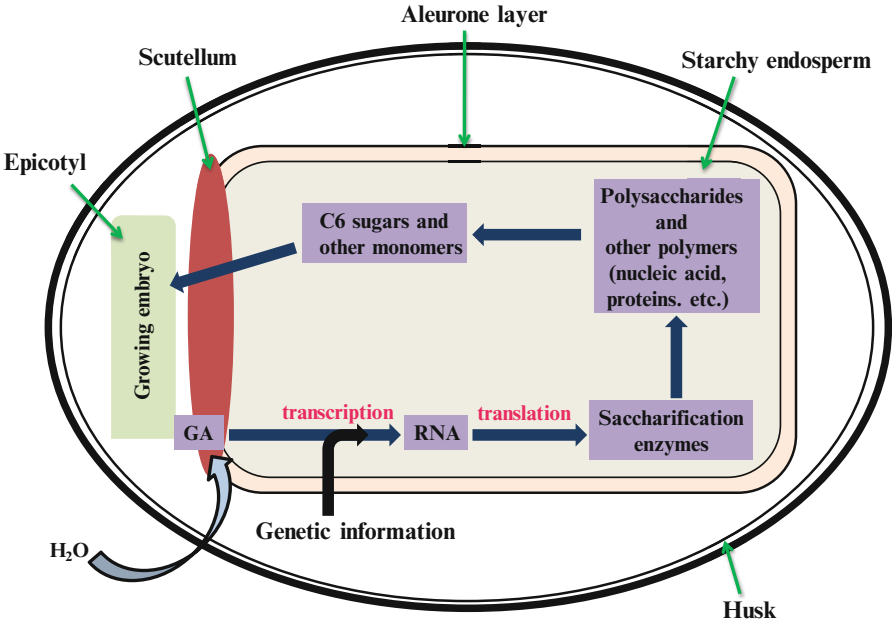

Fig. 1 The generalized scheme representation of the EHEs production in barley grain in response to external stimuli induced during malting process

development of the seed (Khattak et al. 2012). During malting, the expression of proteases involved in the hydrolysis of long-chain peptides into short-chain peptides and amino acids is also essential for seed growth (Celus et al. 2006). The expression of endogenous enzymes inside the seed is primarily involved in the hydrolysis of three major components of the grain: starch, protein, and cell wall polysaccharides (Hayes et al. 2003).

A number of cell wall-hydrolyzing enzymes, including endoglucanase, exoglucanase, arabinofuranosidase, esterase (Bamforth 2009), carboxypeptidase (Bamforth et al. 1979; Sørensen et al. 1989), xylanase (Bamforth 2009; Chithra and Muralikrishna 2008), and β-glucosidase (MacGregor 1987), have been reported to be expressed and obtained from malt. In microorganisms, fungi in particular are often studied with respect to the expression and isolation of endogenous cell wall-hydrolyzing enzymes (Aro et al. 2005; Hasegawa and Nordin 1969). Antagonistic fungi use cell wall-hydrolyzing enzymes as weapons to attack their target fungi and thus maintain stability (Aro et al. 2005). *Trichoderma* and *Aspergilli*, two industrial fungal strains, are capable of producing abundant amounts of cell wall-hydrolyzing enzymes (Durand et al. 1988; Berka et al. 1991). In the case of bacteria, *Streptomyces* strains produce cell wall-hydrolyzing enzymes capable of digesting yeast cell walls (Bacon et al. 1968). Considering the above discussion, we can conclude that endogenous cell wall-hydrolyzing enzymes can be isolated from both plants and microorganisms depending on the nature of the substrate/host.

Malt extract is also a major source of starch-hydrolyzing enzymes. Payen and Persoz (1833) found that precipitates obtained after treatment of malt extract with alcohol have the potential to liquefy starch polysaccharides into glucose (Payen and Persoz 1833). At the time of their study, malt extract was called *diastase*; the functional enzymes within the extract were later classified as α-amylase and β-amylase based on their different activities (Maercker 1878). Similarly, the existence and isolation of other starch-hydrolyzing enzymes, including limit dextrinase and β-glucosidase from malt extract, has also been reported. In recent studies, the activities of starch-hydrolyzing enzymes have been reported in agro-industrial wastes (e.g., wastes from beer fermentation broth [WBFB]), which is thought to be an enriched source of saccharification enzymes (Khattak et al. 2013a, b). Germinated barley seeds possessing abundant quantities of starch-hydrolyzing enzymes have also been processed for enzyme isolation (Sun and Henson 1990). Certain microorganisms, including thermophilic *Archaea*, bacteria (Bertoldo and Antranikian 2002), and yeast (Kelly et al. 1985), have been used for isolation of starch-hydrolyzing enzymes.

Cellulose-hydrolyzing enzymes play a pivotal role in a variety of biotechnologies, particularly biofuel production. Similar to cell wall- and starch-hydrolyzing enzymes, malt has been shown to be an enriched source of cellulose-hydrolyzing enzymes (Khattak et al. 2012). In microorganisms, fungi (Nevalainen and Palva 1978), algae (Mohapatra et al. 2003), and bacteria (Han and Srinivasan 1968) are common sources of endogenous cellulase enzymes, which can be extracted following incubation of the microorganism in its respective media.

Peptidase enzymes are encoded by about 20 % of genes in all types living organisms and play an important role in the viability of living creatures. Germinated seeds are an economical and feasible source of such EHEs. A number of proteolytic enzymes such as endopeptidases and carboxypeptidases are synthesized during seed germination. Drzymała and Bielawski (2009) also reported the isolation of endogenous carboxypeptidase from germinated triticale grains (Drzymała and Bielawski 2009). Similarly, the isolation of endogenous proteolytic enzymes from others plant sources, including barley (Sørensen et al. 1989), wheat (Baulcombe et al. 1987), and triticale (Drzymała et al. 2008), has also been reported. Microbial sources of endogenous proteolytic hydrolyzing enzymes include bacteria (Fujimura and Nakamura 1981; Pacaud et al. 1976), fungi (Kamath et al. 2010), and algae (Matsubara et al. 2000). Certain agro-industrial waste materials, including WBFB (Khattak et al. 2012), cassava wastewater (Barros et al. 2013), slaughterhouse waste, dairy industry effluent, and sewage waste (Boominadhan et al. 2009), have been utilized for the production of proteolytic enzymes through microbial species.

Lipid-hydrolyzing enzymes have been obtained from different sources, including plants, animals, and microorganisms. Based on statistical data, different organisms, including bacteria (45 %), fungi (21 %), animals (18 %), plants (11 %), and algae (3 %), contribute to total lipase production with varying degrees (Patil et al. 2011). Among microbes, fungal strains have always been considered preferable because they usually excrete endogenous enzymes, allowing for simple and inexpensive isolation of enzymes from fermentation broth (Maia et al. 1999).

Agro-industrial wastes (e.g., WBFB, waste from the fish industry, etc.) are composed of complex polysaccharides that can be exploited as economically feasible substrates for microbial growth and are responsible for endogenous enzyme production (Khattak et al. 2012; Rebah and Miled 2013). The availability and cost of these alternative media (agro-industrial waste) are the main determinants of the economics of the enzyme production process. Cereals crops (e.g., malt, wheat, and barley) can be processed for the production and isolation of endogenous lipase enzymes (Khattak et al. 2012). The lipase activity in cereal crops increases significantly, particularly during the initial stage of germination. The requirements for structural carbons and energy to initiate seed growth are met through the hydrolysis of stored food (insoluble tri-acylglycerols) by lipase (Pahoja and Sethar 2002).

Nucleic acid-hydrolyzing enzymes play an important role in certain biological processes. Complete hydrolysis of nucleic acids yields inorganic phosphate, deoxyribose sugar, and four different heterocyclic bases. As mentioned earlier, different types of nucleic acid-hydrolyzing enzymes have been isolated from different sources, including microorganisms, plants, fungi, and highly developed animals. In microorganisms, different bacterial strains (e.g., *Bacillus*, *Brevibacillus*, *Microbacterium*, *Pseudomonas*, and *Stenotrophomonas*) and fungi (e.g., *Alternaria*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Fusarium*, *Gliomastix*, *Humicola*, *Penicillium*, *Scopulariopsis*, *Wardomyces*, and *Periconia*) are commonly used (Balestrazzi et al. 2007; Balabanova et al. 2012). Roberts (2005) reported that more than 3,000 restriction enzymes with over 250 different specificities have been isolated from various organisms (Roberts 2005). These enzymes are thought to have evolved from a common ancestor and likely became widespread via horizontal gene transfer (Jeltsch et al. 1995; Jeltsch and Pinground 1996). Evidence also exists for their evolution as self-genetic elements (Naito et al. 1995). Isolation of restriction nucleases has been reported from *Escherichia coli* (Linn and Arber 1968). Alkaline phosphatases are commonly found in the periplasmic space, external to the cell membrane in gram-negative bacteria, and are usually produced during phosphate starvation (Horiuchi et al. 1959). Additionally, RNase P has been described in several bacterial strains (Evans et al. 2006), Archaea (Hall and Brown 2002), and eukaryotes, such as yeast (Marquez et al. 2006).

Besides the abovementioned enzymes, several other enzymes have also been reported from different sources. Industrial-scale production of glucose oxidase is mainly carried out using *Aspergillus niger* or *Penicillium amagasakiense* (Crueger and Crueger 1990). Moreover, other *Penicillium* and *Aspergillus* sp., including *Penicillium notatum*, *Penicillium funiculosum*, *Penicillium piceum*, *Penicillium purpurogenum*, *Penicillium variabile*, *Penicillium chrysogenum*, and *A. fumaricus*, are also attractive candidates for industrial production of this enzyme (Leiter et al. 2004). Different fungal species, e.g., *Fusarium dendroides* (*Dactylium dendroides*), *Gibberella fujikuroi*, and *G. zeae*, as well as the basidiomycete *Polyporus circinatus*, are widely used for industrial-scale galactose oxidase production. Genes encoding galactose oxidase can also be expressed efficiently in either *Pichia pastoris* (Whittaker and Whittaker 2000) or *A. oryzae* (Xu et al. 2000). Cholesterol oxidases are produced by bacteria, including species from the

Rhodococcus, *Streptomyces*, and *Nocardia* genera. However, several *Brevibacterium*, *Proactinomyces*, *Pseudomonas*, and *Cellulomonas* species also possess enzymes with cholesterol oxidase activity and are suitable candidates for industrial production of this enzyme (Watanabe et al. 1986). Recombinant *E. coli* strains expressing bacterial cholesterol oxidase genes have been reported (Sakka et al. 1994). Different microorganisms, including bacteria (e.g., *Micrococcus*, *Bacillus*, *Microscilla*, and *Alcaligenes* spp.), fungi (e.g., *Aspergillus*, *Penicillium*, *Thermomyces*, *Thermoascus*, and *Acremonium* spp.), and yeasts (e.g., *Saccharomyces*, *Candida*, and *Mycotorula* spp.), are known for their abundant production of catalases. However, catalases from animal sources (e.g., bovine liver) are generally inexpensive, and thus, their production from microbial sources is only economical when better producer strains (preferably recombinant strains) and inexpensive technology can be used or when enzymes with special properties (e.g., functional at high or low temperatures or at alkaline or acidic pH) are produced (Takeuchi and Isobe 1999).

4.2 Methods of Cell Lysis

Obtaining endogenous enzymes from respective organisms without rupturing their cell envelopes is a difficult task. However, certain organisms excrete endogenous enzymes into the surrounding microenvironment for a variety of purposes, most commonly for cell defense. The first attempt to obtain cellular contents, specifically the proteinaceous content of the cell, was made in microbial cells, with attempts to rupture the outer cell envelope by prolonged exposure to water, followed by heating in glycerin solutions. The method was successful only in that it allowed the researchers to obtain fractions of cellular contents; however, most of the contents were altered (Buchner 1966). Over time, a number of specialized, highly efficient techniques have been reported for the rupture of enveloped cells. These techniques are briefly described below.

4.2.1 High-Pressure Homogenizer

High-pressure homogenization was the first successful cell lysis method for obtaining endogenous enzymes from yeast cells. A hydraulic press supplied by Brink and Hubner was used for applying higher pressure to cells to squeeze out the cell contents. Using this mechanical lysis method, almost 1,000 g of yeast was squeezed for a few hours, resulting in about 500 mL of liquid solution that was yellowish brown in color and contained endogenous enzymes (Buchner 1897). Dilution of the yellowish brown solution with water and hydrogen peroxide led to formation of oxygen, inducing violent foaming. Additionally, researchers concluded that the presence of catalase in the solution catalyzed the oxygen reaction. Later studies demonstrated that mixing the yellowish solution with concentrated glucose solution produced bubbles in the form of a thick layer of foam over the surface, clarifying the mechanism of carbon dioxide formation as result of a cell-free fermentation process (Buchner 1966).

4.2.2 Chemical Lysis

Chemical lysis is a well-established method for obtaining endogenous enzymes, particularly from microbial cells (Arnold 1972). Freshly prepared microbial cultures are incubated in organic solvent at room temperature for few days in order to permeabilize the microbial membrane. Addition of organic solvent, primarily toluene, induces the expression of β-fructofuranosidase, classified as a cell wall enzyme; the secretion of this enzyme primarily depends on the action of cell wall-modifying enzymes (endogenous enzymes). The addition of organic solvents (e.g., toluene and ammonium hydroxide) creates hypertonic conditions, resulting in the alteration of the membrane composition, which subsequently activates the EHEs located within the cytoplasm (Arnold 1972). Although the method is well established, certain limitations are associated with chemical lysis, particular the stability of the endogenous enzymes in the presence of endogenous peptidases (Boonraeng et al. 2000).

4.2.3 Enzymatic Lysis

Enzymatic lysis is another approach for the isolation of EHEs that has been used with a great degree of success (Salazar and Asenjo 2007). In enzymatic lysis, specific cell wall-hydrolyzing enzymes are used to digest the cell envelope. The microbial cell wall (e.g., that of yeast) is a highly dynamic structure composed of mannoprotein and fibrous β(1→3) glucans with a few β(1→6) glucan branches. The β(1→6) glucans make connections between the inner and outer cellular components of the yeast cell (Lipke and Ovalle 1998). A number of enzymes, including β(1→3) glucanase, proteases, β(1→6) glucanase, mannanase, and chitinase, can be applied directly in groups and act synergistically to digest the outer cell wall (Salazar and Asenjo 2007). This approach has a major limitation: cellular proteins released into the medium after cell wall digestion are subjected to the activity of the protease.

4.2.4 Freeze-Thawing

Rapid freeze-thawing is an uncommon method used for lysis of the yeast cell wall. A number of traditional methods, mostly mechanical methods, have been used to disrupt the cell wall; however, there are few inherent disadvantages to using these methods. For example, heat generation within a sample is commonly encountered during application of traditional techniques for cell lysis and can cause protein denaturation or aggregation. This problem can be overcome by keeping the sample at a low temperature. Liquid nitrogen is used for rapid freezing of the targeted cells, followed by grinding using a sterilized mortar and pestle prechilled with liquid nitrogen. The freeze-thawing method causes the target cells to swell and ultimately rupture due to the formation of ice crystals during freezing and subsequent contraction during thawing. However, this process takes a long time because several cycles of freezing and thawing are required. However, freeze-thawing has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols. The fine powder obtained from this

method can be stored at −80 °C, and for experimental purposes, cell lysates can be produced by the addition five volumes of buffer solution per volume of cells (Amberg et al. 2006).

4.2.5 Ultrasonication

The process of sonication uses ultrasound pulses, i.e., high-frequency sound waves, for disruption of the cell wall. This approach to cell lysis is an alternative method that is now commonly used for rupturing the cells of bacteria, spores, and even diced tissues. The ultrasonic homogenizer is a common tool used for partial or complete disruption of the cell wall. This tool produces sound waves delivered through a vibrating probe that is immersed in the liquid cell suspension. The device consists of a piezoelectric transducer, temperature probe, horn tip, and digital system for controlling parameters, such as frequency, amplitude, and temperature. Due to the capacity for well-controlled sonication parameters and the comparatively high selectivity for cellular material release and purity, ultrasonication for disintegration of the cell wall has been described as a reliable and efficient approach (Balasundaram et al. 2009).

4.2.6 Bead Beating

Bead beating is one of the most common and frequently used approaches for obtaining cellular proteins through cell lysis. The method involves the mechanical disruption of the cell envelope of microorganisms, plants, and animals (Khattak et al. 2014). Glass beads of different sizes (0.425–0.60 mm) are used, depending on the nature of the cell, and beads are vortexed at high speeds using a vortexer. Because of the nature of this mechanical approach, localized heating within a sample can occur because of the continuous collision of beads with the wall of the container or with one another. Therefore, special measures should be taken to avoid the thermal denaturation of the cellular materials; every cycle of the agitation step must be interspersed with a cooling cycle. The beat beating procedure for cell lysis is depicted in the schematic diagram in Fig. 2. Table 2 summarizes the benefits and limitations of each approach used for the production of cell-free systems.

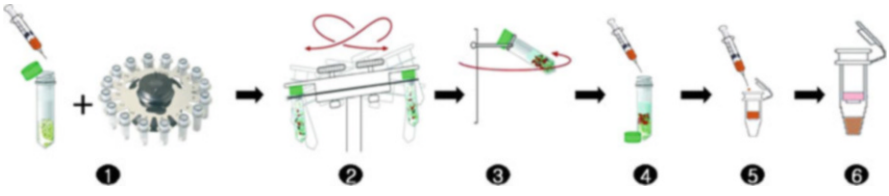

Fig. 2 Bead beating is the most common techniques employed for the cell lysis in order to obtained EHEs from cell lysate. The technique consists of (*1*) isolation of biomass from different sources in a specialized tube, (*2*) vertexing at different speed, (*3*) centrifugation and isolation of supernatant, (*4*) concentration of enzymes mixture through specialized column and obtains highly concentrated endogenous enzyme mixture

Table 2 Comparative study of different cell lysis methods employed for the rupturing of cell enveloped in order to release endogenous enzymes into the medium

Method of cell lysis	Limitations	Advantages	References
Hydraulic press	Need expensive equipment, difficulty in maintenance	Quick and ideal for large-scale lysis, effective for all cells lysis	(Buchner 1966; Goldberg 2008)
Organic solvent lysis	Possibilities of alteration in cellular materials, difficult in purification	Economical as no specialized equipment required and fast process	(Breddam and Beedfeldt 1991)
Enzymatic lysis	Can cause alteration in target protein structure, required expensive exogenous enzymes	Required nonspecialized equipment, highly specialized approach	(Lam and Wassink 1990)
Freeze grinding	Slow process, chances of contamination	Economical, obtained proteins with original activity	(Taskova et al. 2006; Yeng et al. 2013)
Centrifugation	Only effective for weal cell wall, slow process	Economically feasible	(Martz 1966; Lodish et al. 2000)
Cell bomb	Only applicable to specialized cells	Fast, ensured the protein stability and activity	(Goldberg 2008; Simpson 2011)
Ultrasonification	High ultrasound waves show negative effect on endogenous enzyme function	Really effective for large-scale cell disruption, controllable	(Liu et al. 2013; Stathopulos et al. 2004)
Beads beating	Difficulty in target proteins purification, heat produced can cause alteration in target proteins structure	Effective for all kind of cells, economical, sample, fast	(Khattak et al. 2014; Griffiths et al. 2006)

4.3 Separation of EHEs from Cell Lysates

Recent developments in the identification of new enzymes have also facilitated the improvement of recovery and separation methods. Following cell lysis, enzymes are separated from cells or cellular fragments and organelles. This process is rather sensitive due to the small size of microbial cells, which results in slight differences between the densities of cells and the components of the fermentation medium. Moreover, no effective techniques have been developed for recovery of all enzymes, and selection of an appropriate technique based on the type of enzyme and the specific microbial cells used will ensure better separation of enzymes from other constituents. A few of the techniques commonly used today are described below.

4.3.1 Filtration

The rate of filtration can be determined as a function of filter area, pressure, viscosity, and resistance offered by the filter cake medium. Generally, the flow rate becomes more uniform as the medium becomes clearer. Moreover, the cumulative filtrate volume increases linearly with time. The gradual increase in the thickness of the formed filtrate resists the flow rate across the filtration media. This limitation can be overcome to some extent by increasing the pressure applied. However, beyond certain limits, the applied pressure may cause collapsing of the filtrate cake, leading to complete blockage of the filter. The most recent developments in this area have resulted in the application of cross-flow membrane filtration and microfiltration; these techniques have unique advantages, such as enhanced efficacy, ease of operation and cleaning, improved downstream ultrafiltration purification, and, most importantly, cost-effectiveness. During the cross-flow filtration process, the input stream flows parallel to the filter area and thus prevents the accumulation of filter cake and increased resistance to filtration. Additionally, maintaining a sufficiently high filtration rate usually requires large amounts of energy in the form of high flux rates over the membranes.

4.3.2 Centrifugation

EHEs can be separated from components of lysed cells through gentle fractionation techniques, such as centrifugation; often, this method can help to preserve the functions of these enzymes. An effective and economical separation can be achieved only by sedimentation in a centrifugal field based on the molecular weight of the enzyme and the sizes of the media components. The effective operation of centrifugation technique requires a combination of high centrifugal force and short sedimentation distances. Commercially, this is achieved by using either a sieve centrifuge or a solid-wall centrifuge. Another type of centrifuge, called a decanter or scroll-type centrifuge, works with low centrifugal forces and is specifically used for the separation of cells and protein precipitates. These types of centrifuges are feasible for continuously operating processes. Tubular-bowl centrifuges are used for separation of very small-sized particles. However, these centrifuges cannot be employed for continuous operation. Separators or disk-stack centrifuges are used for continuous removal of solids from suspensions.

4.3.3 Extraction

This technique is based on the principle of liquid-liquid extraction in an aqueous two-phase system (Hustedt et al. 1985). Cellular fragments are separated in the first step, followed by subsequent purification using other methods if greater purity is required. This may involve affinity ligand binding, modified chromatography gels, etc.

4.3.4 Flocculation and Flotation

During this process, destabilized particles are induced to come together, make contact, and subsequently form larger aggregates. Separation of cells by filtration or centrifugation is limited by the small sizes and physical properties of microbial

cells and cell debris, as well as the molecular weights of the enzymes. Flocculation of cell suspensions is assisted by both filtration and centrifugation (Bajpai et al. 1991; Sitkey et al. 1992a, b). Flocculating agents are additives capable of increasing the degree of flocculation of a suspension. They can be organic or inorganic and natural or synthetic.

The flocculation of cells or enzymes by polyelectrolytes is a two-step process. In the first step, the surface charge on the suspended material is neutralized. The second step involves the linkage of these particles to form large aggregates. Flocculant selection for a specific cell separation process is a challenge because many factors can affect flocculation. These factors can have their origin in the broth (cell surface charge and size, ionic strength, pH, cell concentration, and the presence of other charged matter), the polymer (molecular weight, charge, charge density, structure, and type), and engineering parameters (mixing and mode and order of addition). The final criteria for flocculant selection should take into consideration all aspects of the flocculation process. These include the cost of the added flocculant, subsequent separation performance, process robustness, and yield. In some cases, flocculation can also provide purification by selectively removing unwanted proteins, nucleic acids, lipids, and endotoxins from the cell broth. However, in cases in which no stable agglomerates are formed, the substances can be separated by flotation. Here, the enzymes or cells are adsorbed onto gas bubbles, rise to the top of the mixture, and accumulate in froth. A typical example of flotation is the separation of single-cell protein (Mayer and Woernle 1985).

4.4 Concentration of EHEs

Protein samples separated from cell debris often contain very small concentrations of enzymes. The volume of the material to be processed is generally very large because large amounts of waste materials must be removed. Thus, there is a tremendous need to concentrate the starting material in order to achieve economic, efficient purification. However, the approaches selected must be such that they do not inactivate or denature the enzymes. Several commonly used approaches are described below.

4.4.1 Thermal Methods

Enzymes are often unstable at elevated temperatures. Thus, only a brief heat treatment can be used for concentration. Evaporators with rotating components that achieve a thin liquid film (thin-layer evaporators and centrifugal thin-layer evaporators) or circulation evaporators (long-tube evaporators) can be employed for the concentration of enzymes.

4.4.2 Precipitation

Enzymes are very complex protein molecules possessing both ionizable and hydrophobic groups, which can both interact with the solvent. Indeed, proteins can be

made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes (Bell et al. 1982) and can be achieved with various reagents, such as salts, organic solvents, and polymers, or at the isoelectric point.

4.4.3 Ultrafiltration

For ultrafiltration, a semipermeable membrane is used; this membrane permits the separation of solvent molecules from larger enzymes under high osmotic pressure. This is the principle on which all membrane separation processes, including ultrafiltration, are based. Ultrafiltration and cross-flow filtration are based solely on the sieve effect. In processing enzymes, cross-flow filtration is used to harvest cells, whereas ultrafiltration is employed for concentrating and desalting.

4.5 Purification of EHEs

Enzymes can be used in the partially purified form (e.g., industrial use) or the highly purified form (e.g., analytical and medical applications). Highly purified enzymes are obtained through various techniques, such as crystallization, electrophoresis, and chromatography. Some enzyme purification techniques are described below.

4.5.1 Crystallization

Crystallization offers a cost-effective, industrial-scale method for purification. Crystallization involves the formation of solid enzyme particles of defined shapes and sizes. An enzyme can be induced to crystallize or form protein-protein interactions by creating solvent conditions that result in enzyme supersaturation. Most studies of enzyme crystallization have been focused on obtaining crystals for X-ray diffraction analysis rather than as a purification process. However, crystallization is attracting increasing interest as a purification process. Some recent advancements have been made concerning the use of self-interaction chromatography for measuring the protein osmotic second virial coefficient to predict protein crystallization behavior (Tessier et al. 2002) and the development of high-throughput screening and analysis systems for establishing robust protein crystallization conditions (Brown et al. 2003). Various enzymes have been crystallized for commercial purposes, including cellulase, glucose isomerase, subtilisin, and alcohol oxidase.

The desired characteristics of industrial-scale enzyme crystallization are product purity, process yield, ease of crystal recovery, and short overall process time. To achieve these aims, the crystallization process must be carefully designed and developed to produce crystals with relatively large sizes and the desired morphology. Many factors, including salt type and concentration, pH, temperature, the presence of variable amounts and types of impurities, mixing, and crystal seeding, can affect enzyme crystallization. Controlling the level of supersaturation throughout the crystallization process is essential for optimization of crystal size, which can be controlled by the use of precipitants, such as salt, pH, and temperature (Scopes 1993; Judge et al. 1995).

4.5.2 Electrophoresis

This involves the separation and analysis of enzymes based on their size and charge. Enzymes are separated by charge in agarose because the pores of the gel are too large to sieve proteins. This technique is mostly employed for the isolation of pure enzymes on the laboratory scale. A few limitations are associated with this approach, including generation of heat and interference caused by convection, as well as the necessity for scale-up due to these two factors.

4.5.3 Chromatography

In chromatographic techniques, enzymes are separated based on various physical properties (such as size, shape, charge, and hydrophobic interactions), chemical properties (such as covalent bonding), and biological properties (such as biospecificity). Several types of chromatographic techniques are described below.

Gel Chromatography

In gel chromatography (also called gel filtration), hydrophilic, cross-linked gels with pores of finite sizes are used in columns to separate biomolecules. Concentrated solutions are necessary for separation because the sample volume that can be applied to a column is limited. Molecules are separated according to size and shape. Molecules larger than the largest pores in the gel beads, i.e., above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules, which enter the gel beads to varying extents depending on their sizes and shapes, require longer times to pass through the column. As a result, molecules are eluted in order of decreasing molecular mass.

Ion-Exchange Chromatography

In this method, enzymes are separated based on charge. Enzymes possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged). The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration. Because of the concentrating effect of ion-exchange chromatography, samples can be applied in dilute form. The ability to process large volumes and the elution of dilute sample components in a concentrated form make ion-exchange methods very useful. The matrix used to produce ion-exchange resins should be sufficiently hydrophilic to prevent enzyme denaturation and must have a high capacity for large molecules with a rapid equilibration. In addition, industrial applications require ion exchangers that give high resolution, allow the use of rapid flow rates, suffer small changes in volume with salt gradients or pH changes, and exhibit high regenerative capacity.

Affinity Chromatography

For affinity chromatography, the enzyme to be purified is specifically and reversibly adsorbed on an effector attached to an insoluble support matrix. Suitable effectors are substrate analogs, enzyme inhibitors, dyes, metal chelates, or antibodies. During

separation, insoluble matrix is contained in a column. The biospecific effector, e.g., an enzyme inhibitor, is attached to the matrix. A mixture of different enzymes is applied to the column. The immobilized effectors specifically bind the complementary enzyme. Unbound substances are washed out, and the enzyme of interest is recovered by changing the experimental conditions, e.g., by altering pH or ionic strength.

5 Mechanism of Action of EHEs

5.1 Cell Wall-Hydrolyzing Enzymes

Cell wall-hydrolyzing enzymes include endoglucanase, exoglucanase, arabinofuranosidases, esterase, carboxypeptidase, xylanase, and β-glucosidase (Khattak et al. 2012). The solubilization of β-glucans is the first step in cell wall hydrolysis. The enzymes involved in this process are termed "solubilases." There are two pathways that lead to the solubilization of β-glucans (Jamar et al. 2011). Specifically, β-glucans are either directly hydrolyzed into glucose by β-glucan exohydrolases or first hydrolyzed by a number of enzymes involved in removal of the outer cell wall layer and then further hydrolyzed into glucose. Enzymes involved in the hydrolysis of β-glucans include (1→3)-β-glucanase, carboxypeptidase, phospholipases, (1→4)-endo-β-glucanase, feruloyl esterase, and arabinofuranosidases (Jin et al. 2004; Georg-Kraemer et al. 2004; Kuntz and Bamforth 2007). Hydrolysis of arabinoxylan is carried out by endo-β-(1→4)-xylanase, exoxylanase, β-xylosidase, and α-arabinofuranosidases (Hrmova et al. 1997). Exoxylanase is involved in the cleavage of the outer β-(1→4) xylosidic linkages, while endo-β-(1→4)-xylanase attacks the inner β-(1→4) xylosidic linkages in arabinoxylan polymers, separating the arabinofuranosyl residues (Egi et al. 2004). β-Xylosidase catalyzes the hydrolysis of β-(1→4) xylosidic bonding, while arabinofuranosidase hydrolyzes the α-(1→2) and α-(1→3) linkage between arabinofuranose units. Arabinofuranosidases consists of two subgroups α-L-arabinofuranosidases and α-D-glucuronidases. α-L-arabinofuranosidases catalyze the cleavage of terminal arabinose residues from the side chains of xylan and other arabinose-containing polysaccharides (Saha 2000). Xylanases are also classified into debranching and nondebranching enzymes, depending on whether or not they produce free arabinose in addition to cleaving the xylan backbone (Matte and Forsberg 1992). However, the reported release of arabinose by xylanases may have been due to the presence of arabinofuranosidases as contaminants. Indeed, the synergistic activity of xylanases and arabinofuranosidases allows small amounts of contaminants to yield detectable amounts of free arabinose (Coughlan et al. 1993). α-D-glucuronidases hydrolyze the linkages between 4-*O*-methylglucuronic/glucuronic acid and xylose residues in glucuronoxylan. Despite the biodegradation of xylan, α-D-glucuronidases also show activity only toward short xylooligomers, while others can release glucuronic acid from polymeric xylan (Puls 1992). Acetyl esterase and ferulic acid esterase also influence the hydrolysis of arabinoxylane (Humberstone and Briggs 2000a, b).

5.2 Starch-Hydrolyzing Enzymes

The two basic steps in the enzymatic conversion of starch are liquefaction and saccharification. Endoamylase (EC 3.2.1.1) protects against the rapid increase in starch solution viscosity caused by the release of amylose during liquefaction (Guzman-Maldonato and Paredes-Lopez 1995). α-Amylase randomly cleaves α-(1→4) glycosidic linkages in starch until the chain lengths of the reaction products are about 10–20 glucose units. At this point, the starch fragments fail to bind to the active site of the enzyme. Hydrolysis of amylopectin produces a mixture of linear malto-oligosaccharides and fragments that contain the α-1,6-bond, which cannot be cleaved by α-amylase. Xylanases and cellulases are glycosyl hydrolases that do not act on starch, but yield improvements in starch processing. Both are involved in the cleavage of the β-1,4-glycosidic bonds in cellulose and xylans, respectively. Xylanases reduce the viscosity of starch slurries by degrading xylans, whereas cellulases positively affect starch hydrolysis when it is contaminated by cellulose fibers. Moreover, β-amylase, an exo-acting enzyme involved in the cleavage of α-(1→4) glycosidic linkages at the nonreducing end of linear chains in starches and other polysaccharides, produces β-maltose and β-glucose successively (Hoseney 1994; Svensson et al. 1985). The catalytic activity of β-amylase continues until α-(1→6) linkages are reached in starch molecules. Glucosidases are also exo-acting enzymes and hydrolyze α-(1→4) glycosidic linkages in starch molecules. The main advantage of glucosidases over β-amylase is their ability to bypass the side chain at α-(1→6) bonds (Lalor and Goode 2010).

Limit dextrinase (EC 3.2.1.41) has the ability to break the amylopectin α-(1→6) linkages of branched dextrins and complete the hydrolysis of starch, producing its monomeric sugar at the end of the hydrolyzing reaction (Bowles 1996). In short, the initial solubilization of starch is catalyzed by α-amylase, while the hydrolysis of the resulting dextrins to oligosaccharides and glucose is subsequently carried out by the synergistic action of α-amylase, β-amylase, limit dextrinase, and glucosidases (Fincher 1989; MacGregor 1996). Figure 3 represents the generalized schematic view of starch hydrolysis.

5.3 Cellulose-Hydrolyzing Enzymes

The hydrolysis of cellulose, the most abundant polysaccharide on Earth, requires the concerted activity of a number of enzymes with different substrate specificities. Cellobiohydrolases (e.g., EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolases) hydrolyze cellobiose units from the ends of long polysaccharide chains. Then, endoglucanases (e.g., EC 3.2.1.4; 1,4-β-D-glucan-4-glucanohydrolase) cleaves the middle, amorphous region of the cellulose chain, thus providing more active sites for the activity of cellobiohydrolases. Finally, the third most important hydrolyzing enzyme participating in the hydrolysis of cellulose is β-glucosidase (EC 3.2.1.21), which cleaves cellobiose to glucose. This enzyme has been available in feedstock as

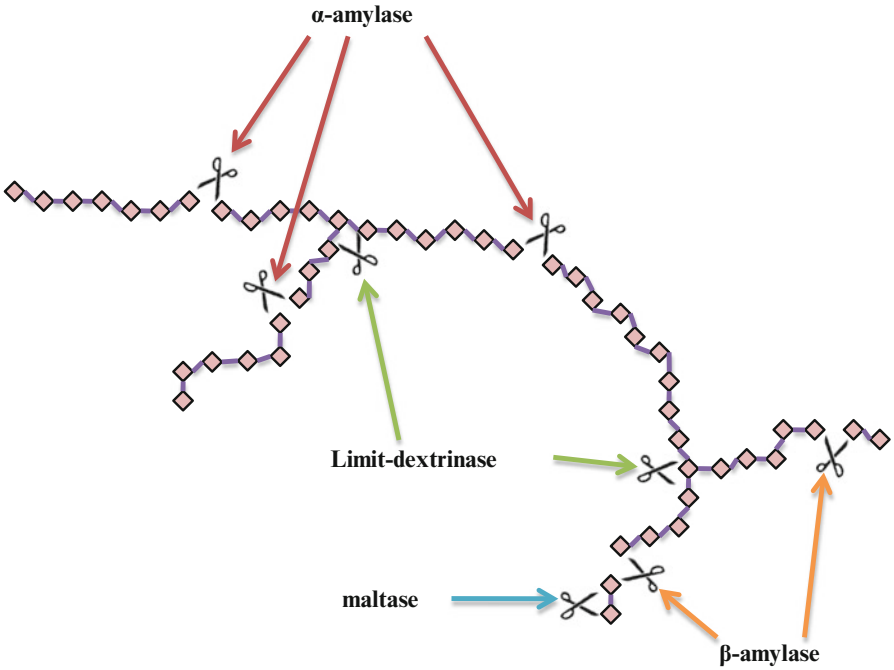

Fig. 3 Starch-hydrolyzing enzymes system consists of four major enzymes that cleave different bonding at different positions and produced glucose molecules as final product

an easily metabolizable carbon source for a number of microorganisms and is known to facilitate the production of desired products.

Another important source of cellulose readily available in complex form is hemicellulose. Hemicellulose is a heteropolymeric structure; however, the process through which hemicellulose hydrolyzes into simple sugar is well understood. Endoenzymes are the major class of hydrolyzing enzymes that cleave the main polymer chain internally, providing active sites for exoenzymes that liberate the simple sugars from the chain. Along with endo- and exoenzymes, a number of other enzymes also contribute to the hydrolysis of hemicellulose by cleaving the side chains of the polymers or oligosaccharides, producing various mono- and disaccharides depending on the type of hemicellulose being hydrolyzed. For example, the breakdown of xylan involves at least endo-1,4-β-D-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37), which act on the main sugar chain, and, depending on the type of xylan, side-chain cleaving enzymes, such as α-glucuronidase (EC 3.2.1.131) and acetyl xylan esterase (EC 3.1.1.72). Similarly, different combinations of endoenzymes, exoenzymes, and ancillary enzymes are required for complete hydrolysis of other types of hemicelluloses into monomeric sugars.

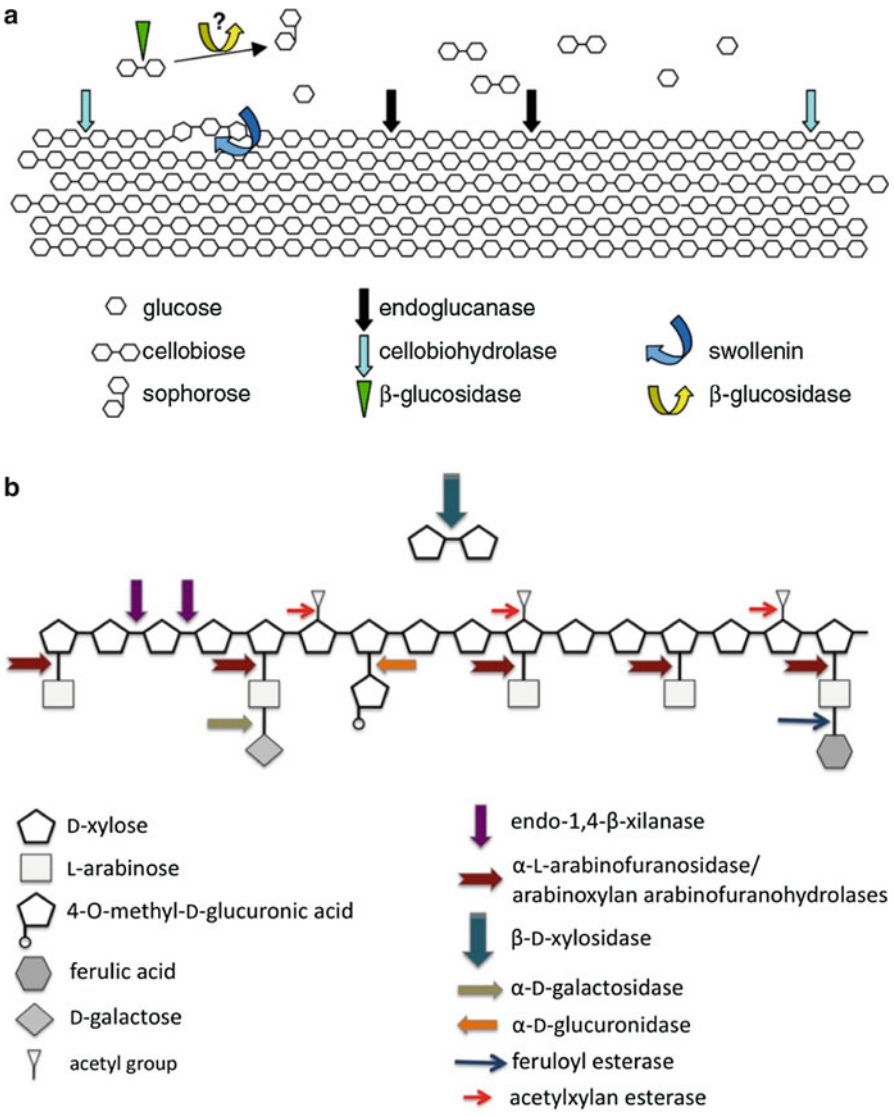

Fig. 4 Hydrolyzing enzyme system for the degradation of cell wall components. A number of hydrolyzing enzymes are participating in the degradation of (**a**) cellulose and (**b**) hemicellulose (Image taken from Aro et al. 2005)

De Vries and Visser (2001) provided a complete list of enzymes involved in the degradation of hemicellulose and their mechanisms of action (de Vries and Visser 2001). Figure 4a, b provides a complete schematic view of the cellulotic and hemicellulolytic system.

5.4 Protein-Hydrolyzing Enzymes

Certain proteolytic enzymes are involved in the production of FAN and di-/tri-amino acids. The enzymes involved in this protein modification system share the same substrate (polypeptides), but have different active sites. The final products, including, FAN, di-amino acids, and tri-amino acids, are produced as result of their synergistic action. Endopeptidases and exopeptidases are two principal groups of protein-hydrolyzing enzymes. Endopeptidases randomly cleave peptide bonds, producing relatively smaller peptide chains, while exopeptidases attack these smaller peptide chains and cleave the bonds between terminal amino acids producing FAN or di-/tri-amino acids. Exopeptidases comprise two major enzymes, carboxypeptidase and aminopeptidase, which have different active sites. Specifically, carboxypeptidase hydrolyzes proteins from the carboxyl end, while aminopeptidase hydrolyzes proteins from the amino end (Lalor and Goode 2010; Evan and Taylor 1990).

Endopeptidases hydrolyze internal, α-peptide bonds in a polypeptide chain. Endopeptidases include chymotrypsin (EC S01.001) (Graf et al. 2004), pepsin (EC A01.001) (Tang 2004), and papain (EC C01.001) (Menard and Storer 2004). Endopeptidases acting on substrates smaller than proteins are termed as oligopeptidases. Thimet oligopeptidase (EC M03.001) is an example of an oligopeptidase (Barrett and Chen 2004). Endopeptidases initiate the hydrolysis of proteins, producing new N- and C-termini, which subsequently act as substrates for exopeptidases that complete the hydrolytic process. Endopeptidases are also involved in the removal of signal peptides from secreted proteins (e.g., signal peptidase I, EC S26.001) (Dalbey 2004) and the maturation of precursor proteins, such as enteropeptidase (EC S01.156) (Sadler 2004) and furin (EC S08.071) (Creemers and Van de Ven 2004).

Exopeptidases require a free N-terminal amino group, C-terminal carboxyl group, or both and hydrolyze a bond up to three residues from the terminus. This group of enzymes is further divided into aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, tripeptidyl-peptidases, and dipeptidases.

Aminopeptidases release a single amino acid residue from the unblocked N-terminus of the substrate. This group includes aminopeptidase N (EC M01.001) (Turner 2004) and aminopeptidase C (EC C01.086) (Chapot-Chartier and Mistou 2004).

Dipeptidases hydrolyze dipeptides when both of the termini are free. They include dipeptidase A (EC C69.001) (Dudley and Steele 2004) and membrane dipeptidase (EC M19.001) (Hooper 2004).

Dipeptidyl-peptidases hydrolyze a dipeptidyl bond, i.e., release an N-terminal dipeptide from the substrate. They include dipeptidyl-peptidase I (EC C01.070) (Turk et al. 2004) and dipeptidyl-peptidase III (EC M49.001) (Chen and Barrett 2004).

Tripeptidyl-peptidases cleave a tripeptidyl bond, producing a tripeptide from the N-terminus of the substrate. Examples of tripeptidyl-peptidases are tripeptidyl-peptidase I (EC S53.003) (Sohar et al. 2004) and tripeptidyl-peptidase II (EC S08.090) (Tomkinson 2004).

Peptidyl-dipeptidases hydrolyze dipeptides from the C-terminus of the substrate. An example is peptidyl-dipeptidase A (EC XM02-001) (Corvol et al. 2004).

Carboxypeptidases hydrolyze a single residue from the unblocked C-terminus of the substrate. Examples include carboxypeptidase A1 (EC M14.001) (Auld 2004), cathepsin X (EC C01.013) (Menard and Sulea 2004), and carboxypeptidase Y (EC S10.001) (Mortensen et al. 2004).

5.5 Lipid-Hydrolyzing Enzymes

Lipid-hydrolyzing enzymes include lipase, lipoxygenase, hydroperoxidase lyase, and hydroperoxide isomerase (Khattak et al. 2012). Lipases hydrolyze esters of long-chain aliphatic fatty acids, producing free fatty acids and glycerol. Lipases are different from all other esterases due to their ability to hydrolyze insoluble fatty acyl esters (Ward 1985). The potential of lipases to act as biocatalysts is associated with their sophisticated selectivity for the modification of triglycerides. Three features are relevant: (1) regioselectivity, i.e., the position of the fatty acid on the glycerol backbone; (2) fatty acid specificity, i.e., concerning the length or unsaturation of the chain; and (3) the class of acylglycerols, i.e., mono-, di-, or triglycerides. Most lipases are 1,3 regiospecific, hydrolyzing primary alcohol 1,3 positions, and only a few lipases are able to hydrolyze the sn-2 position to allow the complete conversion of triglycerides to free fatty acids. With regard to fatty acid selectivity, lipases are able to hydrolyze fats into medium- to long-chain molecules (C4–C18, rarely up to C22), but with different efficiencies. Lipolytic enzymes possessing different selectivity can therefore be used to obtain valuable products, such as structured triglycerides with improved nutritional value, as well as an impressive range of mono-acylglycerols, di-acylglycerols, tri-acylglycerols, fatty acids, esters, and intermediates (Bornscheuer 2000). The enzyme lipoxygenase hydrolyzes the dioxygenation of polyunsaturated fatty acids into hydroperoxy acids. Hydroperoxide lyase (HPL), common in plants, is mainly involved in the hydrolysis of fatty acid hydroperoxides into oxo-acids and aldehydes. Hydroxyperoxide isomerase catalyzes the conversion of hydroperoxylinoleic acid into α- or β-ketols.

5.6 Nucleic Acid-Hydrolyzing Enzymes and Other Hydrolyzing Enzymes

Nucleic acid-hydrolyzing enzymes, called nucleases, can be regarded as molecular scissors and hydrolyze the phosphodiester bonds between the nucleotide subunits of nucleic acids. These enzymes were previously called polynucleotidases or nucleodepolymerases. Nucleases can be classified into two main categories based on the site at which they cleave: endonucleases, which act within the strand, and exonucleases, which act at the terminal end of the strand. Some nucleases have the ability to cleave the phosphodiester bond at both terminal ends and in the middle of

the nucleotide chain, therefore falling into both categories. The most well-known, highly applicable, and well-characterized nucleases are deoxyribonuclease and ribonuclease. Exonucleases have the ability to start cleavage of the nucleotides chain from either the 5′ end or the 3′ end of the nucleotide strand, depending on the encoded information. The generalized mechanism of action of nucleases and their vital roles in DNA repair are summarized in Fig. 5a, b.

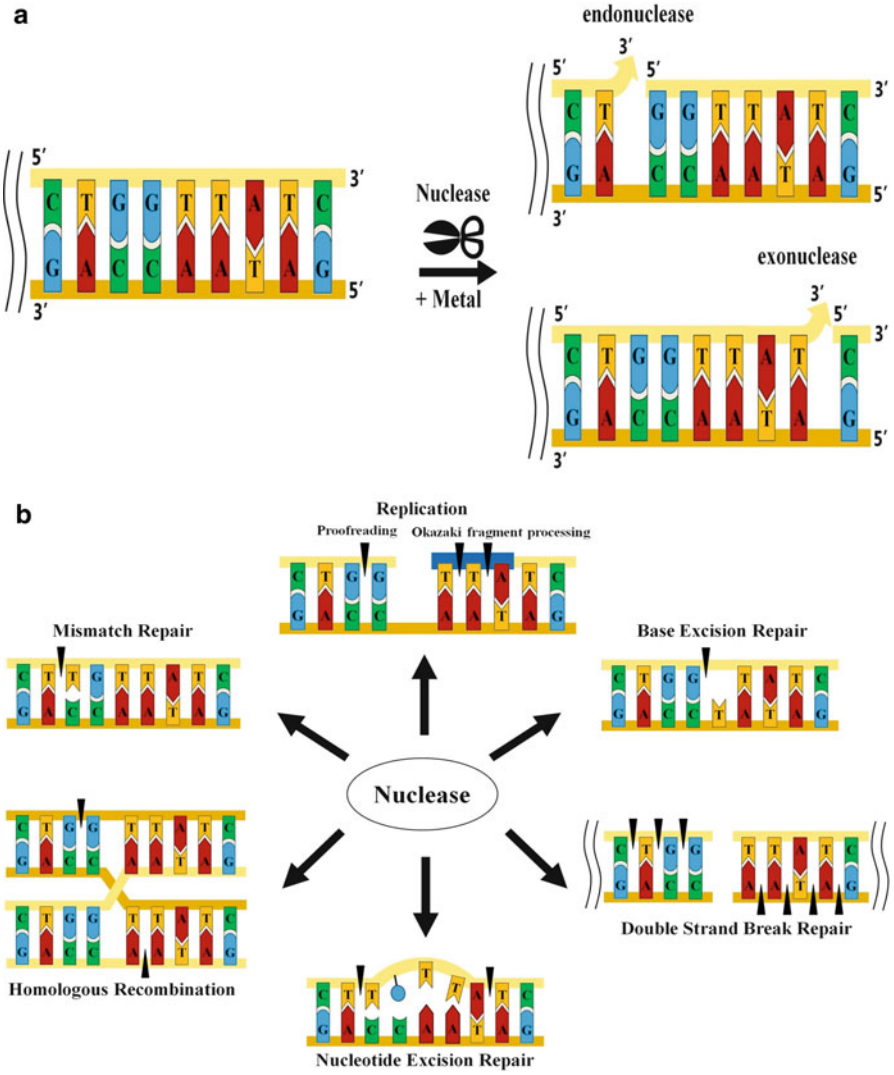

Fig. 5 Schematic diagram of the nuclease enzyme activity. The two strands of DNA are schematically drawn. (**a**) The cleavage made by the nuclease is represented by *arrows*. (**b**) The involvement of nuclease enzymes in the DNA repair system is summarized. Single-stranded region is shown in *blue* color

Beside cell wall-, starch-, protein-, and lipid-hydrolyzing enzymes, phytase, peroxidase, and thiol oxidase (Bamforth 2009) also have hydrolytic activity. Phytase hydrolyzes phytate into phytic acid, inorganic phosphate, and myoinositol (Khattak et al. 2012). Two types of phytases, 3- and 6-phytase, function to dephosphorylate phytate. 3-Phytase is produced by microbes, while 6-phytase is found in seeds of higher plants, including barley. Inorganic phosphate, a constituent of nucleotides, is also utilized by metabolic machinery during in vivo nucleotide synthesis (purines and pyramidines).

6 Applications of EHEs

Endogenous enzymes of various classes have multiple applications in day-to-day life. The major applications are in food, animal feed, textile, paper and pulp, fuel (energy), pharmaceutical, and chemical industries (Carvalho et al. 2003; Hsu and Lakhani 2002). EHEs also have numerous potential applications in advanced biosciences and engineering fields. Some of the potential applications of endogenous enzymes are described below.

6.1 Food Industry

Hydrolyzing enzymes are a vital part of various processes within the food industry. Their hydrolyzing activities are responsible for the formation of different products from the raw food materials. Starch-hydrolyzing enzymes play a significant role in the baking and brewing industries. Almost all hydrolyzing enzymes, such as α- and β-amylases, are found in raw food materials, e.g., flour and malt. Both of these amylases perform individual, but complementary, roles in food processing. Starch is initially broken down into low-molecular-weight dextrins by α-amylases; these dextrins are then further converted to maltose by β-amylase, and maltose is converted into whatever components are necessary for yeast growth (Khattak et al. 2012). Glucoamylases help to facilitate the breakdown of starches into simple fermentable sugars in the baking industry and have been shown to intensify the bread crust color through released glucose (Polaina and MacCabe 2007).

Xylanases hydrolyze hemicelluloses and are used, together with amylases, glucose oxidase, and proteases, in the bread-making process. Wheat flour contains hemicelluloses, which can be broken down to allow the redistribution of water, making the dough softer. Xylanases help to delay the formation of crumbs, thus increasing bread volume (Harbak and Thygesen 2002; Camacho and Aguilar 2003). Xylanase is recommended for making lighter cream crackers and improving the texture and regularity of the wafers. Currently, xylanases have been employed, together with cellulases, amylases, and pectinases, in order to enhance the yield of juice through the liquefaction of fruit and vegetables; achieve better recovery of aromas, important oils, mineral salts, edible dyes, and pigments; reduce viscosity;

and suppress breakdown of materials that obstruct the physiological features or chemical nature of the juice.

Cellulases have multiple applications in the food industry. For example, cellulases can be used for fruit juice and oil extraction. As mentioned earlier, along with other groups of enzymes, cellulases are used for the purification of fruits juices. An important feature of cellulases is their use in the isolation of proteins from soybeans and coconuts. The efficiency of starch isolation is increased with the activity of cellulases. Cellulases can digest ball-milled lignocelluloses, which are commonly used as food additives (Carvalho et al. 2003; Hsu and Lakhani 2002). Cellulases play a vital role in the hydrolysis of cellulosic waste, which results in the production of cello-oligosaccharides and fermentable sugars. These are essential for the digestion of cell wall components, which facilitates the release of flavor-related compounds, enzymes, and polysaccharides (Kuhad et al. 2011).

6.2 Brewing Industry

Enzymes have been used to make beverages since ancient times. Currently, the brewing industry relies on several groups of enzymes for preparing beverages using different feedstocks as substrate materials.

The traditional brewing process is mainly based on the germination and processing of barley grains. The germination period, which lasts about 7 days, involves such enzymes as α-amylase, β-amylase, and proteinases originating from barley grains. The germinated grains are then heated to develop color and flavor. These enzymes further hydrolyze the starches and proteins contained in crushed starchy cereals, resulting in the formation of simple sugars (e.g., mono- and oligosaccharides), which are fermented by yeast to produce ethanol. Amylolytic enzymes are also used for the development of low-molecular-weight carbohydrates (fermentable sugars) used by yeast cells for the production of alcoholic drinks.

Currently, a major focus in the brewing industry is immobilized enzyme technology, which can be used to effectively produce low-calorie beers and other beverages. Conventional fermentation systems produce high quantities of dextrins from starch hydrolysis, which are carried along to the final product. Immobilized glucoamylases are used to convert these dextrins to fermentable sugars for further fermentation. By passing the fermenting beer through a reactor containing immobilized glucosidase, these dextrins can be broken down into glucose, which is then almost completely transformed into alcohol. A great advantage of this technique is that the enzyme does not contaminate the product.

Glucanases obtained from microbes play a vital role in the fermentation processes of beers and wines (Singh et al. 2007; Bamforth 2009). The quality and yield of the fermented products is increased with the use of glucanases and related polysaccharides (Bamforth 2009; Bamforth et al. 2009). Enzymes including glucanase, pectinase, and hemicellulases improve the color extraction, clarification, quality, and stability of wine (Singh et al. 2007; Galante et al. 1998). Wine aroma is

also improved by β-glucosidase, which modifies glycosylated precursors. Currently, many enzymes are commercially available for use in the preparation of wine, contributing to the improvement of wine yield and quality. A number of improved enzymes, such as cellulase and pectinase, can be exogenously added during wine making in order to improve the productivity of accessible brewing processes in the near future (Bamforth 2009; Bamforth et al. 2009).

6.3 Feed Industry

The use of enzymes as feed additives is also well established. A number of enzymes from various enzyme classes are used in various applications in the feed industry. Production of feed stock using enzymes is currently an important part of agricultural business. Most of these types of enzymes are involved in the digestion of cellulose-based feeds.

Xylanases, together with certain other essential enzymes, including glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases, and lipases, are used in the animal feed industry. These enzymes hydrolyze the arabinoxylans contained in feed components and reduce the viscosities of raw materials (Twomey et al. 2003). The addition of xylanase to feed containing maize and sorghum improves the initial digestion of nutrients in the early part of the digestive tract, improving energy utilization. Furthermore, the combined action of the remaining enzymes mentioned above produce a well-digestible food mixture. Endogenous enzymes are produced in much smaller quantities in young fowl and swine as compared to adults. Therefore, their performance as livestock is improved by the added food supplements.

Cellulases are widely used as supplements in the feeds of ruminants and monogastric animals. Additionally, cellulases are used in the pretreatment of lignocellulosic materials and in the dehulling of cereal crops. Their activities influence the digestibility of feeds in monogastric animals and ruminants (Kuhad et al. 2011). Supplementation with cellulase in feedstocks enables dairy animals to consume more feed, resulting in increased milk production. The nutritional values of agricultural wastes and grains can be improved through cellulases and xylanases (Godfrey and West 1996). These enzymes can degrade certain feed constituents and provide additional digestive enzymes, such as proteases, amylases, and glucanases.

Monogastric animals generally cannot digest and utilize plant-based feeds containing cellulose and hemicelluloses. Certain enzymes, such as xylanases and glucanases, are used in cereal-based feeds for these animals, enabling them to digest these feedstocks. β-Glucanases and xylanases are also used in the feeds of monogastric animals to promote the hydrolysis of nonstarch polysaccharides, such as β-glucans and arabinoxylans. Glucanases and xylanases decrease the viscosity of high-fiber rye- and barley-based feeds in poultry and pigs. These enzymes are effective in causing weight gain in chickens and piglets by improving the digestion and absorption of feed materials (Singh et al. 2007; Bhat 2000).

Phytase is currently the most abundant enzyme used in the feed industry. Phytase is used in cereal feeds (containing natural phosphorous bound to phytic acid) consumed by monogastric animals. The addition of phytase to these feeds results in a significant reduction in phosphorus excretion from monogastric animals (Lei and Stahl 2000; Kies et al. 2001).

6.4 Pharmaceutics and Medical Applications

The health-promoting activities of certain enzymes, their derivatives, and combinations of enzymes have facilitated the investigation of the therapeutic potential of these enzymes. For example, certain enzymes have been shown to have antimicrobial effects and have therefore become key elements in various therapeutic drugs and materials. Some of the potential therapeutic applications of EHEs are described below, and additional applications of EHEs in this industry are available in the literature.

Enzymes can be administered orally or by injection for treatment of certain diseases. Sacrosidase (β-fructofuranoside fructohydrolase from *Saccharomyces cerevisiae*) is used in the treatment of congenital sucrase-isomaltase deficiency (CSID). In this disease, patients are unable to use the disaccharide sucrose. Drugs containing sacrosidase hydrolyze sucrose, thus allowing patients with CSID to consume a more normal diet (Treem et al. 1999). Certain pancreatic enzymes, including lipases, proteases, and amylases (in the form of a mixture), have been shown to be effective in the treatment of fat malabsorption in patients with human immunodeficiency virus (HIV) (Carroccio et al. 2001). Furthermore, the same enzymatic mixture has been successfully used to treat pancreatic insufficiency (Schibli et al. 2002).

Lysozyme is a naturally occurring bactericidal agent used in a variety of food and consumer materials. This enzyme has the ability to break down the carbohydrate chains in the bacterial cell wall, thereby prohibiting the growth of bacterial cells and preventing food poisoning and other maladies. Lysozyme has also been shown to possess activity against HIV.

Chitinases are another group of enzymes that possess natural bactericidal potential. Chitin is the basic structural unit of the cell wall in a variety of pathogenic microorganisms, such as protozoa, fungi, and helminths. Chitinases effectively target chitins and possess good antibacterial activities (Fusetti et al. 2002). Certain lytic enzymes derived from bacteriophages target the cell wall of *Streptococcus pneumonia*, *Bacillus anthracis*, and *Clostridium perfringens* (Zimmer et al. 2002). These enzymes have the potential to halt the growth and activities of drug-resistant bacterial strains.

Xylanase and xylan have a few potential applications in the pharmaceutical industry. Xylanases are sometimes added in combination with a complex of enzymes (hemicellulases, proteases, and others) to produce certain medicinal products. Hydrolytic products of xylan, such as β-D-xylopyranosyl residues, can be converted into combustible liquids (ethanol), solvents, and artificial low-calorie

sweeteners. The resulting sweeteners are noncarcinogenic and are suitable for diabetic and obese individuals. A variety of commercial products containing xylitol, such as chewing gum, can be found on the market.

7 Conclusion

EHEs are involved in almost every known biological process. With the advent of novel techniques of enzyme isolation and purification, their applicability is broadening and increasing. Current approaches toward developing cell-free lysates from lysed microbial cells and their isolation, concentration, and purification have generated a great amount of interest in the potential use of EHEs in various industrial applications. Newly developing biotechnological techniques are allowing the identification of various novel applications of purified EHEs, specifically in genetic engineering. Current methods using grouped enzyme approaches for process simplification are providing insight into the broad future applications of EHEs.

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Production of Pullulan by Microbial Fermentation

18

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Contents

Abstract

Pullulan is a microbial exopolysaccharide produced by polymorphic fungus *Aureobasidium pullulans*. Owing to its structure, consisting of α(1→4) and α(1→6) linkages, it has unique properties and hence is suitable for a variety of commercial applications in various field including food, pharmaceuticals, and biomedical. In this chapter, biosynthesis, production through microbial fermentation, and influence of fermentation conditions like pH, temperature, aeration, agitation on microbial growth, pullulan yield, pullulan purity, and molecular weight are discussed. Later, options of using various agricultural wastes as carbon source for pullulan production are considered.

Keywords

Aureobasidium pullulans • Pullulan • Exopolysaccharide • Morphology • Agricultural residues

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Abbreviations

ATP	Adenosine triphosphate
DE	Dextrose equivalent. It is a measure of reducing sugars present in starch hydrolysate. Carbohydrate molecules like starch are polymers of monosaccharide. The extent of polymerization is indirectly measured by DE. In starch hydrolysate DE represents the amount of reducing sugar as dextrose. Dextrose equivalent of starch is 100 and that of glucose/dextrose is 100. Starch hydrolysates with DE ranging between 1 and 13 are termed as dextrins and those with DE in the range of 13–20 are called maltodextrins. DE for glucose syrup is typically greater than 20, and commercial glucose syrup has a DE of 40–42.
UDPG	Uridine diphosphate glucose

1 Introduction

Pullulan is an extracellular α-linked glucan produced by the polymorphic fungus *Aureobasidium pullulans*. It is a linear α-D-glucan. It consists of maltotriose subunits interconnected by (1→6)-α-D-glucosidic linkages (Youssef et al. 1999; Oguzhan and Yangilar 2013). The glucose units in maltotriose are linked by an α-(1→4)-glycosidic bond. Though pullulan mainly consists of repeating units of maltotriose, at random maltotetraose subunits also do appear. The maximum content of maltotetraose is estimated to be 7 % (Singh et al. 2008). Structure of pullulan was first resolved in 1960s (Leathers 2003). The structure of pullulan (Fig. 1) produced by *A. pullulans* is independent of type of strain used (Shingel 2004).

Average molecular weight of pullulan depends on the type of culture used, media composition, and fermentation type. Typically it ranges from 5×10^4 to 4×10^6 (Heald and Kristiansen 1985). From the elemental analysis, chemical formula of pullulan is elucidated as $(C_6H_{10}O_5)_n$ (Cheng et al. 2011). Bioactivity of the polymers is significantly influenced by average molecular weight and molecular weight distribution (Cheng et al. 2011).

In 1938, Bauer observed the polysaccharide production by *Aureobasidium pullulans*, and it was first isolated and characterized by Bernier (1958) from culture broths of *A. pullulans* (Singh et al. 2008). *A. pullulans* is a common plant pathogen found everywhere on the earth. It is found in fresh- and seawater, soil, dead plants, textile, and wood (Moubasher et al. 2013; Shingel 2004). As *A. pullulans* produce melanin also, it is known as black yeast. Apart from *A. pullulans*, *Tremella mesenterica*, *Cyttaria harioti*, *Cyttaria darwinii*, *Cryphonectria parasitica*, *Teloschistes flavicans*, and *Rhodotorula bacarum* also produce pullulan (Oguzhan and Yangilar 2013). Commercial production of pullulan was first started by Hayashibara Company Limited, Japan, in 1976. Hayashibara continues to be the major supplier of pullulan (Farris et al. 2014). Pullulan film was first brought to the market in 1982 (Singh et al. 2008).

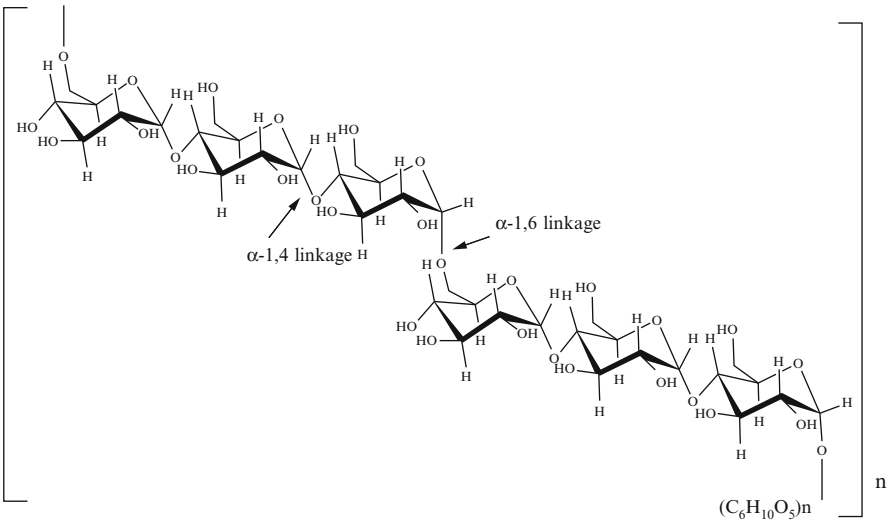

Fig. 1 Structure of pullulan

A. pullulans is an omnipresent organism. It was originally classified as Fungi Imperfecti under the family of Moniliales. Recently, it has been classified under ascomycetous yeast (Singh and Saini 2008). *A. pullulans* exists in five different morphologies. These are yeastlike cells, young blastospores, swollen blastospores, chlamydospores, and mycelia (Cheng et al. 2011; Oguzhan and Yangilar 2013). Though it has been widely accepted that pullulan yield depends on the morphology of the fungus, there is no consensus on which type of cell morphology is responsible for pullulan production (Cheng et al. 2011). Yeastlike cells (Heald and Kristiansen 1985), swollen cells (Campbell et al. 2004), and chlamydospores (Simon and Bouchonneau 1993; Singh et al. 2008) had been claimed to be the major pullulan producers by different group of scientists. Studies had shown that initial pH of the fermentation medium influenced the morphology of the organism and hence pullulan yield (Heald and Kristiansen 1985). It is also reported that higher fraction of hyphae (>3 %) results in poor pullulan yield (Simon and Bouchonneau 1993).

2 Physical Properties

Pullulan is soluble in water and insoluble in organic solvents excluding dimethylformamide and dimethyl sulfoxide (Oguzhan and Yangilar 2013;Youssef et al. 1999). It is nonhygroscopic in nature (Oguzhan and Yangilar 2013). It is edible, nontoxic, and noncarcinogenic. Its aqueous solutions are comparatively less viscous. Two distinct and useful properties, namely, structural flexibility and enhanced solubility, of pullulan result from regular alteration of 1→4 α and 1→6 α linkages (Gniewosz and Duszkiewicz-Reinhard 2008). It has good thermal stability, and it is stable up to 250 °C and decomposes only at temperature above

Table 1 Typical properties of pullulan

Parameter	Specification
Appearance	Whitish or yellowish white (Singh et al. 2008)
Solubility	Easily soluble in water; insoluble in organic solvents excluding dimethylformamide and dimethyl sulfoxide
Specific optical activity	More than +160° in 1 % solution
Molecular weight (kDa)	50–4,000
Polypeptides (%)	Maximum 0.5 %
Solution pH	5–7
Mineral residue ash (sulfated, %)	Maximum 3
Moisture (%)	Maximum 6 %
Taste and odor	Tasteless and odorless

250 °C. Some of the important properties of pullulan are listed below in Table 1 (Singh et al. 2008).

3 Biosynthesis/Elaboration of Pullulan

Polysaccharides are produced by the organisms for their self-protection. Pullulan produced by the *A. pullulans* form a highly hydrated layer around the cells in order to protect them from dehydration and from protozoans (Cheng et al. 2011). The mechanism of pullulan biosynthesis is not yet understood completely in spite of extensive research on this. Biosynthesis of pullulan occurs in cytosol and membrane and then it is secreted out forming a slimy layer on the cell surface.

Aureobasidium pullulans utilizes three enzymes, namely, α-phosphoglucose mutase, uridine diphosphate glucose pyrophosphorylase (UDPG-pyrophosphorylase), and glucosyltransferase, that are involved in the polymerization of glucose units during biosynthesis of pullulan (Cheng et al. 2011). Uridine diphosphate glucose (UDPG) is the main precursor in pullulan biosynthesis from glucose. Biosynthesis of pullulan is described here in two stages. The first stage is the synthesis of essential precursor and the second stage is polymerization of pullulan.

Figure 2 explains the synthesis of uridine diphosphate glucose in *A. pullulans*. For simplicity, the synthesis is explained using sucrose as example raw material. Sucrose is first hydrolyzed into monosaccharides by *A. pullulans*. Isomerase enzyme acts on hydrolyzed glucose units to convert it into its isomer (fructose). The fructose molecules are then phosphorylated by ATP into fructose 1-phosphate which is further converted into fructose 1,6-diphosphate by another ATP molecule. Fructose bisphosphatase enzyme then converts fructose 1,6-diphosphate to fructose 6-phosphate. Subsequently phosphoglucoisomerase converts fructose 6-phosphate into glucose 6-phosphate. Glucose 6-phosphate is transformed by α-phosphoglucose mutase into glucose 1-phosphate which is further acted by UDPG-pyrophosphorylase to convert it to UDP-glucose.

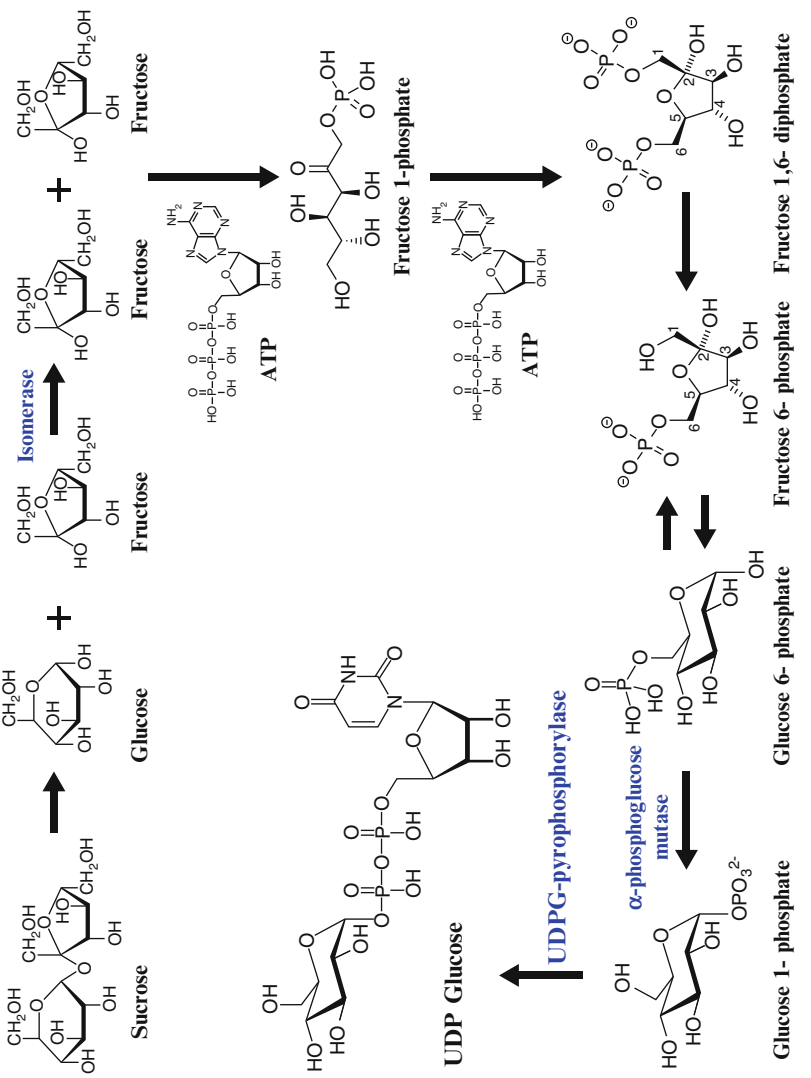

Fig. 2 Synthesis of uridine diphosphate glucose in *A. pullulans*

Polymerization of pullulan starts with the pentasaccharide from UDP-glucose molecule. Glucosyl 1-phosphate from UDP-glucose containing D glucose is attached to lipid hydroperoxide by forming a phosphoester bridge (Fig. 3). Consequently addition of other glucose subunits continues with other UDP-glucose releasing UDP. This initiates assembling of pullulan polymeric unit at α- 1, 6 carbon forming lipid-linked isomaltose unit. Chain elongation continues further with addition of glucose subunits forming isopanosyl precursor. The polymeric chain formation continues with α- 1, 4 maltotriose units linked together at α- 1, 6 position.

However, the biosynthesis of pullulan is not well understood till date. Further research on the metabolic pathway and pullulan formation will be required to increase yield and reduce cost of production of pullulan (Cheng et al. 2011; Shingel 2004).

4 Production of Pullulan

Nature of fungal strain, carbon (C) source, media composition, culture pH, fermentation temperature, dissolved oxygen concentration (Gniewosz and Duszkiewicz-Reinhard 2008), agitation rate (Lazaridou et al. 2002), and fermenter configuration influence pullulan yield (Campbell et al. 2003).

Color variant *A. pullulans* strains which do not produce melanin pigment can be used to improve pullulan yield and to simplify downstream processing steps (Singh et al. 2009). The melanin pigment produced by *A. pullulans* is black in color and has a characteristic absorption wavelength of 400–600 nm (Israilides et al. 1999). It is produced at the end of exponential phase. After released into the medium, they covalently bind to pullulan giving black-colored product. In order to reduce the overall cost of production and to improve the quality of the product, suppression of melanin pigment is very important. Several color variants had been reported in literature. A black yeast strain NG isolated from strawberry fruit produced pigment-free pullulan at pH 4.5 (Li et al. 2009). *A. pullulans* var. *melanogenium* P16 isolated from mangrove system produced pigment-free pullulan (Ma et al. 2014). *A. pullulans* RG-5 (Singh et al. 2012) and *A. pullulans* P 56 (Youssef et al. 1999) are few more examples of strains not producing melanin.

Aureobasidium pullulans produce pullulan from mono- or disaccharides like xylose, glucose, fructose, mannose, galactose, sucrose, and maltose (Thirumavalavan et al. 2009). Commercially pullulan is produced from starch hydrolysate containing 40–50 % dextrose equivalent (DE). The sugar concentration is maintained between 5 % and 15 %. Excess sugar concentration will inhibit the pullulan production (Cheng et al. 2010b; Goksungur et al. 2011). It is reported that excess sugars inhibit pullulan production by suppressing the enzymes like α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glycotransferase which are responsible for pullulan production. Thus, fed-batch reactor will better suit pullulan production.

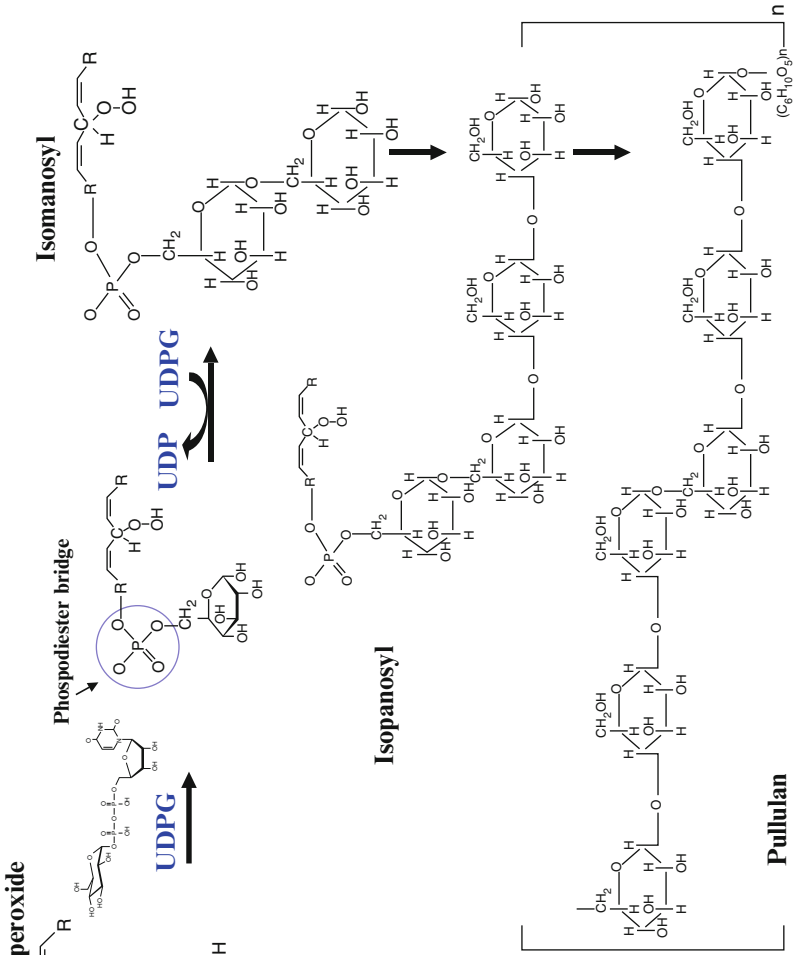

Fig. 3 Pullulan synthesis using UDP-glucose

Other micronutrients like peptone, phosphate, and basal salts are required in sufficient quantity for achieving maximum yield. Up to 70 % of starch hydrolysate used could be converted into pullulan if the fermentation conditions are maintained carefully (Kumar et al. 2012).

pH influences the morphology of the organism and in turn the pullulan yield. The pH of the medium is initially adjusted to 6.5, and during initial 24 h of fermentation, pH gradually reduces (Leathers 2003). Solution pH influences the morphology of *A. pullulans* and, in turn, morphology affects pullulan yield (Wu et al. 2009). At pH 2.5 mycelia form is observed, and at pH 4.5 yeastlike cells are obtained (Ji-Hyun et al. 2002). Optimum pH for pullulan production is between 5.5 and 7 (Wu et al. 2009). Optimum pH for biomass growth and polysaccharide production are different from this (Cheng et al. 2010a; Ji-Hyun et al. 2002). At lower pH biomass growth is high, but pullulan production is low (Israilides et al. 1999). Yeastlike morphology was maximum (60 %) at pH 6.3 while Asian palm kernel was used as solid substrate for *A. pullulans* and hence pullulan production was maximum (Sugumaran et al. 2013a). According to published reports, pullulan production was higher if the medium pH was allowed to drop naturally rather than maintained constant (Ji-Hyun et al. 2002; Sevious et al. 1992). If the pH is not controlled, fraction of high molecular weight of pullulan gradually decreases due to pullulanase activity (Ji-Hyun et al. 2002). However, if the pH goes below 2.5, it can cause severe acid stress and reduce pullulan yield (Wang et al. 2013). For maximum pullulan production, mild to moderate acid stress (pH 3.5–4.5) is preferential according to Wang et al. (2013). On the other hand, if pH is controlled at 6.5, fraction of high molecular weight pullulan is nearly constant (Ji-Hyun et al. 2002).

In addition to pH, nutritional level and dissolved oxygen also affect the morphology and hence pullulan yield (Li et al. 2009).

Temperature of the medium is to be maintained at 30 °C. Typically at 75 h the culture growth reaches a maximum. At the end of the logarithmic growth phase, when there is a depletion of nutrients, biosynthesis of pullulan begins. Maximum pullulan synthesis is obtained between 70 and 90 h (stationary phase) of cultivation. Nitrogen starvation favors pullulan synthesis. Depletion of ammonium ion concentration plays a major role in diversion of carbon source to polysaccharide production by suppressing biomass accumulation. However, *A. pullulans* also synthesizes pullulan-degrading enzyme (pullulanase) at later stages of growth. Thus, beyond stationary phase pullulan yield drops due to the action of pullulan-degrading enzymes (Campbell et al. 2003). Typically pullulan yield of about 70 % and above is achievable (Leathers 2003).

Often two-stage pH control or two stage pH and temperature control improves both cell growth and pullulan production as different conditions are required for cell growth and pullulan production. Low pH and high temperature are preferential during initial period, while relatively higher pH (~4–6.5) and low temperature will be required for pullulan production during the stationary phase later (Wang et al. 2013; Wu et al. 2010; Xia et al. 2011).

A. pullulans grows well under aerobic condition (Gniewosz and Duszkiewicz-Reinhard 2008). Aeration and agitation both stimulate the production pullulan (Survase et al. 2007). Agitation rate, impeller speed, and shear conditions influence the morphological changes of the culture and metabolite production and sometimes cause cell damage (Lazaridou et al. 2002). Strong aeration and agitation promotes yeastlike morphology which is more efficient in pullulan production. At low shear rate the amount of yeast cell is more and hence pullulan production (Wecker and Onken 1991). As the polymer accumulates, the broth becomes non-Newtonian and becomes highly viscous. This leads to increased demand for oxygen. A combination of low shear rate and low dissolved oxygen level maximizes pullulan yield (Sevious et al. 1992; Wecker and Onken 1991).

Addition of uracil is reported to improve pullulan yield. Both the time of addition and amount of uracil added affect the pullulan yield. Uracil added at zeroth hour of fermentation was utilized as a nitrogen source by the organism to stimulate the cell growth. When added after 48 h, uracil promotes pullulan production by 32 % (Sheng et al. 2014). Uracil enhances pullulan production by acting as a precursor for UDP-glucose which plays a vital role in the biosynthesis of pullulan (Sheng et al. 2014).

5 Reactors

Pullulan can be produced in batch reactor. As discussed earlier, pullulan is produced during the stationary phase and under nitrogen-limiting conditions. As excess sugar concentration can inhibit/decrease pullulan production, fed-batch fermentation is preferred over batch reactors. However, reports indicate that upon prolonged fermentation, say 7th day, further feed addition did not improve pullulan yield (Cheng et al. 2011). Continuous pullulan production is preferred for large-scale commercial production. At low dilution rate pullulan yield is increased in continuous fermenters. In order to improve the yield, various other designs of reactors had been proposed by several researchers.

Cheng et al. (2010b) had demonstrated that biofilm reactor enhances pullulan production. In this reactor 12 plastic composite tubes were employed. They were tied to the agitator of the reactor as shown in the figure. Plastic composite support employed was made of mixture of polypropylene and nutritious compounds. According to the authors, moderate leaching of nitrogen from the support maintains nitrogen level low in the reactor, favoring pullulan production, and no further addition of nitrogen is required. Another advantage of using plastic composite support, as reported by the authors, is that the cells were maintained in the stationary phase and hence the higher pullulan yield (Cheng et al. 2010b).

During the cell growth, the viscosity of the medium gradually increases. Typically in a stirred tank reactor, as the culture reaches its exponential phase, viscosity gradient appears in the radial direction. Near the agitator viscosity is lowest and near the wall it is highest (Gaidhani et al. 2003). Thus, the shear rate is maximum

near the impeller and minimum near the walls. As viscosity increases, both oxygen transfer and mixing become more difficult. This results in the formation of stagnant zones and impeller flooding in continuous stirred tank reactors. Since both oxygen transfer and mixing are influential factors of pullulan production, reactor systems with better oxygen transfer and mixing are preferential. Oscillatory baffled reactor developed by Gaidhani et al. (2005) overcomes these bottlenecks. It ensures uniform mixing with significantly lower shear strain rates. Up to 75 % higher mass transfer coefficients as compared to conventional stirred tank reactors are achievable in this reactor (Gaidhani et al. 2005). Uniform bubble size and enhanced gas holdup are considered to be responsible for improved mass transfer (Gaidhani et al. 2005).

Airlift reactor can also be used for pullulan production. In airlift reactor height to diameter ratio is high. Thus, process control is better and easy when compared with conventional reactor.

Recently, solid-state fermentation is gaining importance in bioconversion processes. Solid-state fermenters are superior to submerged fermenters as they consume less energy and less power. They are suitable for bioconversion of solid wastes into value-added products. However, there could be operational difficulties like heterogeneity in the reactor and heat and mass transfer limitations.

6 Downstream Processing

Typically purification of pullulan would require removal of cells from the culture, removal of melanin from the culture, removal of melanin pigment from the broth, precipitation of pullulan by addition of suitable solvents, resuspension of the precipitate, and final purification by ultrafiltration/chromatography.

Cell biomass can be removed by centrifugation or by cross-flow filtration (Cheng et al. 2011). The melanin pigment can be removed by activated carbon adsorption or by using a mixture of salt and alcohol (Singh et al. 2008). As pullulan is soluble in water and insoluble in most of the organic solvents, the product can be recovered from the purified supernatant by adding sufficient amount of organic solvent. Isopropyl alcohol, acetone, and ethanol are frequently employed. Upon addition of organic solvent addition, the polysaccharides begin to precipitate. The above two steps are repeated to achieve better purification. To further purify the product, pullulan solution is subjected to ultrafiltration and/or ion exchange (Kumar et al. 2012). Purified pullulan can be precipitated, dried, and mechanically ground to powder form (Cheng et al. 2011).

7 Pullulan Production from Agricultural Wastes

As stated earlier, *A. pullulans* utilize sugars like sucrose, glucose, fructose, and maltose and produce pullulan (Thirumavalavan et al. 2009). Alternatively, pullulan can also be produced from agricultural wastes like Asian palm kernel

(Sugumaran et al. 2013a), sweet potato (Wu et al. 2009), grape skin pulp (Israilides et al. 1998), hydrolyzed potato starch waste (Goksungur et al. 2011; Israilides et al. 1998), deoiled Jatropha seedcake (Choudhury et al. 2012), coconut by-products (Thirumavalavan et al. 2009), beet hydrolysate (Leduy and Boa 1977), deproteinized whey (Roukas 1999), carob pod (Roukas and Biliaderis 1995), jackfruit seed (Sugumaran et al. 2013b), etc. Pullulan yield obtained from several agricultural wastes under different formation conditions is listed below in Table 2.

Sweet potato is rich in starch content. As starch cannot be directly utilized by *A. pullulans*, saccharification pretreatment is necessary. However, since sweet potato contains β-amylase enzyme in it, external addition of saccharifying enzyme is often not required. Hydrolyzed sweet potato with a DE of 45 (containing approximately 54 % of maltose) yields 28.57 g/L (Wu et al. 2009). However, higher dextrose equivalent actually reduces pullulan yield. This is because of the fact that pullulan synthesis depends on the maltose concentration and not on glucose concentration (Wu et al. 2009).

While producing pullulan from agrowastes, the composition of saccharified intermediate affects pullulan yield. Treatment with more than one enzyme is often very useful to improve pullulan yield. While producing pullulan from hydrolyzed potato starch waste, Barnett et al. (1999) obtained maximum yield by using a two-step enzyme treatment (Barnett et al. 1999). In the first step, the starch waste was liquefied with α-amylase enzyme. For liquefaction, α-amylase was added to the starch waste, and the mixture is maintained at 65 °C and pH 6.5 for 30 min. Later the mixture was maintained at 90 °C for 2 h to complete liquefaction. Later in the second step, saccharification of the waste is carried out using an enzyme mixture consisting of pullulanase and β-amylase/amyloglucosidase. Saccharification with amyloglucosidase results in glucose-rich hydrolysate. Thus, extended saccharification to yield higher dextrose equivalent did not improve pullulan yield. On the other hand, when β-amylase was used for saccharification, maltose sugars were obtained as end product. Thus, extended period of saccharification yielded more maltose and ultimately resulted in higher pullulan yield (Barnett et al. 1999).

Israilides et al. (1998) reported that supplementation of NH_4NO_3 and K_2HPO_4 reportedly improves pullulan yield obtained from hydrolyzed potato starch from 12.9 to 30.8 g L^{-1} (Israilides et al. 1998).

Beet molasses is a sugar industry waste which is rich in sugar content. Typical beet molasses contain approximately 50 % sucrose. Thus, it is a potential substrate for pullulan production. However, it also contains high concentration of heavy metals like zinc, copper, magnesium, etc., which may inhibit pullulan production (Roukas 1998). Heavy metals can be removed by ion exchange to keep them below inhibitory concentration. Sulfuric acid pretreatment can increase polysaccharide concentration and yield by 79 % and 73 %, respectively.

Carob pod is another sugar-rich agrowaste. It contains 40–50 % sugars like glucose, fructose, sucrose, and maltose. Apart from sugar, it contains 7 % cellulose,

Table 2 Comparison of pullulan production from various agrowastes

Agrowaste	Strain	Polysaccharide concentration	Fermentation type	References
Coconut water	*A. pullulans*	38.3 g/L	Submerged fermentation	Thirumavalavan et al. 2009
Coconut milk	*A. pullulans*	58.0	Submerged fermentation	Thirumavalavan et al. 2009
Sweet potato	*A. pullulans* AP329	29.35	Submerged fermentation	Wu et al. 2009
Asian palm kernel	*A. pullulans* MTCC2670	18.43	Solid-state fermentation	Sugumaran et al. 2013a
Grape skin pulp extract	*A. pullulans* NRRLY 6220	22.3	Submerged fermentation	Israilides et al. 1998
Hydrolyzed potato starch	*A. pullulans* NRRLY 6220	30.8	Submerged fermentation	Israilides et al. 1998
Hydrolyzed potato starch	*A. pullulans* NRRLY 6220	58	Submerged fermentation	Barnett et al. 1999
Hydrolyzed potato starch	*A. pullulans* P56	19.2	Submerged fermentation	Goksungur et al. 2011
Deoiled Jatropha seedcake	*A. pullulans* RBF 4A3	83.98		Choudhury et al. 2012
Mixture of deoiled Jatropha seedcake, jaggery, and corn steep liquor	*A. pullulans* RBF 4A3	66.25	Submerged	Mehta et al. 2014
Beet hydrolysate	*A. pullulans* 2552, 140B and 142	12–14	Submerged	Leduy and Boa 1977
Cassava starch residue	*A. pullulans MTTC 1991*	27.5	SSF	Ray and Moorthy 2007
Beet molasses	*A. pullulans* P56	32	Submerged	Roukas 1998
Deproteinized whey	*A. pullulans P56*	11	Submerged	Roukas 1999
Carob pod	*A. pullulans* SU No 18	6.5	Submerged	Roukas and Biliaderis 1995
Sucrose + soybean oil	*A. pullulans NRRL Y-2311-1*, *A. pullulans NRRL Y-6220*	19–30	Submerged	Sena et al. 2006
Jackfruit seed	*A. pullulans* NCIM 1049	34.22 g/L	SSF	Sugumaran et al. 2013b

5 % hemicellulose, 3–4 % protein, 1–2 % pectin, etc. It may contain up to 20 % phenolic components. The kibbles obtained from carob pod after removing the seeds need to be comminuted. Sugars can be extracted from the comminuted kibbles by mixing with sufficient quantity of water. Later the sugars can be used in fermentation (Roukas and Biliaderis 1995).

Asian palm kernel is a potential feedstock for the production of pullulan. *A. pullulans* grown on solid mass of Asian palm kernel produced 18.3 g/L of pullulan (Sugumaran et al. 2013a).

Pullulan could also be produced from a mixture of deoiled Jatropha seedcake, corn steep liquor, and jaggery without addition of any further nutrients. The yield was reported as 62.25 g/L under optimized condition (Mehta et al. 2014).

Cassava is used for the commercial production of starch. Cassava residue contains up to 60 % of starch and can be effectively utilized for the production of various fermentation products. It serves as potential feedstock for the production of pullulan in solid-state fermentation (Ray and Moorthy 2007).

Sena et al. (2006) had shown that supplementation of sucrose medium with soybean oil had enhanced pullulan yield. They had achieved 25–50 % increase in pullulan yield through the addition of soybean oil (Sena et al. 2006). Similarly, supplementation of sucrose with olive oil had also reportedly improved pullulan yield (Youssef et al. 1998).

8 Applications of Pullulan

By virtue of unique structure consisting of regular alteration of α (1→4) and α (1→6) linkages, pullulan exhibits distinctive properties (Cheng et al. 2010c; Oguzhan and Yangilar 2013). Owing to its unique properties, pullulan can be used in numerous applications in food and pharmaceutical industries (Oguzhan and Yangilar 2013). It can be used as low-calorie viscosity enhancer in the food and blood plasma substitutes (Cheng et al. 2010c). It is used as starch substitute in low-calorie formulations (Gaidhani et al. 2005). It is also used as low-viscosity filler in some beverages (Leathers 2003). Pullulan functions as a prebiotic by promoting beneficial bifidobacteria growth. Thus, it can be used as a dietary fiber in humans and rats (Leathers 2003).

It has the ability to form thin fibers and thin films. Pullulan films are transparent, strong, and impermeable to oxygen, oil, and grease (Leathers 2003; Sevious et al. 1992). As the pullulan film is oxygen impermeable, it can be employed as food protective coating to protect flavor and aroma and to improve the shell life (Israilides et al. 1999). Commercial use of pullulan film is very common in various breath freshener or oral hygiene products.

9 Conclusion/Prospects

Pullulan is a commercially important microbial exopolysaccharide produced by polymorphic fungus *Aureobasidium pullulans*. Biosynthesis of pullulan involves UDPG and glucose residues. Microbial fermentation is influenced by fermentation conditions like pH, temperature, aeration, agitation on microbial growth, pullulan yield, pullulan purity, and molecular weight. In order to reduce the raw material cost, pullulan can be produced from several agricultural

wastes after proper pretreatment. Through careful control of operating conditions and choice of reactor, pullulan yield and molecular weight can be controlled.

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Biosynthesis of Extracellular Matrix Components, Glycosaminoglycans, Proteoglycans, Collagens, Elastin and Structural Glycoproteins

19

Leslie Robert

Contents

Abstract

Most polysaccharides of higher vertebrates are in the extracellular matrix (connective tissues) either free or bound to proteins. Their composition and biological role will be described in decreasing order of their polysaccharide content.

Keywords

Extracellular matrix • Hyaluronan • Proteoglycans • Glycosylated collagens • Structural or matrix glycoproteins

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1 Introduction

Extracellular matrix (ECM) is the macromolecular component of connective tissues (CT), which comprise also the cellular elements, essential fibroblasts, and some other cell types which produce ECM rich in carbohydrates, unevenly distributed among its macromolecular components: glycosaminoglycans (GAGs), proteoglycans (PGs), structural or matrix glycoproteins (SGPs), collagens, and elastin. All four types of macromolecular components play essential roles in tissue structure and function to be described in the next sections, in decreasing order of the importance of their carbohydrate components. The biological role of ECM components depends on their chemical composition, their quality, quantity, and type of linkage of carbohydrates. Several of these macromolecules are used since immemorial times (as collagen for leather and for other industrial purposes). The emphasis is on vertebrate – mammalian tissues, first of all human connective tissues. We have to remind here that polysaccharides and carbohydrate-rich macromolecules appeared early during evolution, from prokaryotes, bacteria, algae, etc., both in the plant and animal kingdoms. The best known among the plant polysaccharides is cellulose, a polymer of glucose, of industrial use since antiquity. These polysaccharides were also intensely studied and will be mentioned in other chapters of this book.

2 Connective Tissue Polysaccharides

Hyaluronic acid, or better hyaluronan as called by scientists specialized in its study, is a high molecular weight polysaccharide composed of alternative units of a hexosamine (*N*-acetylglucosamine) and a uronic acid (D-glucuronic acid). Its very long chains can reach several millions of molecular weight (Balazs 1970). Its most interesting and unique properties are a high capacity to retain water and viscoelasticity. These properties are closely linked. It is the high water content which confers to this polysaccharide its remarkable viscoelasticity. This quality determines also its physiological roles in tissues. Young skin is rich in hyaluronan and confers to skin elasticity and hydration, component together with resistance to pressure. With age, the hyaluronan content of the skin decreases, with it decreases also the hydration of the skin. This contributes to the loose, wrinkly aspect the skin takes on old people. The long hyaluronan polysaccharide chains are susceptible to degradation by enzymes, called hyaluronidases, and also by free radicals (or better reactive oxygen species, abbreviated as ROSs, comprising also hydrogen peroxide (H_2O_2)). A simple experiment to demonstrate this physiologically important property of hyaluronan is to add to its solution a small amount of vitamin C (ascorbic acid) with some ferrous iron (Fe^{++}) to see the viscosity of the polysaccharide to decrease rapidly – measured with a viscosimeter – as a result of the rapid degradation of the polysaccharide by free radicals generated by this reagent. A testicular extract, rich in hyaluronidase, will exhibit the same effect. Both processes, degradation by ROSs and by hyaluronidases, are of great biological

importance. Because of its exceptional physicochemical properties, hyaluronan is industrially produced for medical purposes as, for instance, injection in arthritic articulations (as, for instance, the knee joint) for lubrication. A cross-linked hyaluronan preparation is used to fill wrinkles on the face. High molecular weight hyaluronan is used also in eye surgery. Injected before opening the eyeball, it will prevent the dislocation of intraocular structures during the surgical intervention. The inventor of this method, E.A. Balazs in the USA, called this type of surgery under the protection of hyaluronan "viscosurgery."

3 Polysaccharides of Proteoglycans

Proteoglycans (PGs) are also important macromolecular components of connective tissues. They are composed of a protein backbone carrying from one to hundreds of polysaccharide chains, called, according to their composition, chondroitin sulfates or dermatan sulfates. They are composed, as hyaluronan, of alternating units of a hexosamine and uronic acid, with one exception, keratan sulfate to be described later. There are several types of chondroitin sulfates: chondroitin A sulfate or better chondroitin 4-sulfate is composed of a succession of alternating hexosamines and uronic acids, different from those in hyaluronan. Chondroitin 4-sulfate is composed of an acetylgalactosamine-4-sulfate linked to 2-sulfated glucuronic acid. Chondroitin 6-sulfate is similar, but its *N*-acetylgalactosamine components are sulfated on position 6 as the only difference. Dermatan sulfate, another component of proteoglycans, is a polysaccharide chain composed of 4-sulfated *N*-acetylglucosamine linked to iduronic acid which can be also sulfated on position 2 of its ring, but not all iduronic acids in the polysaccharide chains are sulfated. Keratan sulfate, mentioned above, is a different polysaccharide, differing from the chondroitin and dermatan sulfates by the absence of uronic acid. In its chains, *N*-acetylglucosamine is 6-sulfated and linked to a unit of galactose (an isomer of glucose), being therefore different from chondroitin or dermatan sulfates, all containing uronic acids.

4 Heparin and Heparan Sulfates

These polysaccharides differ both in composition and localization from chondroitin or dermatan sulfates. Their hexosamine component is glucosamine which can be acetylated and/or *N*-sulfated, linked to an iduronic acid which also can be 2-sulfated. These polysaccharide chains are located on cell membranes where they play important roles in "helping" the fixation of growth factors enhancing cell proliferation. Heparin is well known for its anticoagulant capacity, used in medicine.

In the proteoglycans, these polysaccharide chains are mostly linked by an O-glycosidic linkage to the OH – group of the amino acids serine and threonine. Some polysaccharides are however linked to asparagine residues by an N-glycosidic linkage of the hexosamine component of the polysaccharide.

When bound to the central protein of proteoglycans, these polysaccharide chains are sticking out laterally and interact with collagen fibers, oriented parallel to the protein part of the proteoglycans. These interactions consolidate the orientation of collagen fibers as well as the construction of fiber bundles, important for the solidity of tissues.

5 Matrix or Structural Glycoproteins (SGPs)

Glycoproteins were first isolated from blood plasma or serum. Most plasma proteins are glycated, with the exception of serum albumin which is not, at least in bovine and human blood. The glycan content and composition are variable in quantity – from a few % to 20 % or more of the molecular weight – as well as in quality. For most, but not all, glycoproteins, the glycan chains are linked to the protein by an N-glycosidic linkage. The usual monosaccharide constituents are glucosamine, mannose, galactose, fucose, and sialic acid, at least in vertebrates and in human plasma. The detailed structure of these glycan chains was elucidated and described in great detail, among others by Jean Montreuil in Lille. The interest in blood glycoproteins increased considerably when it was demonstrated that some of them, as orosomucoid or better α_1-acid glycoprotein as well as haptoglobin, increase in blood during inflammatory processes. For this reason, determinations were described and widely used in hospital laboratories to follow the course of such diseases. One of the precursors of this medical research, M.F. Jayle at the Paris Medical School, who discovered haptoglobin – a glycoprotein forming a 1 to 1 complex with hemoglobin – proposed that connective tissues, actively participating in the inflammatory process, are the source of the glycans attached to inflammatory glycoproteins. Although this hypothesis could not be confirmed – most circulating glycoproteins are elaborated in the liver – we started to look for similar glycoproteins in connective tissues. Using an avascular tissue, cornea, we could show that it contains and synthetizes glycoproteins, similar in composition to circulating glycoproteins (Labat-Robert et al. 1986). Their glycan portion was of a comparable importance (of ~12 %) compared to circulating glycoproteins. The composition of the glycan chain was also similar; for the cornea preparation, it contained mannose, galactose, glucosamine, fucose, and sialic acid, and the only difference with plasma glycoproteins is that it was sulfated. To distinguish circulating glycoproteins from tissue glycoproteins, we called them structural glycoproteins (SGPs). We also demonstrated, using radioisotope-labeled precursors, that SGPs, present in all tissues investigated, were synthesized locally by connective tissue cells, fibroblasts, and others. A large number of such tissue-originated glycoproteins were described, forming a large part of the matrisome, the molecular component of ECM. The most studied component of these tissues – glycoproteins – is fibronectin (FN), which acts as a molecular linkage fastening cells to the surrounding ECM. Only adherent cells survive and detached cells die by apoptosis, a sort of cellular suicide. FN links ECM components to cells by interacting with cell receptors called integrins (Hynes and Yamada 2012). This adhesion process enables

cells to exchange "informations" with ECM components and is also "informed" of the behavior and fate of ECM components. This exchange of information, called "inside-out" and "outside-in" by R.O. Hynes, who discovered integrins, is of crucial importance for the homeostasis (normal physiology) of tissues.

6 Collagens

Collagens are quantitatively the most important components of ECM. They appeared with the sponges during the "Cambrian explosion"; the original, ancestral gene coding for the typical collagen sequences X–Y–glycine, where X and Y are often proline or hydroxyproline, was demonstrated in fungi (mushrooms) and even in some large viruses. Collagen molecules are composed of three peptide chains of the above composition, forming a triple helix. These peptide chains contain lysine and hydroxylysine residues. Some of the hydroxylysine residues are glycosylated, containing O-linked glucose or galactosyl-glucose residues. Therefore, collagen molecules are also part of the glycoprotein family. There are a number of different collagen types coded by different genes and differing also as far as their glycan content is concerned. Among the collagen types in vertebrates, only type VI is rich in glycans; an important part of this molecule has the typical composition of a glycoprotein, quite different from collagen composition. Therefore, it can be considered as a structural glycoprotein. Some tissues, as the cornea, are rich in type VI collagen (about 20 %), suggesting an important structural role for this molecule. As a matter of fact, the perfectly regular arrangement of collagen fibers is the condition of corneal transparency. This regular arrangement is created by the interaction of collagen type VI and proteoglycans of the cornea during its development in the embryo.

7 Elastin

As stated above, elastin is the only nonglycated macromolecule of the ECM. Its subunit, tropoelastin (TE), is not glycated and remains unglycated after its cross-linking by the formation of specific cross-link – amino acids, desmosine and isodesmosine, catalyzed by an enzyme called lysyl oxidase (LOX), as elucidated by Miles Partridge in GB. In order to form elastic fibers in tissues as the large, "elastic" blood vessels, elastin must interact with "microfibrils." These are composed of a number of SGPs, among them the fibrillins, which all are glycated. Such microfibrils (MFs) were shown to be present also in tissues, except elastin, and were present at earlier stages of phylogenesis, before the appearance of elastin with the vertebrates. Microfibrils, rich in fibrillins, replace elastin in invertebrate tissues. With aging, and even more during the development of the atherosclerotic process, the elastic fibers are degraded by elastolytic enzymes, elastases, liberating elastin peptides in the blood circulation reaching an average concentration in human blood serum of about 10 μg/ml. This is not a neutral process. The liberated elastin peptides

interact with an elastin receptor on cell membranes, triggering a series of reactions, some of which are harmful, as the release of more elastases and free radicals. This is a vicious circle, playing an important role in tissue aging.

Finally, let us say a few words on the biological importance of glycoconjugates, in particular of their glycan portion. As they exhibit specific carbohydrate sequences with their stereospecific conformations, they can specifically interact with complementary surfaces on other macromolecules and especially with lectins. These are proteins possessing stereospecific conformations enabling them to interact specifically with some carbohydrates. One example is the class of galectins specific for galactose end groups on glycan chains. A component of the elastin receptor acts as a galectin. Lectins are widespread in plants; also, the best known is concanavalin which found some practical applications in biochemistry. Glycan chains on most if not all glycoconjugates interact with stereo-compatible conformations on other molecules to fulfill their biological roles.

8 Conclusions

Oligo- and polysaccharides attached to proteins (glycoproteins) formed an important family of biological macromolecules. Most, but no all, proteins of the body are glycoproteins. The glycan chains, of specific composition, impart characteristic properties to glycoproteins and define their biological roles. Glycoproteins are present in tissues as well as in the blood circulation. Another family of polysaccharides, hyaluronan, and protein–polysaccharide complexes, proteoglycans, are important tissue components especially in connective tissues, designated as the Matrisome. They fulfill specific functions of vital importance for the body.

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Polysaccharides from Bioagro-Waste for New Biomolecules

20

Paola Di Donato, Annarita Poli, Valentina Taurisano, and Barbara Nicolaus

Contents

Abstract

Polysaccharides are the main chemical components of several kinds of agro-wastes including food processing residues and agricultural wastes. These types of biomass are produced in huge amounts every year and therefore represent a global environmental issue. The exploitation of agro-wastes as rich sources of valuable polysaccharides, according to the biorefinery approach, could afford both a strategy for waste minimizing and a more sustainable production of energy and chemicals. As a matter of fact, indeed polysaccharides are traditionally used in a wide range of production chains, for example, in food, pharmaceutical, material, or packaging industries.

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In this chapter, besides a brief overview of more abundant agro-wastes produced in the world and of their annual production, some remarkable examples of already implemented or possible biotechnological uses of waste polysaccharides are presented.

Keywords
Boimass • Biopolymers • Biorefinery • Bioagro-waste • Biomaterial

Abbreviations

AGs	Arabinogalactans
AGXs	Arabinoglucuronoxylans
Ara*f*	Arabinofuranose
AXs	Arabinoxylans
Fru*f*	Fructofuranose
GalA*p*	Galactopyruronic acid
Gal*p*	Galactopyranose
GaMs	Galactomannans
GAXs	Glucuronoarabinoxylans
GGMs	Galactoglucomannans
Glc*p*	Glucopyranose
GMs	Glucomannans
GXs	Glucuronoxylans
HG	Homogalacturonan
Man*p*	Mannopyranose
MeGlcA	4-*O*-methyl-α-D-glucopyranosyl uronic acid
RG	Rhamnogalacturonan
Rha*p*	Rhamnopyranose
Suc	Sucrose
XGs	Xyloglucans
Xyl*p*	Xylopyranose

1 Polysaccharides from Bioagro-Wastes: A Sustainable Source of Chemicals and Energy

Polysaccharides' market is continuously increasing because of the wide range of potential applications of this class of natural polymers that indeed, as such or by means of chemical and/or biological transformations, can constitute the main feedstock in many industrial activities such as food, materials, chemicals, and energy production.

Polysaccharides are produced by animals, microorganisms, and plants, but the latter are the main source of these biomolecules: indeed about 90 % of total natural

polysaccharides produced on Earth can be found in the vegetables. Polysaccharides are the main constituents of vegetable biomass that, in turn, is currently exploited for the production of chemicals, materials, and energy: many examples of biorefinery facilities (a new production system resembling the petroleum refinery for the production of chemicals and fuels, but that is based on renewable feedstock like vegetables) can be found such as corn or sugarcane refineries that produce food and biofuel.

Notably polysaccharides are massively processed for different production chains; they are indeed the sources of the sugars that in turn are the starting materials for the production of those chemical compounds that have been included in the list of the so-called "building block" molecules, according to the National Renewable Energy Laboratory (NREL) of the United States. These "building block" substances (i.e., succinic, fumaric, and malic acids; 2,5-furandicarboxylic acid, 3-hydroxypropionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol, and xylitol/arabinitol) are fundamental feedstock for the production of secondary and intermediate chemicals that are then used in several industrial sectors such as cosmetic, pharmaceutical, material, food, transportation, industry, etc. (Pacific Northwest National Laboratory 2004).

For these reasons in the last years, an increasing demand of plant polysaccharide-derived goods (energy and foods) has caused an increase in the intensity of acreage, agricultural production, and agro-industrial activities that in turn generate million tons of wastes. Such wastes, i.e., bioagro-wastes, comprise all the residues produced from horticulture and/or agricultural activities during cultivation, postharvest, and processing of plants. Indeed all the different phases of manufacturing of vegetable materials (e.g., selection of fruits and vegetables for the whole market or food industries, industrial processing of crops for the production of chemicals, energy, food, and so on) produce every year huge amounts of highly heterogeneous residues. Depending on the feedstock and on the production chain considered, such waste biomass is constituted by unemployed parts of plants, like roots, straw, leaves, cobs, etc., or by vegetable transformation residues like exhausted pulps, peels, and seeds, etc.

The proper disposal of such residual biomass represents a critical environmental concern and an economical problem that all agro-industries have to face with. Nonetheless, since bioagro-wastes still contain a variety of value-added chemicals, they could be considered as starting material for other production chains rather than residual matter. Indeed significant amounts of proteins, polysaccharides and fibers, polyphenols, carotenoids, fatty acids, etc., that are lost in the discarded materials could be used for the production of a variety of goods like biomaterials, food additives, nutraceuticals, antioxidant and antimicrobial agents, bioenergy, etc. The reuse and then the valorization of bioagro-wastes could be implemented in the frame of an integrated biorefinery approach to biomass exploitation, in which the exploitation of full plant feedstock could be a solution to the issues of sustainability and of waste disposal.

Therefore, on these bases, vegetable biomass like bioagro-wastes is under investigation as sources of biologically and biotechnologically useful polysaccharides (Sanchez-Vazquez et al. 2013).

In the next sections, after a brief overview of main plants' polysaccharide structures, some of the principal agro-waste sources of polysaccharides and the most diffused and promising biotechnological applications of such biomolecules are discussed.

2 Polysaccharides of Vegetable Origin: Chemical Structure and Classification

Polysaccharides are carbohydrate polymers made up of different monosaccharide units and are mainly located in plants that indeed produce more than 90 % of the total polysaccharides on Earth.

Generally referred to also as "glycans," polysaccharides are very complex polymers: indeed they can be defined as "polydisperse" molecules (due to the wide range of molecular weights they can have) and as "polymolecular" since they exhibit different fine structures, depending on their natural source.

Their classification can be made on the basis of chemical and structural features or depending on their biological role.

On the basis of structural criteria, the following groups can be distinguished:

- Homoglycans (made up of one type of monomer unit) and heteroglycans (made up of two or more types of monomer units)
- Linear and branched (with different degrees of branching, i.e., with few and very long branches regularly or irregularly spaced, with short branches grouped to form clusters, or with branch-on-branch structures, i.e., with bush-like structures)
- Neutral or charged (cationic or anionic)

Finally, on the basis of their biological role, they can also be distinguished in:

- Structural elements
- Energy-reserve polysaccharides

Following the latter classification, in this section the main polysaccharides that can be found in higher plants and therefore in the different kinds of bioagro-wastes are described.

2.1 Structural Polysaccharides in Higher Plants

The main and most abundant structural polysaccharide that is found in plant kingdom is cellulose, a homoglycan constituted by β-(1→4)-linked

D-glucopyranose (Glc*p*) units. Cellulose is a high-molecular-weight polysaccharide made up of repeating cellobiose units (n) forming a linear structure in which both intra-chain and interchain molecular hydrogen bonds occur to link the chains.

The intermolecular hydrogen bonds give a sheetlike nature to the native polymer that possesses a crystalline structure whose organization varies from source to source (Klemm et al. 2005).

The cellulose chains are organized to give supramolecular structures, namely, elementary fibrils (with a length between 1.5 and 3.5 nm), microfibrils (between 10 and 30 nm), and microfibrillar bands whose length can be on the order of several hundred nm (Klemm et al. 2005). Nevertheless, the physical organization of cellulose polymers into such fibrillar structures varies depending on the plant source (Reddy and Yang 2005).

Cellulose is found both in primary and secondary plant cell wall in tight association with other polysaccharides, namely, the group of hemicelluloses. Historically identified as those polysaccharides extractable from higher plant tissues by means of hot aqueous alkaline solutions, hemicelluloses are a group of structurally different polysaccharides that can be distinguished in xylans, mannans, β-glucans, and xyloglucans (Ebringerova et al. 2005).

Xylans are the most abundant polysaccharides that can be found in the secondary cell walls of both mono- and dicotyledon (hardwood, grasses, cereals), and they include both homopolymers (the less frequent ones) and heteroglycans containing glucuronic acid and arabinose units. Xylans can be distinguished in: glucuronoxylans (GXs), arabinoglucuronoxylans (AGXs) and glucuronoarabinoxylans (GAXs), and arabinoxylans (AXs).

GXs possess a linear backbone of β-(1→4)-linked D-xylanopyranose (Xyl*p*) units decorated by single 4-*O*-methyl-α-D-glucopyranosyl uronic acid (MeGlcA) residues linked at C-2 of Xyl*p* monomers (Figs. 1 and 2).

AGXs (Fig. 3) and GAXs are made up of a linear β-(1→4)-D-Xyl*p* unit chain branched with MeGlcA at position 2 and α-L-arabinofuranose (L-Ara*f*) at position 3 of the backbone units.

Finally AXs are polysaccharides consisting of a linear chain of β-(1→4)-D-Xyl*p*-linked units bearing branches of α-L-Ara*f* at position 2 or 3 of xylan residues (Fig. 4).

The second group of xylans, namely, mannans, are the main components of the secondary cell walls of softwoods, although some of them can be found also as reserve polysaccharides. Mannoglycans can in turn be divided in: galactomannans (GaMs), glucomannans (GMs), and galactoglucomannans (GGMs).

GaMs (Fig. 5) are composed of linear chains of β-(1→4)-linked D-mannopyranose (Man*p*) units substituted at position 6 with galactopyranose (Gal*p*) residues with the Man:Gal ratio ranging from 1:1 to 5.7:1.

GMs (Fig. 6) possess a backbone consisting of β-(1→4)-D-Man*p* and β-(1→4)-D-Glc*p* units that, like in the case of Konjac's mannans, can be acetylated at two or three position of some Man*p* residues.

GGMs are characterized by a backbone made up of alternating segments of β-(1→4)-linked D-Man*p* and segments made up of both β-(1→4)-linked D-Man*p*

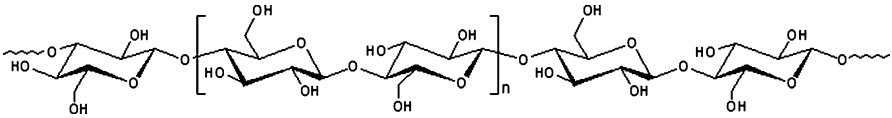


Fig. 1 Structure of cellulose backbone with the cellobiose repeating unit in brackets

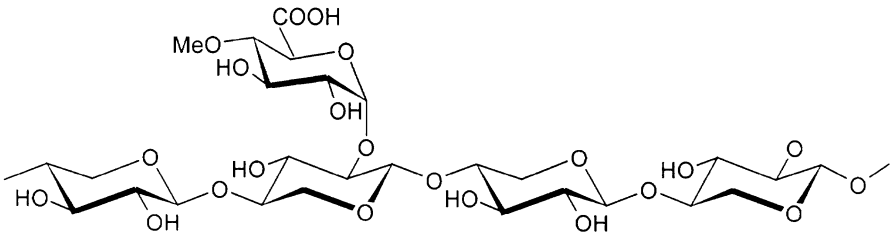


Fig. 2 Structure of glucuronoxylans (*GXs*) (Ebringerova et al. 2005)

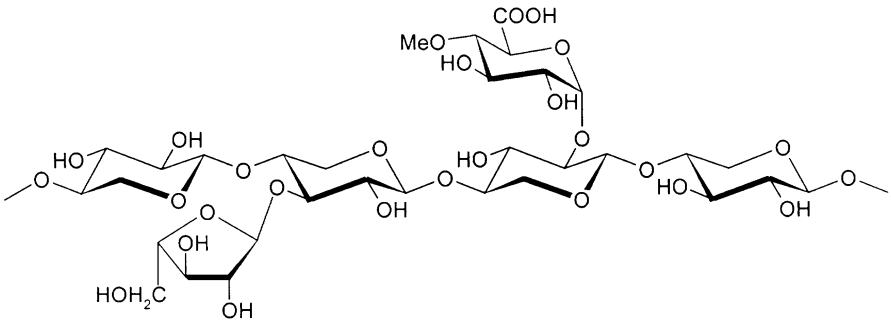


Fig. 3 Structure of arabinoglucuronoxylans (*AGXs*) (Ebringerova et al. 2005)

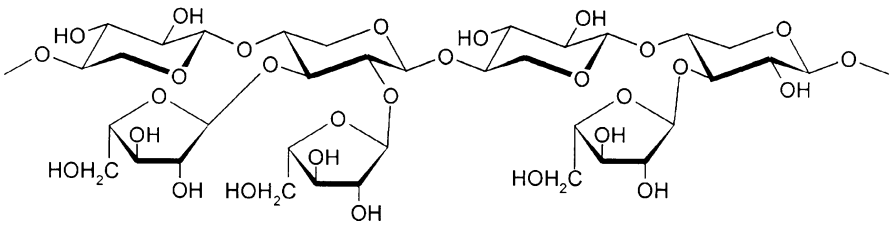


Fig. 4 Structure of arabinoxylans (*AXs*) (Ebringerova et al. 2005)

and (1→4)-D-Glc*p* units, with branches of Gal*p* units linked at two, three, or six position of Man*p* and at three or six position of Glc*p* units. Either GMs or GGMs are also present as storage carbohydrates in the seeds or roots of some plants such as Konjac.

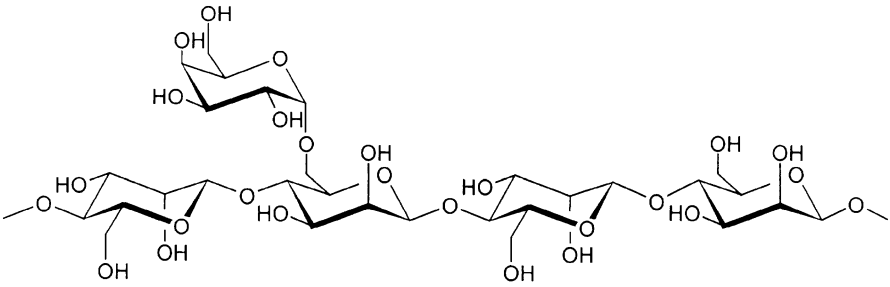


Fig. 5 Structure of galactomannans (*GaMs*) (Ebringerova et al. 2005)

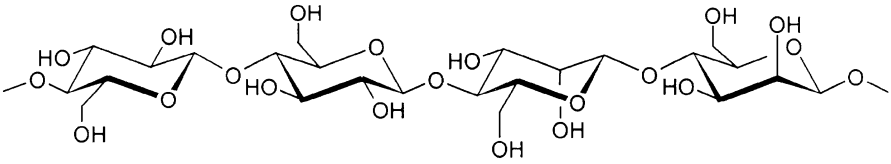


Fig. 6 Structure of glucomannan (*GM*) (Ebringerova et al. 2005)

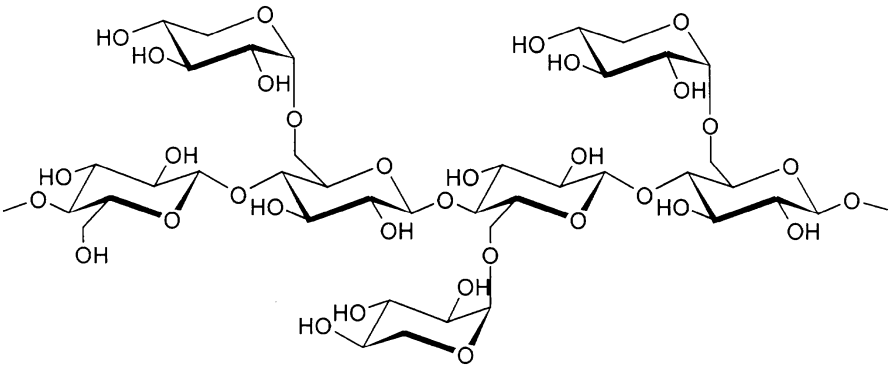


Fig. 7 Structure of xyloglucan (*XG*) (Ebringerova et al. 2005)

β-Glucans, also named (1→3, 1→4)-β-D-glucans, are the hemicellulose components of cereal grains. They are unbranched homopolymers in which about 70 % Glc units are (1 →4) linked and the remaining 30 % are (1→3) linked, mainly assembled as repeating units of cellotriosyl, cellotetraosyl, or less frequently of chains of 9–14 Glc residues, separated by single (1→3) links.

Xyloglucans (XGs) are the most abundant polysaccharides that can be found in the primary cell walls of dicotyledon. They present a cellulose-like backbone made up of (1→4) β-D-Glc*p* units decorated with α-D-Xyl*p* residues linked at 6 position of Glc monomers (Fig. 7), with a degree of xylosylation ranging from 30–40 % in grasses to 60–75 % in some dicotyledons.

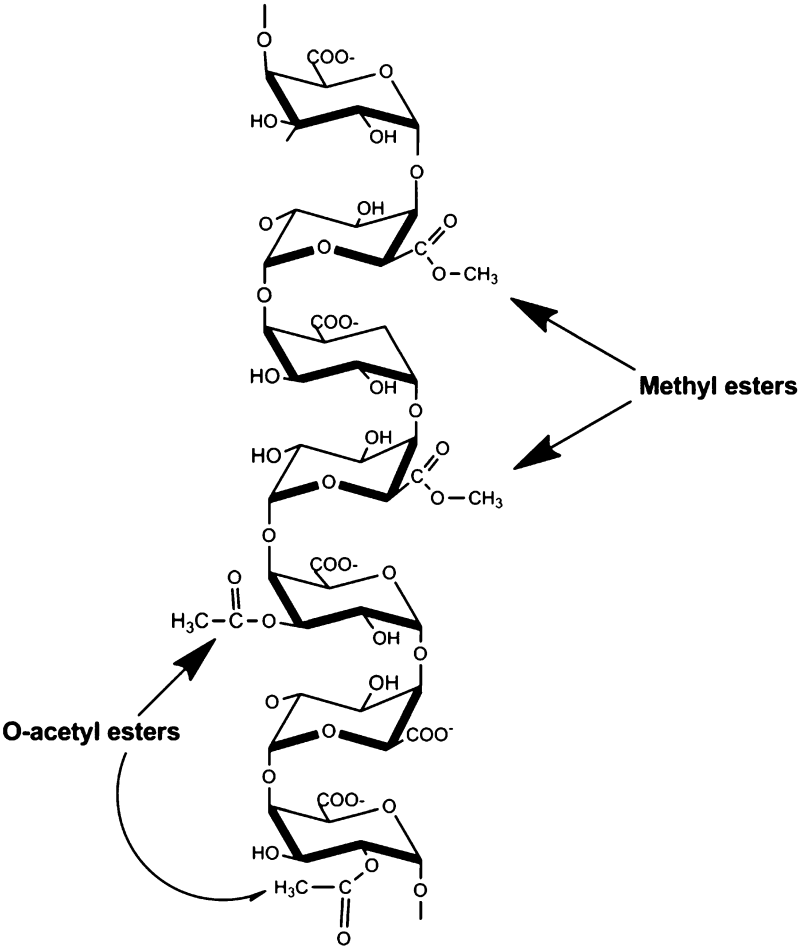


Fig. 8 Structure of homogalacturonans (*HGs*) (Ochoa-Villarreal et al. 2012)

XGs can be distinguished in two types: XXXG, in which three xylosylated Glc units are separated by one free Glc unit, and XXGG, in which there are alternating sequences of two xylosylated and two non-xylosylated Glc units.

The other main group of structural polysaccharides that can be found in plants is represented by the complex of pectic polysaccharides that are constituted by both neutral and acidic sugars. They comprise the homogalacturonans (HGs) also called the "smooth region" of pectins, and the heteropolymeric rhamnogalacturonans (RGs) and arabinogalactans (AGs), the "hairy regions" of pectins.

Homogalacturonans (HGs), the most abundant members of pectins, are homopolymers of α-(1→4)-D-GalA*p* units partially methyl esterified at C-6 and *O*-acetyl esterified at C-2 or C-3 (Fig. 8), depending on the source. Depending on the degree

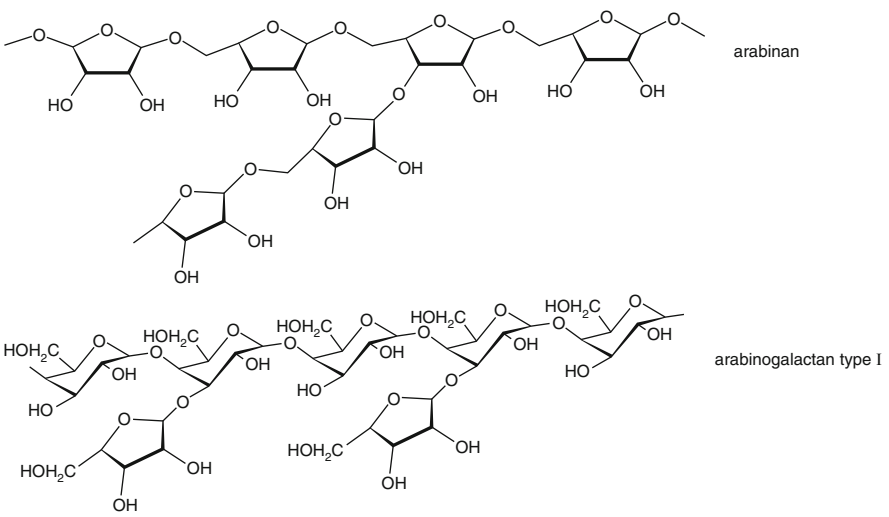


Fig. 9 Structure of arabinogalactans I (*AG-I*) (Wong 2008)

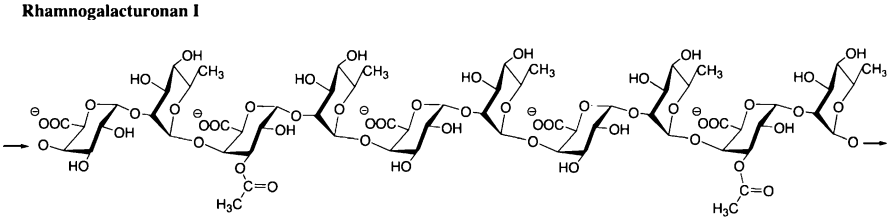


Fig. 10 Structure of rhamnogalacturonans I (*RG-I*) (Wong 2008)

of methyl esterification (DM), HGs can be distinguished in "high methyl-esterified HGs" (DM >50 %) or "low methyl-esterified Hgs" (DM <50 %) (Yapo 2011).

The "hairy region" of pectins is composed of arabinogalactans (AGs) linked to rhamnogalacturonans (RG) chains. AGs can be distinguished in two groups: type I arabinogalactan (AG-I) (Fig. 9) and type II arabinogalactan (AG-II) (Fig. 10). AG-I, also defined as arabino-4-galactans, are constituted by a β-(1→4)-Gal*p* backbone (Fig. 9) with side chains, linked at C-3 of Gal, made by arabinans. Arabinans are linear or branched chains of 1→5-linked L-Ara*f* units (Fig. 9): linear ones are substituted by single L-Ara*f* residues at C-2 or C-3 of the chain.

Type II arabinogalactans (AG-II), also defined as arabino-3,6-galactans, present a linear backbone of 1 →3- and 1→6-linked Gal*p* units, branched at 1, 3, or 6 position with arabinan chains. Both AG-I and AG-II are the constituents of RG-I pectins.

Rhamnogalacturonans (RGs), also defined as the real pectins, are heteropolymers of galactopyruronic acid (GalA*p*) and rhamnopyranose (Rha*p*) branched with AGs chains. They also are divided in two groups:

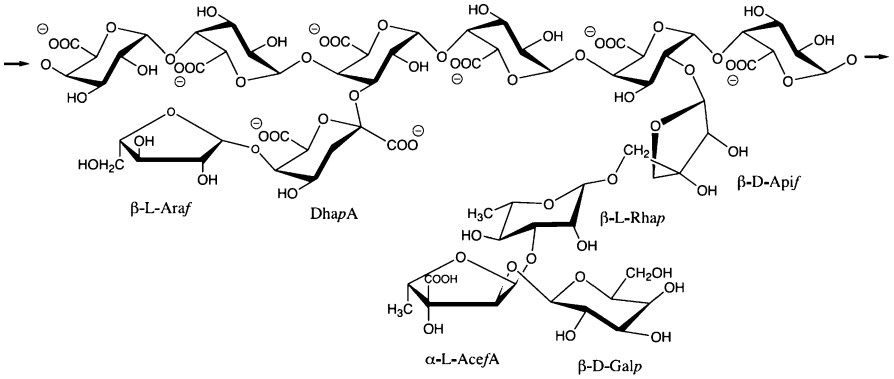


Fig. 11 Structure of rhamnogalacturonans II (*RG-II*) (Wong 2008)

rhamnogalacturonans I and II (RG-I and RG-II). RG-I possess a linear backbone of alternating α-1,4-linked GalA*p* units and α-1,2-Rha*p* units, the latter being the branching point to which (on position 4) arabinans or AGs chains are linked (Fig. 10).

Rhamnogalacturonans II (RG-II) are constituted by a homogalacturonan (HG) backbone of about 9–10 GalA*p* monomers, partially methyl esterified, and branched at position 3 or 4 by four different types of oligosaccharide (Fig. 11).

The side chains of RG-II present some glycosyl residues rarely found in other polysaccharides such as apiose (3-C-hydroxymethyl-β-D-erytrose), AceA (3-C-carboxyl-5-deoxy-L-xylofuranose), 3-deoxy-D-manno-octulosonic acid (KDO), 2-*O*-methylfucose, 2-*O*-methylxylose, 3-deoxy-D-lyxo-2-heptulosaric acid (DHA), aceric acid, and L-Gal (Paulsen and Barsett 2005).

2.2 Energy-Reserve Polysaccharides in Higher Plants

Starch is the main energy-storage polysaccharide that can be found in higher plants: it is composed of two glucose homopolymers, namely, the linear amylose and the branched amylopectin. Amylose is a linear chain of α-(1→4)-linked Glc*p* units, while amylopectin has a linear backbone of α-(1→4)-linked Glc*p* units with branches at C-6 made up of linear chains similar to amylose chains (Fig. 12). The degree of branching of amylopectin can vary, depending on the source of starch, from 4.2 in maize to 5.2 in oats. Similarly, the ratio of amylose vs. amylopectin component is different in the diverse natural sources: in general the branched component is the prevailing one since it can range from 75 % in maize or 77 % in wheat or 78 % in potatoes or 80 % in rice till up to 100 % in the mutant waxy maize (Robyt 2008).

Although starch is the main energy-reserve carbohydrate, other glycans such as fructans can be found as reserve in about 15 % of flowering plants. Fructans include both linear and branched polymers made up mainly of fructofuranose (Fru*f*) units

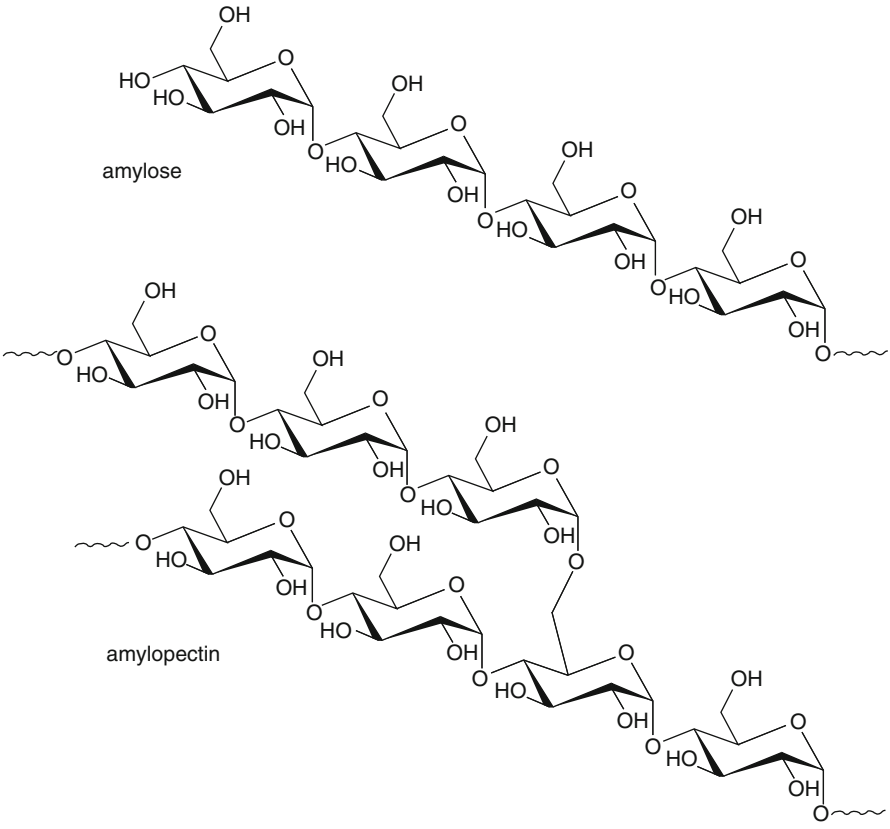


Fig. 12 Structure of amylose and amylopectin

that can be linked by β-(2→1) or β-(2→6) or both glycosidic bonds. The most studied fructans are the inulin-type fructans that possess a linear backbone of β-(2→1)-linked Fru*f* units attached to an initial sucrose (Suc) unit (Fig. 13).

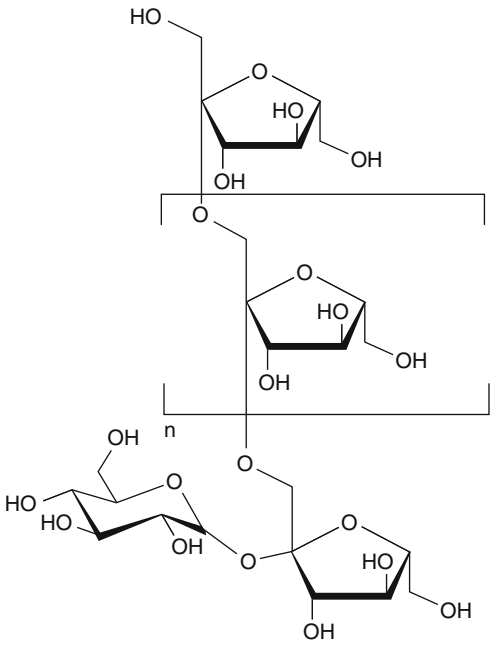
Notably, as mentioned in the previous section, some structural glycans can also serve as energy-reserve polysaccharides in higher plants, such as galactomannans in some legumes, mannans and glucomannans, and β-glucans in cereal grains.

3 Main Bioagro-Waste Sources of Polysaccharides

Agro-industrial wastes' chemical composition includes a complex of diverse polysaccharides that, in the frame of the biorefinery approach, could be converted in chemicals and/or energy by means of chemical and/or biochemical treatments.

Agro-wastes that potentially could afford sources of polysaccharides can be divided in two groups, namely, food wastes, i.e., residues of processing (canning, squeezing, peeling, milling, etc.) of fruits, cereals, and vegetables for

Fig. 13 Structure of inulin



food production, and agricultural residues, i.e., residues of harvesting and postharvesting phases.

3.1 Food Wastes

Food wastes that are currently used or that could potentially be exploited for polysaccharide production include mainly vegetable processing residues like seeds, peels and skins, husks, exhausted pulps, unripe or damaged fruits, etc. (Fig. 14).

Such residues are rich in structural carbohydrates (cellulose, hemicellulose, or pectins) or in other kinds of glycans and/or dietary fibers (i.e., a mixture of nondigestible carbohydrates comprising cellulose, hemicelluloses, pectins, and inulin in association with other nonsugar molecules like lignin, waxes, and polyphenols).

Several examples are available in literature concerning the chemical investigation of polysaccharide fraction of different vegetable wastes: in Table 1 some of them that are already exploited for industrial purposes and others that could be promising sources of valuable polysaccharides are listed.

Apple pomace annual production can rise up to 3.42 Mtons since nearly 50 % of the fruit is discarded after industrial processing for fruit juice production (Min et al. 2011; Walia et al. 2013). Depending on the extraction method employed, such wastes can be an interesting source of pectins and arabinans that can account from

Fig. 14 Some main food wastes: **a** apple pomace, **b** lemon residues, **c** tomato peels, **d** potato peels

4.6 % to 11.7 % or of hemicellulose and cellulose (Nawirska and Kwasniewska 2005) that in turn can be employed as soluble dietary fibers.

Banana is among the main agricultural resources in tropical and subtropical countries, and its cultivation and consumption generate about 10.9 Mtons of wastes per year (Sanchez-Vazquez et al. 2013). Banana wastes, i.e., peels, account for about 40 % of the fresh fruit weight and are composed of 50 % of dietary fibers of which 21.7 % are pectin polysaccharides, 3.8 % are hemicellulose, and 1.3 % are cellulose (Das and Singh 2004).

Pomace is also the main kind of residue remaining after the industrial processing of other fruits: some examples are constituted by black currants, cherries, chokeberries, and pears that are used in several European countries for the production of juices and beverages. These wastes are almost composed of dietary fibers (comprising besides polysaccharides like pectins, hemicellulose, and cellulose also lignin as main noncarbohydrate fraction) whose amount for the above-listed fruits is equal to 90.8 %, 91.37 %, 90.3 %, and 94 % of dry matter weight, respectively. The pomaces of the listed fruits present variable amounts of pectins, hemicellulose, and cellulose (Nawirska and Kwasniewska 2005), as reported in Table 1.

World citrus fruit production reaches more than 80 Mtons every year [Pourbafrani et al. 2010], and about 50–60 % of this biomass is discarded after squeezing for juices or peeling for liquor or jams production. Such wastes

Table 1 Main food waste sources of polysaccharides (Partially adapted from Poli et al. 2011)

Waste		Polysaccharides		
Source	Amount (Mtons y^{-1})	Chemical composition	Content (% dry weight)	Reference
Apple pomace	3.42	Pectins (arabinans)	4.6–7.7	Min et al. 2011; Walia et al. 2013
		Pectins/ hemicellulose/ cellulose	11.7/24.4/43.6	Nawirska and Kwasniewska 2005
Banana peels	10.9	Pectins/ hemicellulose/ cellulose	21.7/3.8/1.3	Sanchez-Vazquez et al. 2013; Happi Emaga et al. 2008; Das and Singh 2004
Black currant	≈ 0.5	Pectins/ hemicellulose/ cellulose	2.73/25.3/12.0	Nawirska and Kwasniewska 2005
Cherry		Pectins/ hemicellulose/ cellulose	1.51/10.7/18.4	
Chokeberry		Pectins/ hemicellulose/ cellulose	7.85/33.5/34.6	
Pear		Pectins/ hemicellulose/ cellulose	13.4/18.6/34.5	
Orange peels	7.4	Pectins	18/25	Kratchanova et al. 2004; Pourbafrani et al. 2010
		Pectins/ hemicellulose/ cellulose	23.0/11.0/37.1	Sanchez-Vazquez et al. 2013
Mandarin peels	3.1	Pectins/ hemicellulose/ cellulose	16.0/6.0/22.6	Sanchez-Vazquez et al. 2013
Lemon residues	≈ 2.5	Pectic polysaccharides	14.3	Poli et al. 2011; Sanchez-Vazquez et al. 2013
Carrot residues	≈11	Pectins/ hemicellulose/ cellulose	3.88/12.3/51.6	Vazquez et al. 2013; Nawirska and Kwasniewska 2005
Coffee husk	5	Pectins	12.4	Sanchez-Vazquez et al. 2013; Passos et al. 2014
Corn bran	7.8	Starch/ hemicellulose/ cellulose	4–11.2/28/70	Rose et al. 2010; Sanchez-Vazquez et al. 2013
Corn fiber	11.4	Starch	15–20	
Peanut husk	10	Arabinans/ galactans/ mannans	0.7/0.2/0.1	Sanchez-Vazquez et al. 2013

(*continued*)

Table 1 (continued)

Waste		Polysaccharides		
Source	Amount (Mtons y^{-1})	Chemical composition	Content (% dry weight)	Reference
Potato	>80	Starch/ hemicellulose/ cellulose	67.5/9.2/1.2	Sanchez-Vazquez et al. 2013; Das and Singh 2004
Tomato	11	Xyloglucan	7.5	Tommonaro et al. 2008
		Hemicellulose/ cellulose	11/9.1	Das and Singh 2004

(mainly coming from oranges, mandarins, grapefruit, lemons, and limes transformation) include peels, seeds, and pulps that contain different polysaccharides among which pectins are the most abundant. The total pectins' amount that can be recovered from citrus fruit residues depends upon both the extraction method employed and the citrus species: as shown in Table 1, the extraction yields can vary from 18 % to 25 % of dry matter for orange fruits (Kratchanova et al. 2004; Pourbafrani et al. 2010) to 23.0 % of mandarin peels (Sanchez-Vazquez et al. 2013) or finally to 14.3 % in the case of lemon residues (Poli at al. 2011). With regard to lemon residues, they account for about 50 % of starting material; therefore, since the total world production of lemons is ≈5 Mtons y^{-1}, a potential quantity of ≈2.5 Mtons of such residues could be exploited as source of pectins. In addition also other structural glycans could be recovered from citrus fruits, for example, hemicellulose and cellulose that share a significant portion of the polysaccharides extracted from orange and mandarin wastes (Table 1).

Exhausted pulps are produced also in the conversion of vegetables like carrots whose world production reaches about 33.6 Mtons per year (Sanchez-Vazquez et al. 2013): about 30–40 % of such amount is lost as by-products. Notably, 54.2 % of carrot pulp, on weight basis, is constituted by dietary fibers in which the main polysaccharides are represented by pectins, hemicellulose, and cellulose (see Table 1).

Corn is one of the main cereal crop productions indeed; according to data from the Food and Agriculture Organization of the United Nations (FAO, http://faostat3.fao.org/faostat-gateway/go/to/home/E), its world production is more than 800 Mtons per year. Corn bran and fibers are the main residues from processing of grains for food production: bran is the residue of dry milling for meals and floor production, while fibers are left after wet milling of grains for starch and oil recovery. Since corn grains account for about 15 % of total harvested maize, and considering that bran represents 6.5 %, while fiber is 9.5 % of total corn grain, a total production per year of about 7.8 Mtons and 11.4 Mtons of bran and fibers can be hypothesized, respectively. Corn fibers still contain interesting quantities of starch, while bran can afford besides starch also significant yields of cellulose and hemicellulose.

Among the most abundant vegetable food wastes, also potato and tomato residues have to be mentioned. Potato wastes, mainly peels, are about 80 Mtons y^{-1} and contain besides starch, as main polysaccharide, also appreciable quantities of cellulose and hemicellulose polymers. Tomato residues from canning industry (more than 11 Mtons of peels, seeds, and exhausted pulps, according to FAO data), besides the structural glycans of cell wall (cellulose and hemicellulose), are also an interesting source of new polysaccharides like the xyloglucan fraction isolated from peels and seeds by means of alkaline extraction (Tommonaro et al. 2008).

Husks from coffee and peanuts are also food waste sources of polysaccharides. About 55 % of coffee wastes are produced in Latin America, and husks contain pectins as main polysaccharide, accounting for more than 10 % of waste dry weight. Peanut is also cultivated and used in several countries; indeed its world production is about 34 Mtons: the by-products, mainly husks, are a rich source of arabinans, galactans, and mannans accounting in total for about 1 % of residues.

3.2 Agricultural Residues

Agro-wastes that are potential sources of polysaccharides encompass all the residues that are produced during the cultivation, harvesting, and postharvesting steps of several crops such as sugarcane or cereal crops like corn, barley, rice, wheat, etc. (Fig. 15). Such wastes are also classified as "lignocellulosic residues": lignocellulose is the complex of cellulose, hemicellulose, and lignin that are characteristic of plant cell wall.

Among the most abundant agricultural and lignocellulosic residues, sugarcane bagasse and cereal straw are particularly interesting polysaccharide sources because of their total annual production and significant lignocellulose content.

According to FAOSTAT data, the world total sugarcane production in 2012 has been more than 1,000 Mtons. Sugarcane bagasse, i.e., the residue of crushing of canes for sugar juice extraction, represents about 30 % of the plant, and it is composed of cellulose (40–45 %) and hemicelluloses (30–35 %), besides lignin (Cardona et al. 2010).

Cassava is a tuber crop that represents the basic food for more than 700 million people in Asia (where it is known as tapioca), Latin America, and Africa. According to FAO data, its world total production in 2012 has been about 263 Mtons y^{-1}. Cassava processing for flour production and starch extraction from tubers results in the production of peels and of a solid residue, the bagasse, that still retains high levels of starch that indeed (on a weight basis) constitute about 50 % of the biomass (Pandey and Nigam 2009).

With regard to cereal crops, the main residues left after harvesting are represented by straws that comprise more than 50 % of the crops: since in late years the world production of cereals on average has been about 2600 Mtons (FAO data), it can be estimated that potentially 1,300 Mtons of these lignocellulosic residues are produced and can be exploited as valuable carbohydrate polymer sources.

Fig. 15 Some main agricultural residues: **a** corn stover, **b** rice straw, **c** wheat straw, **d** sugarcane bagasse

Corn world production, as mentioned in the previous section, equals about 800 Mtons per year: only the grains are harvested and used for food applications, while the other components (leaves, shell, stalks) are left on the ground. Corn residues from harvesting, i.e., corn stover, account for about 85 % of the plant; therefore, the total amount of such wastes is nearly 700 Mtons per year. These wastes are particularly rich in cellulose and hemicellulose thus representing the main renewable source of world lignocellulose (Sanchez-Vazquez et al. 2013).

Barley, oat, rice, sorghum, and wheat world production in 2012, according to FAOSTAT data, were ≈13, 21, 719, 57, and 670 Mtons, respectively.

Barley grain is used as animal feed, as malt, and for human food: after harvesting, the main residue is constituted by straw whose chemical composition reveals that more than 60 % of this biomass is made of valuable polysaccharides, namely, cellulose and hemicellulose. Since cereal postharvest residues account on average for 50–75 % of crop, therefore in the case of barley potentially, 5.6–9.8 Mtons could be available for polysaccharide recovery.

Table 2 Main agro-industrial waste sources of polysaccharides (PS)

Waste source	Waste amount (Mton y^{-1})	Chemical composition	PS content (% dry weight)	Reference
Sugarcane bagasse	300	Hemicellulose/ cellulose	30–35/40–45	Cardona et al. 2010
Cassava bagasse	≈200[a]	Starch	50	Das and Singh 2004
Corn stover	696	Hemicellulose/ cellulose	32.0/27.8	Sanchez-Vazquez et al. 2013; Li et al. 2012
Barley straw	≈5.6–9.8	Hemicellulose/ cellulose	27/44	Das and Singh 2004
Oat straw	≈10.5–15.7	Hemicellulose/ cellulose	16/41	Das and Singh 2004
Rice straw	731	Hemicellulose/ cellulose	26/33	Das and Singh 2004; Binod et al. 2010
Sorghum straw	≈28–43	Hemicellulose/ cellulose	30/31	Das and Singh 2004
Wheat straw	550	Hemicellulose/ cellulose	36/39	Kuan and Liong 2008; Das and Singh 2004

[a]85 % moisture, not on dry weight basis

Oat is diffused in temperate regions, and it is mainly used as breakfast cereal as flakes or for porridge; indeed it is not suitable for baking since it lacks gluten. Its by-products still retain significant quantities of both cellulose and hemicellulose, as reported in Table 2. The potential waste from oat that is yearly produced should be about 42 Mtons.

Together with corn and wheat, rice is one of the main cereal crops in the world with China as the main producer; notably it also represents the principal staple food for more than 50 % of the world's population. After harvesting, the rice is dehusked, and the resulting straw is usually left onto the soil or burned: nevertheless it is an interesting lignocellulose biomass since it still retains appreciable amounts of cellulose and hemicellulose (Table 2).

Sorghum is native to Africa, but nowadays it is cultivated in Africa, the United States, and Asia where Nigeria, Mexico, the United States, and India are the main producers, respectively. Sorghum is mainly exploited as animal feed or for alcohol production, but in poorest areas it also serves for human nutrition. Sorghum straw is among the lignocellulosic biomass under investigation due to its polysaccharide fraction that is mainly constituted by cellulose and hemicellulose, as shown in Table 1. On the basis of sorghum total world production, its straw yield is estimated to be between about 28 and 43 Mtons per year.

Wheat is mainly cultivated in Asia (43 %) and Europe (32 %) followed by North America as the third producer with 15 % of total global production. The main part of wheat is used for food production, with a minor fraction for animal feeding. The remainder part is estimated to be about 550 Mton y^{-1} and is also a biomass rich in cellulose and hemicellulose that could be further exploited for other purposes.

4 Brief Phytochemistry

In the assessment of polysaccharide structure, two stages can be distinguished: firstly, the determination of primary structure, which means the polysaccharide composition, the configuration, and position of glycosidic linkages and the ring configuration, and, secondly, the determination of spatial structure that can be obtained through the knowledge of bond lengths, bond angles, and torsional angles overall regarding the glycosidic torsional angles and the exocyclic torsional angle. Besides the primary structure, a detailed knowledge of the spatial structure is essential in order to assess structure-function or structure-property relations. The clarification of polysaccharide structures is very important to explain the physicochemical and biological properties of these biopolymers and to attribute and in some cases to predict the biotechnological applications of these biomolecules. The rheological properties of these polymers are surely influenced by the primary conformation. Furthermore, the ordered secondary configuration frequently takes the form of aggregated helices. Moreover, the presence or also the absence of specific acyl groups, for example, O-acetyl or O-succinyl esters or pyruvate ketals, can influence the formation of ordered helical aggregates (Poli et al. 2011). Several chemical and physical techniques are used to determine the primary structure of polysaccharides: chemical degradation and derivatization, in association with chromatographic methods and mass spectrometry analysis, are used to determine the sugar composition, their absolute configuration, and the presence and the position of possible substituents. In details, molecular size analyses are carried out by Sepharose CL-6B column using a mixture of dextrans for calibration curves (Pazur 1994). Fourier transform infrared (FT-IR) spectroscopy spectra of EPS are obtained with FT-IR spectrometer between 400 and 4,000 wave numbers (cm^{-1}). Thermogravimetric analysis (TGA) of EPS is obtained with TGA apparatus where a known amount of polysaccharide sample in H_2O is heated from 30 to 400 °C at a rate of 20 °C min^{-1} under a constant flow of nitrogen. For carbohydrate analysis, the purified polysaccharide fractions are hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. Sugar components are firstly detected by thin-layer chromatography (TLC), using a mix of acetone/butanol/H2O (8:1:1, v/v/v) as mobile phase and standard monosaccharides for qualitative determination. Afterward monosaccharide composition is defined by high-pressure anion exchange-pulsed amperometric detector (HPAE-PAD). Sugars are eluted isocratically with 16 mM NaOH and identified by comparison with reference standards (Poli et al. 2011). The linkage positions of the monosaccharides are determined by methylation analysis: the samples are methylated with CH_3I in dimethyl sulfoxide and NaOH, and the products are hydrolyzed using TFA 1 M at 70 °C for 45 min. After reduction with $NaBD_4$, the samples are acetylated, and then the sugar derivatives are analyzed by GC-MS. The absolute configuration of the sugars is determined by gas chromatography of the acetylated (S)-2-octylglycosides (Manzi and van Halbeek 2009).

The scrutiny of unknown structures still lies in the crucial point of polysaccharide research, and the nuclear magnetic resonance (NMR) technique persistently

continues leading this procedure. Even today, when important technological advances occur, the unambiguous identification remains a challenging task (Halabalaki et al. 2014). Toward this direction, computational tools might hold a key role in the future. Expert systems equipped with versatile algorithms enabling structure or spectra prediction are able to give recourses for the structure elucidation of natural molecules in complicated mixtures. Comprehensive and qualified open access natural products databases support considerably the identification of unknown molecules (Halabalaki et al. 2014). NMR contribute to the determination of the repeating unit of polysaccharides by using in particular two-dimensional ^{1}H- and ^{13}C-NMR. For conformational analysis it is important that the primary structure of polysaccharide being studied is known and that as many signals as possible have been assigned unambiguously. In order to make possible NMR or mass spectrometry analysis, it is necessary to carry out a chemical or enzymatic digestion of polysaccharides to obtain smaller fragments that are more easily analyzed. Two types of homonuclear 2D NMR spectroscopy could be used for structural analysis of oligosaccharides: the first is characterized by magnetization transfer through scalar coupling (COSY type); in experiments of the second type, the magnetization is transferred through space (NOESY type) at short interproton distances. The most useful method of the COSY type is the 2D homonuclear Hartmann-Hahn (2D HOHAHA) spectroscopy. Advantages of 2D HOHAHA over other COSY-type techniques are higher sensitivity; pure in-phase magnetization, which avoids signal canceling by antiphase magnetization in conjunction with large line widths, as in 2D double-quantum filtered COSY (DQF-COSY); and the possibility to obtain complete subspectra by multistep magnetization transfer. In some cases also NOESY-type experiments are useful for assignment purposes often in combination with COSY-type experiments (Halabalaki et al. 2014).

Other assignment techniques exploit the large spread in chemical shift in ^{13}C NMR spectra. Since the proton-detected heteronuclear multiple-quantum coherence (HMQC) experiment renders information about direct (one bond) ^{1}H-^{13}C connectivities, it is used for the assignment of either the ^{1}H or the ^{13}C spectrum. Long-range heteronuclear correlation experiments such as the proton-detected heteronuclear multiple-bond correlation (HMBC) spectroscopy afford linkage information and therefore useful for the determination of carbohydrate sequences. In addition, the HMBC experiment makes it possible to assign carbon atoms that are not accessible by HMQC spectroscopy, namely, quaternary carbon atoms (Halabalaki et al. 2014).

In addition, a solid-state NMR has been adopted as uniquely suited for the examination of insoluble and complex macromolecule, or, for example, to study whole-cell systems and also to check the integrity of the polysaccharide structures (Finore et al. 2014). Solid-state NMR spectroscopy allows investigation of molecules in the solid state and reveals information about crystal packing using a noninvasive and nondestructive analysis. This method can provide not only chemical information but also chemical environment and ultrastructural details that are not easily accessible by other nondestructive high-resolution spectral techniques

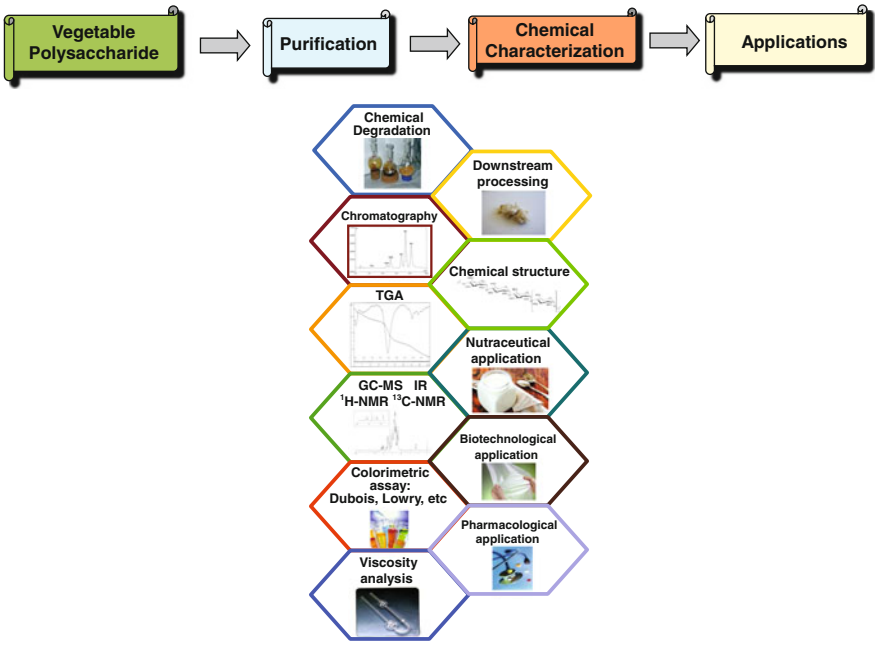


Fig. 16 Main steps and methods for purification and structural characterization of polysaccharides

(Foston 2014; Serra et al. 2012). The solid-state NMR methodologies result is particularly useful when studying structural problems in complex biological systems, for example, in the study of the mechanisms of biosynthesis and deconstruction for lignocellulosic biomass or individual plant cell wall components. The most frequently used solid-state NMR techniques are ^{13}C CPMAS, a double-bearing cross-polarization magic-angle spinning NMR spectroscopy that is the combination of three techniques: firstly, cross polarization; secondly, magic-angle spinning; and, thirdly, high-power decoupling (Foston 2014).

Recently, a bioanalytically probing technique has been developed to study biomaterials at cellular and molecular level within intact tissue (Yu 2011). This technique named synchrotron radiation Fourier transform infrared microspectroscopy (SR-IMS) takes advantage of bright synchrotron light which is a million times brighter than sunlight (Marinkovic and Chance 2006). It is able to have simultaneous information about tissue chemistry, tissue composition, tissue environment, and tissue structure by a noninvasive and nondestructive tool. An appropriate example of this technique comes from the study of microstructural features of the embryo (germ) in sorghum (*Sorghum bicolor* L.) seeds in which the intensity and distribution of the various chemical functional groups (including cellulosic compounds) could be chemically mapped (Yu 2011) (Fig. 16).

5 Biotechnological Approaches to Waste Polysaccharide Exploitation

Polysaccharides from renewable and sustainable sources, like food and agricultural wastes, can play a central role in the emerging biobased economy, i.e., the new economy system shifting from fossil resources of energy and chemicals to renewable resources such as biomass. Indeed the wide variety of structures and biological functions of polysaccharides that can be recovered from agro-wastes make them biotechnologically useful biopolymers that either are already exploited for several purposes or are under investigation for new applications (Fig. 17).

Since a great share of industrial production of chemicals and energy is based on the exploitation of plant polysaccharides, like starch, the search for new biotechnologies and strategies for exploiting and employing waste materials in several industrial fields is therefore the object of growing interest. Such an approach to polysaccharide waste biomass exploitation comes under the frame of the so-called biorefinery, i.e., a new production system in which a variety of goods (fuels, chemicals, and biomaterials) can be recovered from renewable feedstock by using a single or a combination of chemical, physical, and biological treatments.

Biorefinery of biomass is a multistep process that can be divided in: choice of starting material and of suitable pretreatment method, in order to render it more prone to the following processes, and conversion of the pretreated biomass by means of one or combined biological, chemical, and physical techniques to obtain

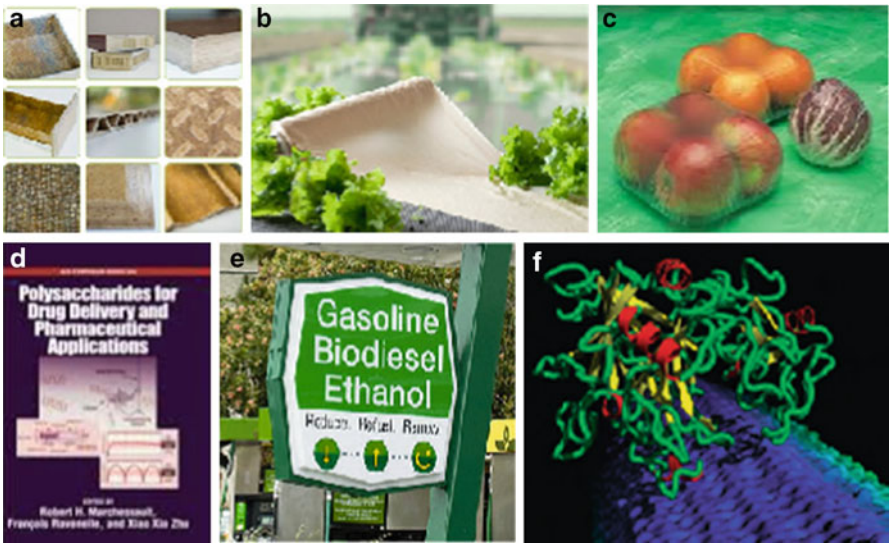


Fig. 17 Main industrial applications of polysaccharides for biomaterials and biodegradable plastics (**a**, **b**), for food packaging (**c**), for pharmaceutical industry (**d**), for fuel ethanol (**e**) and enzyme (**f**) production (Photos' web sources: (**a**) www.jetsongreen.com; (**b**, **c**) www.novamont.com; (**d**) www.bookdepository.com (**e**) http://www.indianaenvironmentallaw.com/agriculture/)

energy or value-added chemicals and/or building blocks for further production processes.

Polysaccharide rich waste biomass is the ideal feedstock for biorefinery since they are renewable, not in competition with the food chain and not impacting on land use and biodiversity.

In addition, such materials are available in significant amounts every year (see Tables 1 and 2) and therefore are under investigation for several industrial applications, for example, production of green chemicals, biomaterials, second-generation biofuel, etc.

In the following section some remarkable examples of useful products that can be obtained from polysaccharides recovered from agro-industry waste biomass by means of physical, chemical, and biological transformation are described.

5.1 Building Blocks for Composites and Biomaterials

Waste polysaccharides like cellulose and starch are valuable building blocks for the material's preparation like composites or bioplastics.

Cellulose is the main glycan found in the lignocellulosic agro-wastes that in turn are emerging as potential renewable sources of fibers for paper, material, and textile industry. Cellulose can be recovered by waste biomass fractionation by means of different pretreatment techniques such as steam explosion, ionic liquid-based fractionation, and acidic treatment followed by shear mechanical treatment. The cellulose polymers that in such ways can be recovered, for example, from wheat (Dufresne et al. 1997), rice straw (Ping and Hsieh 2012), and from sugarcane bagasse (Mandal and Chakrabarty 2011), are essentially nanocrystalline cellulose, i.e., a cellulose polymer that is formed by rigid rodlike particles whose widths and lengths are from 5–70 nm to 100 nm up to micrometers in range. Cellulose nanofibers, extracted from wheat straw by these methods, have been used for the reinforcing of polypropylene composites, for the preparation of biocomposites and of thermoplastic starch-based nanocomposites, and for the production of composition panels that have shown to be particularly resistant to earthquake (Kalia et al. 2011). Also other several applications have been identified for nanocrystalline cellulose from wastes such as in nanocomposites as filler, improving mechanical and barrier properties, as building block for selectively permeable membranes, as foams or aerogels, as adhesive or to prepare adhesive materials, and as a reinforcing agent for polymer electrolytes in lithium batteries (Brinchi et al. 2013), but further and maybe still unknown applications in these fields are possible.

Other examples of application of waste cellulose for composites are represented by: wheat straw cellulose that has been used as a natural filler to reinforce composites of a polyolefin and of a biodegradable polyester (Le Digabel et al. 2004); by rice straw cellulose that has been used to prepare composite boards for construction that afforded good acoustical and electrical insulation, besides possessing anti-caustic and anti-rot properties (Yang et al. 2004a); by banana

peels fibers that have been employed to prepare composites with polyester matrix (Pothan et al. 2007); by rice residual cellulose that has been investigated as reinforcing filler for polypropylene-based thermoplastic composites (Yang et al. 2004b); and finally by bagasse wastes that, like the previously mentioned waste sources of cellulose, were employed to produce composite materials by mixing with low-density polyethylene, acid stearic, or maleated low-density polyethylene (Habibi et al. 2008).

Starch is also very promising for new biodegradable polymers that can find several applications in materials science; after blending with other natural polymers like gutta-percha or natural rubber, they can afford more suitable composite materials (Yu et al. 2006). With particular regard to cassava bagasse starch, it has been used with different approaches to produce biocomposites: it has been mixed with Kraft paper to obtain a composite similar to cardboard that showed mechanical properties resembling the recycled paper (Matsui et al. 2004); it has also been used to produce thermoplastic starches using glycerol as plasticizer (de Morais Teixeira et al. 2005).

Also food wastes like tomato wastes showed to be potential sources of polysaccharides for bioplastics: indeed a glucan isolated from tomato canning wastes, after addition with glycerol as plasticizer agent, showed to possess promising properties as bioplastic for mulching or solarization applications (Tommonaro et al. 2008).

5.2 Biomaterials for Food Packaging

Several examples of polysaccharide-based edible films and coatings are available in literature, but most of them are based on the exploitation of polysaccharides from non-waste sources. Recently several examples of the reuse of agro-industrial residues for the preparation of edible and biodegradable films appeared: in this section a survey of very recent examples of applications of waste polysaccharides to this issue will be presented.

Hemicellulose polymers from both food wastes and agricultural residues can find applications in packaging; indeed, for example, arabinoxylans (AXs) isolated by alkali extraction from barley residues were used to prepare edible films by water casting and showed to have interesting mechanical properties (Mikkonen and Tenkanen 2012). On the other hand also AXs recovered from corn residues and blended with glycerol, propylene glycol, or sorbitol were used to produce stable films that showed to be able to afford moisture barrier in a preservation experiment with grapes samples (Zhang and Whistler 2004); AXs from corn bran were emulsified with fats (palmitic acid, oleic acid, triolein, a hydrogenated palm oil) and then used to prepare water vapor-permeable films (Peroval et al. 2002).

Cellulose from sugarcane bagasse has been exploited to prepare a cellulosic film fully biodegradable, with very high tensile strength and good water vapor permeability that potentially could be used for food packaging (Ghaden et al. 2014); cellulose nanocrystals obtained from the same agro-waste were used to reinforce

starch-based films that showed in this way higher water resistance and water barrier properties (Slavutsky and Bertuzzi 2014).

Residual starch from cassava bagasse proved to have potential application in bioactive food packaging since it was used to prepare biodegradable films impregnated with antimicrobial agents and possessing favorable water sorption and permeability properties (de Souza et al. 2014); cassava bagasse polysaccharides (cellulose and starch mixture, see Table 2 for chemical composition) have also been mixed with cassava starch to prepare a thermoplastic starch matrix (de Morais Teixeira et al. 2009).

Also pectins from food wastes have been studied for their potential exploitation in food packaging: pectins indeed have been used to produce bioactive films and coatings that are useful for prolonging food's shelf life. Such materials, besides providing a semipermeable barrier to oxidizing agents, can also be added with antimicrobial, antioxidant, and anti-softening agents, thus preventing food deterioration (Raybaudi-Massilia and Mosqueda-Melgar 2012).

5.3 Human Nutrition, Food Additives, and Prebiotics

Pectins, cellulose, and hemicellulose are part of human nutrition since they constitute the so-called dietary fibers: thanks to their chemical properties, during human digestion, they reach the small intestine where they have beneficial effects on the microflora.

High dietary fiber powders can be obtained from both food and agricultural residues. Dietary fibers can be recovered from orange peels, in peach residues, orange and lemon peels, or apple pomace. Agricultural residues like corn, wheat, and rice bran are also a good source of such valuable compounds. Thanks to their physicochemical features, dietary fibers can find application in food industry for improving the texture, viscosity, and shelf life of foods: several reports are found in literature concerning their beneficial effects when added to jams, bread, pasta, frozen foods, dairy products, etc. (Elleuch et al. 2011).

Pectins are the most popular polysaccharide from food wastes that, for generations, have been used as food ingredients or additives in all countries of the world. Their chemical structure, characterized from the coexistence of polar and nonpolar regions, makes them suitable for incorporation in different kinds of foods. Commercially employed pectins are mainly derived from apple pomace and citrus peels, and their principal application is in food industry as gelling agents, thickeners, water binders, and stabilizers. Pectins are indeed exploited for the industrial preparation of jam and jellies, to which they are added as a powder during the cooking step; fruit juices, where they act as clouding agents; soft drinks, where they counterbalance the deprivation of sugar; yogurts, where they prevent floatation of fruit pieces; frozen foods, where they exert a great firming effect and retard ice crystal growth thus improving food texture (Thakur et al. 1997).

Polysaccharides from food wastes have also a great biotechnological potential as sustainable sources of prebiotics. The latter are a group of food ingredients that

resist digestion in the intestine and stimulate the growth and activities of indigenous bacteria (commonly *Bifidobacteria* and *Lactobacilli*) that constitute the beneficial gut microflora. Oligosaccharides are among the most known molecules that are able to exert a prebiotic effect, and some of them can be produced starting from food wastes by means of enzymatic modifications. Pectic oligosaccharide with prebiotic properties has been prepared from commercial pectins by using an enzyme membrane reactor and proved to be effective against toxins produced by *Escherichia coli* and to be able to induce apoptosis in human adenocarcinoma cells. Moreover, pectic oligosaccharides recovered from orange peel were able to favor *Bifidobacteria* sp. and *Escherichia rectale* growth (Pandey and Nigam 2009). Cellulose oligosaccharides also showed prebiotic properties: indeed the cellulosic fraction of apple pomace was used to produce prebiotic oligosaccharides like glucooligosaccharides, xylooligosaccharides, and arabinooligosaccharides by digestion using an enzyme cocktail (cellulose and cellobiase from *Trichoderma reesei* and *Aspergillus niger*) (Pandey and Nigam 2009).

5.4 Feedstock for the Production of Chemicals, Enzymes, and Biopolymers by Microbial Fermentation

The polysaccharide components of residual agricultural raw materials can be used, by means of bioconversion, for the production of chemicals like organic acids (citric acid and lactic acid) or building block molecules, enzymes, and biopolymers that in turn are employed in a wide range of industrial fields.

With regard to organic acids, citric acid has a great commercial importance since it has many applications in food industry (as antimicrobial, preservative, or pH adjuster for soft drinks, confectionery, dairy, marmalades, fats, and oil production), in pharmaceutical industry (as anticoagulant, acidulant, or effervescent), in cosmetic industry (as antioxidant, metal chelator, or pH adjuster), and finally for other miscellaneous sectors (textile, paper industry, tobacco production, waste treatment, etc.) (Pandey and Nigam 2009). Thanks to their high polysaccharide content, citrus wastes and apple pomace have been evaluated as carbon sources for the production of citric acid employing *A. niger* strains in both submerged fermentation (SmF) and solid-state fermentation (SSF) conditions (Pandey and Nigam 2009). Citric acid can be produced by means of SmF with different *A. niger* species using several agro-residues including apple pomace, orange and carrot wastes, coffee husks, and sugarcane and cassava bagasse. Lactic acid is another organic acid that can be produced using waste polysaccharides. It is also used for several purposes in food industry (as preservative, for production of fermented foods and dairy products), in pharmaceutical industry (as blood coagulant or for preparation of anti-inflammatories), and for the synthesis of biodegradable and biocompatible plastics (by polymerization to polylactic acid, PLA). Lactic acid is produced in SSF conditions by means of fungi (*Rhizopus* species) or bacteria using cassava bagasse, wheat straw, or carrot wastes (Pandey and Nigam 2009).

With regard to the building block molecules production (Pacific Northwest National Laboratory 2004), some hemicellulosic wastes have been investigated for the production of xylitol, a natural sweetener substitute of sucrose that is widely used in food industry because it is anticariogenic and also suitable for diabetes patients (Canilha et al. 2005). Xylitol can be produced by fermentation of xylose recovered from hemicellulose hydrolysates from several food and agricultural wastes such as: barley and corn wastes, by *Debaryomyces hansenii* fermentation (Cruz et al. 2000); corn cobs, by a xylitol-producing yeast (Domínguez et al. 1997), *Candida* sp. 11–2; and peanut husks by *A. niger* fermentation (Mudaliyar et al. 2011).

With regard to enzyme production, agro-wastes are also valuable feedstock for their industrial production by means of microbial fermentation. A remarkable example is represented by residual cellulose polysaccharides that can be employed as sole carbon sources for the fermentation of biotechnological strains that in turn are usually exploited for the production of industrially useful enzymes. This is the case, for example, of cellulose and hemicellulose from sugarcane bagasse that have been exploited to produce: cellulase enzymes by fermentation of *Streptomyces*, *Trichoderma*, and *Aspergillus* species, xylanase that can be generated in SSF conditions by cocultured *T. reesei* and *A. niger* or *A. phoenicis* or by *Penicillium janthinellum* and *Trichoderma viride*, and inulinase, by fermentation of *Kluyveromyces marxianus* (Pandey and Nigam 2009). Also pectins in food wastes like citrus and apple pomace can be exploited as fermentation medium for the production of useful enzymes. Such wastes have been investigated, both in SmF and SSF conditions, as growth substrate for the production of bacteria, yeasts, or fungi producing pectinase activities. Such enzymes are the object of interest since they are used in different fields like in food and alcoholic drinks production, in textile and paper industry, for wastewater treatment, etc. (Jayani et al. 2005). Moreover polysaccharide fraction in apple pomace has been exploited also to produce by fermentation other useful enzymes like β-fructofuranosidase, xylanase, β-glucosidase, peroxidase, and cellulose activities. Similarly it has been reported for citrus waste polysaccharides that they can be used to produce α-amylase, protease, xylanase, and cellulose enzymes (Pandey and Nigam 2009).

Finally, with regard to microbial biopolymers' production, an interesting example is given by residual starch from cassava that has been employed also for xanthan gum's production by means of *Xanthomonas campestris* SmF on cassava acid hydrolysate as fermentation medium. Other studies showed the benefit in using residual pectin, cellulose, and hemicellulose recovered from citrus wastes to produce xanthan, by means of *X. campestris* fermentation (Pandey and Nigam 2009). Commercial uses of xanthan gum are mainly as food additives in salad dressing and sauces or for the preparation of low-fat and gluten-free foods. Acid hydrolysates from sugarcane bagasse have been used for the synthesis of microbial biopolymers like polyhydroxyalkanoates (PHAs), a biopolyester that is the object of interest as biodegradable plastic.

Bagasse polysaccharides have been used to produce PHA by aerobic bacterium *Ralstonia eutropha*.

5.5 Pharmaceutical Applications

Several kinds of polysaccharides already find numerous applications in the pharmaceutical industry, for example, cellulose that since many years is usually used for drug delivery, or starch that is the starting material for cyclodextrins' production. Notably, also polysaccharides from food and agricultural residues recently have received attention for their biological properties that make them potentially useful for different pharmaceutical applications.

Pectins can be exploited for pharmaceutical purposes, for example, for the preparation of mucoadhesive polymers for gastrointestinal adhesion, in combination with other polymers such as Carbopol and chitosan; against poisoning by lead and mercury; as antihemorrhagic and coagulating agent; for overeating control; and for drug delivery, for controlled release of active principles in the treatment of diseases like ulcerative colitis, Crohn's disease, or colon carcinomas (Srivastava and Malviya 2011). Pectic polysaccharides from citrus wastes are able to bind and thus inhibit galectin-3 (GAL3), a prometastatic protein that is overexpressed in many cancer types.

By means of enzymatic and/or alkali treatment, it is possible to produce chemically modified pectins that have also been claimed to be potential anticancer agents. Some modified citrus pectins, patented as PectaSol (commercially available as supplement) and as GCS-100 (Maxwell et al. 2012), showed indeed to be active against human prostate cancer and to be effective in the treatment of patients showing solid tumors, respectively. The encouraging results obtained in human studies have been confirmed also in animals and tumor cell lines. Oral intake of modified citrus pectins in mice caused a decrease in colon cancer; on the other hand modified apple pectins showed to be able to lower inflammation and to prevent tumor formation in a mouse model of colitis-associated colon cancer. Several studies performed with cancer cell lines showed that modified pectins from citrus fruits are able to inhibit GAL3 and thus to induce apoptosis in murine endothelial cells (Maxwell et al. 2012); moreover, modified citrus pectins have been shown to be active against cultured leukemic cells (Ramachandran et al. 2011).

Hemicellulose and in particular xylans from agricultural wastes can be useful for biomedical applications: xylans recovered from corn stover and wheat straw have indeed been reported to inhibit the growth rate of sarcoma-180 and other tumors, and on the other hand water-soluble AGXs isolated from corn residues showed to possess in vitro mitogenic and co-mitogenic activities (Ebringerova et al. 1999).

Finally a glucan isolated from tomato wastes showed interesting biological properties as a potential anti-inflammatory agent. Indeed in an in vitro experiment of J774 macrophages stimulation with bacterial lipopolysaccharide, the tomato waste polysaccharide proved to be able to inhibit NF-κB activation and iNOS

expression by preventing the production of reactive oxygen species. Therefore it could be a promising molecule for controlling oxidative stress and/or inflammation processes (De Stefano et al. 2007).

5.6 Feedstock for the Production of Second-Generation Bioethanol

Polysaccharides from wastes and more specifically from cellulosic agricultural residues represent the main and most important feedstock for the production of second-generation bioethanol, since they are not in competition with food chain. Lignocellulosic wastes could afford a less environmentally impacting production of ethanol because their production do not require further exploitation of land (they are the residues of "primary crops") and also because they are available at zero cost in significant amounts (see also Tables 1 and 2).

The most studied waste biomass for second-generation bioethanol are some lignocellulosic residues, like cereal straw or sugarcane bagasse: the biotechnological potential of cellulosic and hemicellulosic residues for renewable energy production has been indeed the object of various researches carried out in the last 40 years. Due to the complex chemical composition and to the recalcitrance of lignocellulosic biomass, such studies have been focused on the problems connected to the different stages of the industrial process for conversion of biomass to fermentable sugars and finally to ethanol, i.e., the initial delignification treatment step, necessary to remove the nonsugar lignin fraction from the carbohydrate complex matrix, the fractionation of the complex polysaccharide matrix made of cellulose embedded in the hemicellulose envelope, the production of highly concentrated monomer sugar mixtures by means of enzymatic hydrolysis of polysaccharides (the so-called saccharification), the fermentation of sugar syrups, and the final distillation phase to gain pure ethanol.

The pretreatment step that is necessary to enable enzymes to degrade the glycosidic bonds in the polymer, by enhancing the surface area, can be accomplished by steam explosion, ammonia fiber explosion (AFEX), SO_2/H_2SO_4 or CO_2 explosion, hot liquid water, dilute acid or alkaline treatment, or finally ionic liquid or ligninolytic enzyme treatment. After the first step, hydrolysis of polysaccharides (saccharification) is carried out by using cellulose enzymes: the most studied include cellulose and β-glucosidase enzymes produced by fungi such as *Trichoderma reesei*, *Trichoderma viride*, and *Aspergillus niger*. The mixture of sugar resulting from cellulose and hemicellulose depolymerization is therefore fermented by ethanologenic microorganisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The main challenge associated to the fermentation in bioethanol production based on cellulosic feedstock is represented by the conversion of pentoses (deriving from hemicellulose fraction degradation) that are not fermented by the conventional yeast species. For this reason many recent researches have been devoted to identify a microorganism able to ferment both C6 and C5

sugars generated from hydrolysis of lignocellulosic biomass. Among the most interesting species is also *Z. mobilis* that has shown to be able to ferment both glucose and xylose derived from corn stover.

The biotechnological process by which biomass after pretreatment is converted in ethanol can be accomplished in different configurations, and it is the object of study too. The basic process is represented by separate hydrolysis and fermentation (SHF) that allows the optimal temperature conditions either for cellulose enzymes or for fermenting microorganisms, but that can be less efficient due to the glucose inhibition of cellulose enzymes; therefore, a simultaneous saccharification and fermentation process is under investigation to afford higher saccharification yields. Although the latter could be a more sustainable and efficient process, some problems are still to be solved, such as the different temperature operation's conditions required from enzymes and yeasts. For these reasons recently the so-called consolidated bioprocessing (CBP) has received increasing attention: in such a biotechnological process, biomass would be converted in ethanol by using a single engineered strain able to carry out the cellulase enzyme production, the saccharification of cellulose, and the fermentation of monomer sugars to ethanol (Cheng and Wang 2013). It is noteworthy to underline that a commercially available microorganism able to perform a CBP process has not yet been reported, but research in this field is still going on (Pandey and Nigam 2009).

6 Conclusion

Vegetable biomass is one of the most promising renewable sources of energy and chemicals. Its exploitation for biofuel and commodity chemicals is indeed the focus of continuous researches since many years. In the last decades the gradual shift toward the so-called biobased economy (an economy system based on exploitation of renewable resources such as biomass) has determined a global increasing demand for goods derived mainly from polysaccharide components of vegetable biomass. However this phenomenon is causing many environmental, social, and economical problems such as the intensive exploitation of land (e.g., for energy crops), the competition with food chain, and the consequent increase of foods prices, especially dramatic for developing countries. In order to ensure a more sustainable productivity and the global food security, wastes coming from biomass cultivation, harvesting, and processing of vegetables are more currently considered as an alternative source for polysaccharides that are used for energy and chemical production.

Indeed different kinds of agro-wastes such as food residues (coming from industrial processing of cereals, fruits, and vegetables) or such as agricultural wastes (resulting from harvest and postharvest operations of sugar or cereal crops) are massively produced every year. Therefore several million tons of such residual biomass are available as valuable sources of polysaccharides that in turn

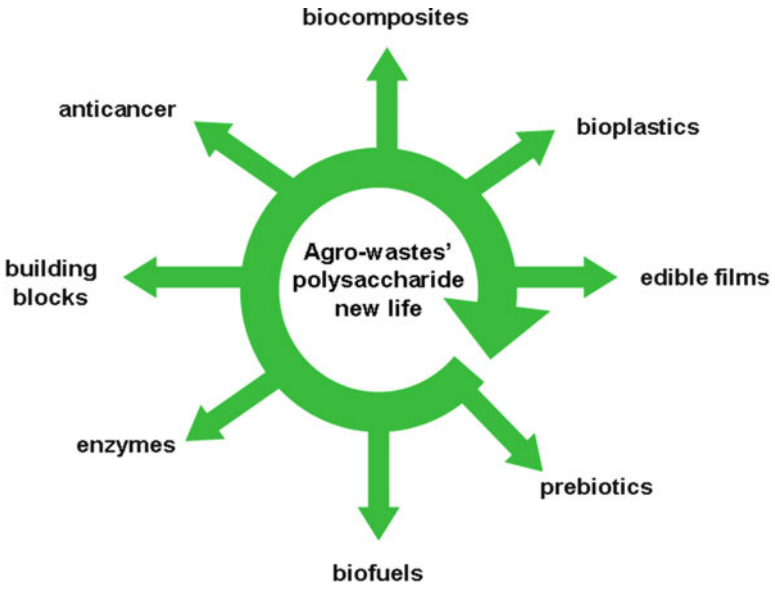


Fig. 18 Schematic representation of potential biotechnological uses of agro-wastes

could be exploited as feedstock for the production of a wide range of value-added chemicals and for renewable energy generation.

Several biotechnological strategies for the conversion of agro-waste polysaccharides are available for the production of food additives (dietary fibers, stabilizers, preservatives, sweeteners, etc.), of biomaterials (biocomposites, edible films for food packaging, or biodegradable plastics), of bioactive compounds (prebiotic or anticancer agents), of enzymes and value-added compounds (organic acids or building block chemicals), and of renewable energy (second-generation bioethanol) (Fig. 18).

Some of these production systems are already an industrial reality, while others are under investigation for the scaling up of laboratory scale processes. A remarkable example of the biotechnological potential of agro-wastes is represented by polysaccharides from tomato canning residues. Indeed in a recent study, it showed promising features (Fig. 19) since it was able to inhibit inflammatory processes, but also it could be used as a building block for biodegradable plastic suitable for agricultural uses.

The future biobased economy development will be limited only by the availability of biomass; therefore, in such a scenario polysaccharides from agro-wastes can represent not only a more environmentally friendly and sustainable source of goods but also the economical solution to supplementing feedstock for a wide range of industrial activities.

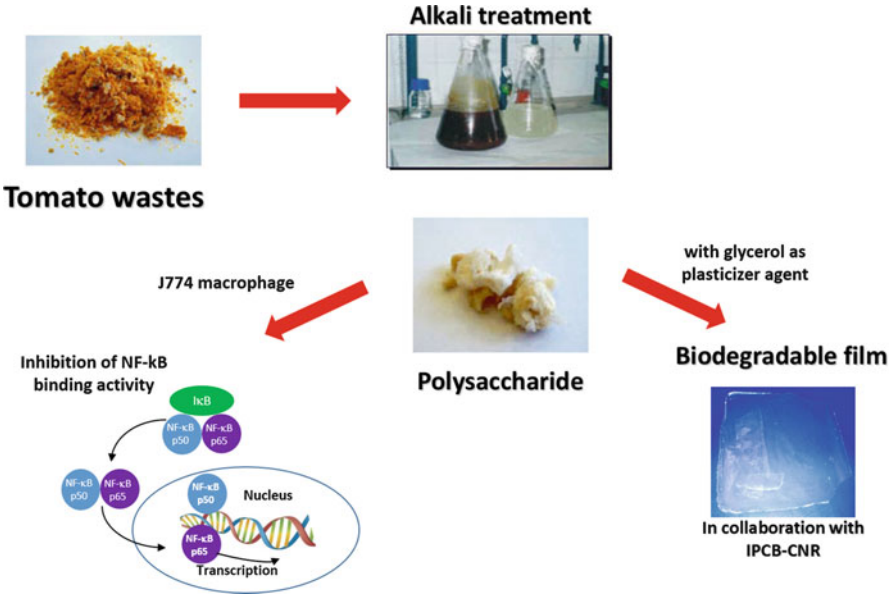


Fig. 19 Schematic representation of potential uses for tomato waste polysaccharides

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Part II

Food

Gum of *Prosopis*/*Acacia* Species

21

Yolanda L. López-Franco, Francisco M. Gooycolea, and Jaime Lizardi-Mendoza

Contents

Abstract

This chapter presents the structural and physicochemical characteristics, functional properties, and main applications of two exudate gums: gum arabic (*Acacia senegal*) and mesquite gum (*Prosopis velutina*). Gum arabic is the oldest and best known of all natural gums. It possesses physicochemical characteristics and functional properties that make it unique in the international trade of food, pharmaceutical, textile, and other industries. Mesquite gum is other exudate less known in the international market, but it has potential applications in food and other industries. The chemical structure and functional properties of mesquite gum are comparable with gum arabic. It is a good emulsifier and stabilizer of oil–water emulsions, and it may be compared with gum arabic for such application. The information contained in this chapter considers that mesquite gum can be a suitable replacement of gum arabic in arid regions of the world where *Prosopis* trees have widespread occurrence.

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Polysaccharide • Exudates • Gum arabic • Mesquite gum • Chemical structure • Functional properties • Industrial applications

1 Introduction

Gums represent one of the most abundant raw materials due to their sustainable and bio-safe characteristics. The term gum is used to describe a group of natural polysaccharides that have widespread industrial applications due to their ability either to make the viscous solutions or stabilize the emulsion systems. The plant gum exudates are complex polysaccharides produced by species of trees of the genus *Acacia* and *Prosopis*. Exudation is produced under conditions of heat and drought stress, as part of the normal metabolism of plants or result of the protection mechanisms against mechanical or microbial injury.

Plant gum exudates are highly heterogeneous complex polysaccharides with variable quantities of protein and other minor components (i.e., lipids, polyphenols). The chemical composition, physicochemical characteristics, and quality of these gums differ from botanical species and from cultivar to cultivar. Also, there is a considerable variation in gums from the same species collected from trees grown under different climatic conditions or even from the same tree in different seasons (Boothby 1983; Nussinovitch 2010). These variations can also be attributed to factors such as size and age of tree, soil type, rainfall, drought, temperature, storage conditions, and processing method (Nussinovitch 2010; Al-Assaf et al. 2012).

Chemically, gum arabic and mesquite gum are known as macromolecular complexes of type II AG and proteoglycans (arabinogalactan protein, AGP) comprising ca. 2.0–2.5 % of protein to gum arabic (Randall et al. 1989; Osman et al. 1993a, b) and 3–7 % to mesquite gum (López-Franco et al. 2004, 2008). The gums are soluble in water but not in organic liquids such as benzene, chloroform, ether, turpentine, and fixed oils (Howes 1949). These polysaccharides are capable to produce highly concentrated solutions without large viscosity increase. The considerably growing interest in plant gum exudates is due to their diverse structural characteristics and functional properties in food, pharmaceutical, cosmetic, textile, and biomedical products and colloid science. Gums can be used as a dietary fiber, texture modifiers, thickeners, emulsifiers, stabilizers, coating agents, and packaging films.

2 Gum Arabic (*Acacia senegal*)

2.1 Source and Production

There are approximately 1,373 *Acacia* species distributed throughout tropical and warm areas of the world. It is possible to find 955 species in Australia, 185 species in America, 144 species in Africa, and 89 species in Asia (Al-Assaf et al. 2007).

Many of these species produce and exude gum. This gum is a polysaccharide hydrocolloid with many practical uses. Sudan is currently the largest gum producer in the world with a gum arabic belt zone of approx 520,000 km^2 (Nussinovitch 2010). *Acacia senegal* and *Acacia seyal* are the most commercially exploited species and referred to as gum arabic.

The gum formation occurs in the cambial region of the stems or branches by a process named gummosis. The tree must be 5 years and older for gum production to happen. The exudation can be caused either by natural causes such as heat, drought, and attack by insects or pathogens or by injuries caused intentionally (tapping). Distinctively, in Sudan gum arabic tapping process is well established, and it is carried out by farmers during the dry season (November/December). The gum yield is influenced by environmental circumstances as rainfall or maximum temperature and also by harvesting conditions like the time of tapping or its intensity.

Superficial incisions are commonly made on the branches by using axes, but a more sophisticated tool, called a sonki (sharp spear), is available (Elrayah et al. 2012). The gum is exudated as sticky fluid and grown up to 1.5–7.5 cm in diameter and gradually dried and hardened in the sun and collected in the wound within 4–6 weeks depending on the weather condition. The tapping is done once a year, although in some areas there are two tapping seasons. However, it has been suggested that a second tapping has a significant damaging to the health of the trees (Elrahay et al. 2012: Al-Assaf et al. 2012); therefore one tapping season with more than one picking is recommended.

The production of gum arabic suffers large seasonal fluctuations depending primarily on the weather and also on its price. The production and trade of gum arabic is dominated by Sudan, accounting for 80–90 % of the world market (Chikamai et al. 1996). Nigeria is the second largest producer and exporter of this hydrocolloid. The production of gum arabic in these regions faces a number of problems, such as weather conditions, plagues (e.g., locusts), scarce funding for smallholders, and political instability. However, the production in Sudan has been estimated between 25,000 and 95,000 ton, averaging 50,000 ton per year (Williams and Phillips 2000). Other African countries as Ethiopia, Tanzania, Cameroon, and Senegal produced and exported volumes below 1,000 ton a year.

The gum is frequently exported raw, and only manual cleaning, sifting, and sorting operations are carried out to remove large impurities that could occur. Clean, large, and light color nodules make the handpicked selected (HPS) grade. This grade is the most expensive and tends to be of highest quality compared to the rest of the gum (Al-Assaf et al. 2012). Also, gum arabic is processed at industrial scale by companies such as Dansa Food Processing Co. Ltd in Nigeria, or Sanimex in Chad, or Khartoum Gum Arabic Processing Co (GAPC) in Sudan. These companies produce spray-dried gum arabic powder of various grades for direct use without further processing, and it is exported to global market. The international market for gum arabic has been usually unstable, due to an increasing demand, social and political volatility in the producing regions, and large variability in price, quality, and supply. Europe (France, the United Kingdom, Germany, Italy, Belgium, and Ireland) is the biggest importer of gum arabic, and the United States is its

second largest market. France and the United States are the mayor countries for both importing and reexporting gum arabic products (Verbeken et al. 2003).

2.2 Collection

Partially dried nodules of gum arabic are collected manually on multiple harvests every 2 weeks. A yield of 0.5–2 kg is obtained per tree annually, although production may range from few grams up to 10 kg (Elrahay et al. 2012). The collected gum is cleaned manually, usually by women who remove foreign matter and sort the gum according to the size of the pieces.

2.3 Regulatory Status

Gum arabic originating from the *Acacia senegal* and the *Acacia seyal* is recognized by the Codex Alimentarius (food additive E414). The gum is safe for human consumption based on a long history of usage as well as toxicological studies. The exudate is also used in nonfood applications, such as: pharmaceuticals, cosmetics, textiles and lithography, and minor forest products (Wang and Anderson 1994).

Gum arabic contains no pathogens and no more than 10^3 microorganisms per gram (Blake et al. 1988). Because of the high temperatures involved, spray-dried preparations contain no more than ~40 % of the usual microorganism count (~4×10^2 microorganisms per gram). The number of viable bacteria contained in the gum can also be reduced by treatment with ethylene oxide (no longer permitted for food use), or propylene oxide (less efficacious). Heating carried out during manufacture to reduce the microflora can lead to precipitation of the arabinogalactan protein complex (Anderson and McDougall 1987a), which promotes stabilization and emulsification in a range of food products (Randall et al. 1989).

Recent European Community legislation has approved gum arabic as an ingredient which can be labelled as a food dietary fiber. This recent change has created an opportunity and demanded for *A. seyal* due to its lower viscosity, which can be used at higher concentration compared to *A. senegal*. The low viscosity is due to its highly branched structure and therefore can be dissolved up to 50 % (wt %) to produce a solution of moderate viscosity.

2.4 Structure

The physicochemical characteristics of gum arabic are listed in Table 1. These characteristics may vary depending on the geographical origin and age of trees, weather conditions, soil environment, and the place of exudation on the tree (Islam 1997).

The chemical structure of gum arabic consists of (1→3)-linked β-D-galactopyranose backbone with branches of (1→6)-linked galactopyranose units.

Table 1 Physicochemical characteristics of gum arabic from *Acacia senegal*

Characteristics	Gum arabic
Appearance	Amber transparent nodules
Nodule size (cm)[a]	1–6
Nodule color[a]	Light
Moisture (%)[b]	8.08
Total ash (%)[b]	3.80
Tannin (%)[b]	0.38
Protein[e] (%)[b]	3.73
pH[b]	4.32
Specific rotation $[\alpha]_D^{25}$	−28.77
AEW (g mol^{-1})[b]	1,121
Viscosity 5 % (mL g^{-1})[f]	17.7
Arabinose (%)[c]	24
Galactose (%)[c]	45
Rhamnose (%)[c]	13
Glucuronic acid (%)[c]	16
4-*O*-Me-glucuronic acid (%)[c]	1.5
Structural parameters[d]	
M_w (g mol^{-1})	4.39×10^5
R_h (nm)	22.36
R_g (nm)	35.02
Ratio R_g/R_h	1.57

[a]From Al-Assaf et al. 2012
[b]From López-Franco et al. 2012
[c]From Anderson et al. 1990
[d]From López-Franco et al. 2004
[e]Protein = N × 6.60
[f]At 25 °C in 0.1 M NaCl

Both the main and the side chains contain residues of α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl, and 4-*O*-methyl-β-D-glucuronopyranosyl. It also contains 2–3 % of protein as an integral part of the structure (Randall et al. 1988).

The gum has been studied extensively by different techniques such as: sequential Smith degradation (Anderson and McDougall 1987a, b, c), enzymatic degradation (Connolly et al. 1988), hydrophobic interaction chromatography (Randall et al. 1989; Osman et al. 1993a; Ray et al. 1995; Fauconnier et al. 2000), anion exchange chromatography (Osman et al. 1995), gel permeation chromatography (Osman et al. 1993b; Idris et al. 1998; Picton et al. 2000; Al-Assaf et al. 2005a, b), and asymmetrical flow field-flow fractionation (Alftrén et al. 2012).

Using hydrophobic interaction chromatography, it has demonstrated the heterogeneous complex nature of this polysaccharide by the presence of three main fractions: arabinogalactan (AG), arabinogalactan protein complex (AGP), and glycoprotein (GP). Each fraction contains a range of different molecular weight components with different protein contents (Randall et al. 1989; Williams et al. 1990b).

These components represent 88 %, 10 %, and 1 % of the molecule. The protein is covalently attached to the carbohydrate and demonstrated the presence of hydroxyproline-oligoarabinose and serine-carbohydrate linkages (Akiyama et al. 1984).

The molecular structure of AG fraction of gum arabic was unknown until Sanchez et al. (2008) proposed a thin disk model based on small-angle neutron scattering and microscopy. The model consisted of an inner branched structure and a ring both mainly composed by sugar residues (98 %). Polysaccharide domains were linked to a central short polypeptide backbone, forming a spheroidal random coil (Sanchez et al. 2008).

The AGP fraction conformation is described by the "wattle blossom" model in which approx. five AG blocks, ~200,000 Da each, are arranged along the GI polypeptide chain which may contain up to 1,600 amino-acid residues (Connolly et al. 1987). Other alternative model proposed for the structure of AGP of gum arabic is the twisted hairy rope (Qi et al. 1991). Using preparative gel permeation chromatography and transmission electron microscopy, it was possible to find that the molecule consisted of ~400 amino acids containing ~130 hydrohyproline residues with a 10–12 residue repetitive motif. The polypeptide backbone of the gum arabic GP is composed of repeating sequences of 19 amino acids (Goodrum et al. 2000).

2.5 Physicochemical Properties

Gum arabic functional properties are closely related to its structure, which determines, for example, solubility, viscosity, degree of interaction with water and oil in an emulsion, and microencapsulation ability, among others. Table 1 gives some physicochemical characteristics of the gum arabic from *Acacia Senegal*.

Solubility: Gum arabic is highly soluble in hot and cold water in concentration up to 50 %. The gum has the ability to produce concentrated solutions without causing an excessive increase in viscosity, because principally to the branched and compact structure and a small hydrodynamic volume (Williams et al. 1990a; Goycoolea et al. 1995; Verbeken et al. 2003).

Viscosity: Gum arabic solutions are distinguished by their low viscosity (0.19 dL g^{-1}) at high concentrations (50 % w/w) (Goycoolea et al. 1995), enabling the use of high gum concentrations in various applications (Dziezak 1991; Imeson 1992). Solutions display Newtonian behavior at concentrations of up to 40 % and become pseudoplastic at higher concentrations. Above ~30 %, the hydrated molecules effectively overlap and steric interactions result in much higher solution viscosities generating the pseudoplastic behavior (Nussinovitch 1997). The pH of the solution is usually around 4.5–5.5, but maximal viscosity is found at pH 6.0. At still higher pH, ionic strength of the solution increases until the repulsive electrostatic charges are masked, yielding a compact conformation with lower viscosity (Anderson et al. 1990; Williams et al. 1990a, b; Imeson 1992). The use of prolonged heating causes the denaturation and precipitation of the proteins from AGP and GP fractions, and these cause a reduction in the emulsification capacity and solution viscosity.

Molecular Association: The physicochemical characteristics of gum arabic (i.e., molecular weight, shape, size, etc.) determine the way how the molecules interact with each other in aqueous media. The resulting molecular associations can affect its function in specific applications. There are several factors such as hydrogen bonding, associations mediated by ions, and electrostatic or hydrophobic interactions, which depend on the gum concentration, and the presence of protein components that affect the ability to form supramolecular complexes.

Acacia gum (gum arabic) can be modified in the dry state in a range of physical forms. The treatment involves maturation under strictly controlled conditions of temperature and humidity of the dry gum (Al-Assaf et al. 2007). The process does not involve change in the basic structural components, and while the maturation takes place, the level of association increases giving way to AGP with higher molecular weight and protein content. Subsequently, the role of protein components in gum arabic to promote molecular association when the gum is subjected to different processing treatments such as maturation, spray drying, and irradiation was analyzed (Al-Assaf et al. 2009). Results demonstrate the ability of protein components to promote hydrophobic associations that influence the size and proportion of the high-molecular-weight component AGP. When gum arabic undergoes maturation, there is an increase in the hydrophobic nature of the gum and therefore an increase of its emulsifying properties. Spray drying involves not only the aggregation through hydrophobic associations but also changes in the surface properties of protein increasing gum arabic hydrophilicity compared with the association promoted by the maturation treatment in solid state. Ionizing radiation in both aqueous solutions and solid state induces cross-linking between polysaccharide blocks by the formation of C−C bonds (Aoki et al. 2007).

Emulsification Capacity: It is now widely accepted that gum arabic is an excellent emulsifier, thanks to its amphiphilic character due to the presence of protein and polysaccharide moieties. It works by reducing the oil–water interfacial tension, thereby facilitating the disruption of emulsion droplets during homogenization. The protein is strongly adsorbed onto the oil droplet surface, while the polysaccharide chains extend out into aqueous phase, preventing droplet flocculation and coalescence through electrostatic and steric repulsion forces. The interfacial membrane formed around the oil droplets prevents them from aggregating (flocculating and/or coalescing). However, only 1–2 % of the gum is absorbed into the oil–water interface and participates in the emulsification; thus, over 12 % of GA content is required to stabilize emulsions with 20 % orange oil (Randall et al. 1988). Randall et al. (1988) reported that the AGP complex is the main component responsible for gum arabic ability to stabilize emulsions, and after treatment with protease, the gum significantly reduces its emulsifying properties.

Encapsulation Capacity: In the food industry, encapsulation is an important process to improve the chemical stability of sensitive compounds that need to be protected, isolated, or slowly released over time. The encapsulating material must preserve and protect the encapsulated compounds during manufacture, storage,

and handling to release them into the final product during manufacture or consumption. Gum arabic is an effective encapsulant agent because it produces stable emulsions with most oils in a wide pH range, and it has the ability to form films (Kenyon 1995).

Nutraceutical Properties: Gum arabic can be categorized as a nondigestible carbohydrate or dietary fiber. It is fermented by intestinal bacteria to short-chain fatty acids, particularly propionic acid in the large intestine, leading to a wide range of potential health benefits (Phillips and Phillips 2011).

Compatibility: The gum arabic is compatible with most gum, starches, and proteins, but it is incompatible with sodium alginate and gelatin. However, gum arabic in combination with gelatin at low pH gives coacervates that can be used for oil encapsulation (Imeson 1992).

2.6 Food Applications

Gum arabic has been used in a wide range of food products for many years. It is a multifunctional food additive being emulsifier, flavoring agent, humectant, stabilizer, thickener, and retards sugar crystallization. In the food industry, gum arabic is primarily used in confectionery, bakery, dairy, beverage, and as a flavor microencapsulating agent (Imeson 1992).

Confectionery: Gum arabic has a major role in the confectionery industry for many centuries, especially in products in which sugar content is high and moisture content comparatively low, from soft lozenges to hard gums (Whistler and BeMiller 1973). The gum may influence crystallization in three different ways: the hydrocolloid can attach itself to a growing crystal surface and thus alter its normal growth pattern; the hydrocolloid and the crystal may compete for the same building blocks; the gum may combine with impurities that affect crystal growth (Shuman 1960; Glicksman 1969). Low levels of gum arabic (up to 2.0 %) are included in chewy sweets based on gelatin to improve product adhesion, reduce elasticity, and produce extra-fine sugar crystallization with a smooth texture. Chewy gels with desirable texture can be produced by creating special conditions of pH, protein concentration, and heating, among others (Anson and Pader 1958). Concentrated gum acacia solution (up to 25 % w/w) is applied over snacks as an adhesive coating to retain seasoning, e.g., dry-roasted peanuts (Idris and Haddad 2012). It is also used in toffees as an emulsifier to maintain a uniform distribution of the components throughout the product (Verbeken et al. 2003).

Bakery Products: Gum arabic is extensively used in baking due to its moisture adsorption properties. Baking properties of wheat and rye flours can be improved by adding a small amount of gum arabic since its capacity for retaining moisture reduces the hardening of bread. It also has favorable adhesive properties to be used in glazes, toppings, and meringues. Another useful property is that gum arabic provides softness when used as emulsion stabilizer (Glicksman 1983).

Beverages and Flavors: Gum arabic has a unique combination of excellent emulsifying properties and low solution viscosity, making it very useful as a stabilizer of citrus oil emulsion concentrates in soft drinks and as a flavor encapsulator. Gum arabic ability to stabilize foams is used in the manufacture of beer. In addition, it can be used to clarify wines (Idris and Haddad 2012) or as a source of soluble fiber in low-calorie and dietetic beverages (Phillips 1998).

2.7 Nonfood Applications

Gum arabic is being widely used for industrial purposes such as a suspending agent, stabilizer, thickener, adhesive and binder, emulsifier, and encapsulator in textiles, ceramics, lithography, cosmetic, and pharmaceutical industry (Verbeken et al. 2003).

In ancient Egypt, gum arabic was used as a binder in cosmetics and inks and as an agent in the mummification process. In cosmetics, gum arabic functions as a stabilizer in lotions and protective creams, where it increases viscosity, imparts spreading properties, and provides a protective coating and a smooth feel. It is used as an adhesive agent in blusher and as a foam stabilizer in liquid soaps (Whistler 1993).

Gum arabic is also used as a suspending agent or protective colloids in soluble inks, watercolors, and quick-drying and typographic inks. In these types of ink, the gum helps to reduce the sedimentation rate of particles in suspension. In lithographic processes, the gum is used to protect images during etching. During printing, water adheres to the gum arabic surfaces and avoids the oily parts, while the oily ink used for printing does the opposite (Whistler and BeMiller 1973).

Sizing is the application of various materials to a fabric to produce stiffness or firmness. Gum arabic is applied as a sizing agent for cloth, particularly in silk or rayon, where the gum imparts body to the fabric without interfering with its transparency (Whistler and BeMiller 1973).

It is used as a dispersant in paints and insecticidal/acaricidal emulsions, respectively, keeping the pigments and active components uniformly distributed throughout the product (Fuyama and Tsuji 1981). Other applications are corrosion inhibitor (Umoren et al. 2006), pigment manufacture, ceramics, and polishes (Nussinovith 2010).

Also, gum arabic is recognized by its ability to sustain colloidal stability for systems of carbon nanotubes in aqueous solutions due to nonspecific physical adsorption (Bandyopadhyaya et al. 2002). It is also used as a steric stabilizer in the preparation of colloidal silver particles (Velikov et al. 2003), and it has probed for the coating and increased biocompatibility of iron oxide magnetic nanoparticles (Williams et al. 2006; Banerjee and Chen 2007a, b, 2008; Roque and Wilson 2008; Wilson et al. 2008) and gold nanoparticles (Kannan et al. 2006; Kattumuri et al. 2007).

3 Mesquite Gum

3.1 Source and Production

Mesquite trees are leguminous plant trees that are widespread in arid and semiarid regions of the world. The genus *Prosopis* comprises about 44 species that grow mostly in North America (9 species) and South America (31 species) and also in northern Africa (1 species) and eastern Asia (3 species). Around ten species of *Prosopis* are found in Mexico being *P. juliflora* the most abundant (Vernon-Carter et al. 2000), which has been suggested to correspond with *P. laevigata*. In northwestern state of Sonora, *P. velutina* and *P. pubescens* are found, although it is possible to find hybrid species. The case of *P. chilensis* is notorious, that is, a mesquite species that was introduced and promoted as garden ornamentals or as shade trees in schools and private gardens. Now it could be found in the Sonora desert among other native species.

The bark of *Prosopis* produces an exudate known as mesquite gum as response to insect attack, wounding or weather conditions (temperature), drought, age, and health of the tree that can affect the exudation. The production of mesquite gum is not known around the world. However in Mexico, there are two main regions where mesquite gum is produced, in the desert plains of northwestern state of Sonora and in the lowlands of northeastern state of San Luis Potosi.

Mexico can be considered as the main producer of mesquite gum, and in the absence of national statistics, the information available on the production of mesquite gum from wild plantations comes only from few field studies that have estimated the availability of the gum in the two regions in Mexico since time ago. In central region of Sonora with mean tree density of 80 trees per ha, only 10 % of the trees produced gum. The amount of gum exudates was calculated in per tree equivalent to 240 g gum ha. The gum production spans over 5 weeks in hot–dry weather time (May–June) (Goycoolea et al. 2000). In San Luis Potosi, there is a higher tree density (200 trees ha), and 70 % of them produce gum in a production time of 24 weeks. With these data the estimated potential of production reach over 2,000 ton per year (Vernon-Carter et al. 2000). This production is sufficient to fulfill the demand of gum arabic that during the last 3 years was 1,500 ton·per year with an importation total price of six million of USD (Table 2) (INEGI 2012). However, mesquite gum is collected and traded at very low level, just to cover the demand of local commodities of markets. There are neither commercial plantations nor established processing facilities.

There is the possibility that the production of mesquite gum oscillates by the different factors mentioned above. Therefore, alternative production methods have been investigated. In vitro studies of the culturing of *P. laevigata* have demonstrated that the application of combined environmental conditions (temperature increase) and biotic elicitors can be utilized for increasing mesquite gum production with similar characteristics to those produced in situ by wild trees (Orozco-Villafuerte et al. 2003, 2005).

Table 2 Gum arabic imported by Mexico

Year	Imported quantity (kg)	Imported value (USD)
2010	1,500.130	5,951.875
2011	1,560.598	6,177.042
2012	1,572.775	6,106.625

Source: Statistical data from the Instituto Nacional de Estadística y Geografía (INEGI)

3.2 Collection

The production season of mesquite gum in Sonora begins during the late spring and early summer months May–July, ending with start of the rain season at late July or August. The collection of mesquite gum is done by hand in hard environmental conditions as low humidity, high temperature, and thorny vegetation and is not properly organized or established as the gum arabic. There is no standard quality grading system available to sort the mesquite gum nodules. However, they can be classified by size, color, and the contents of bark and foreign matter. This classification procedure is a method for the selection of mesquite gum that meets the quality requirements of the Joint FAO/WHO Expert Committee on Food Additives for gum arabic intended for foods (López-Franco et al. 2012).

3.3 Regulatory Status

Mesquite gum is not permitted as food additive neither by the FDA nor by the Codex Committee on Food Additives. However, in Mexico the gum can be used as processing aids in food and beverages (Secretaría de Salud 2006, 2012). The authorization was given after a multigeneration study to three generations in Wistar rats. The study evaluated and compared the toxicity and mutagenicity of mesquite gum (*P. laevigata*), commercial gum arabic and cellulose included in the diet. In general, the tests proved that the rats' growth, development, and survival rate were not affected by the inclusion of mesquite gum in the diet and that mesquite gum did not induce any kind of mutagenicity; thus it did not present any carcinogenic activity (Flores-Dominguez et al. 2000).

Mesquite gum is not an established hydrocolloid in the world market. However, the gum was widely used by the native cultures of the northwest of Mexico and southwest of the United States since pre-Columbian times (Felger 1977). It is used mainly as hard candy and as medicinal aid, i.e., to prepare eye drops or to treat sore throat (Felger and Moser 1974). Similar uses prevail in regions where mesquite tree grows, and this gives account of its safety for human consumption.

3.4 Structure

Mesquite gum is a neutral salt of a complex acidic branched polysaccharide formed by a core of β-D-Gal residues comprising a (1→3)-linked backbone with

(1→6)-linked branches and bearing L-Ara (pyranose and furanose rings form), D-glucuronic acid, and 4-*O*-methyl-β-D-glucuronic acid (White 1946, 1947a, b, 1948; Cuneen and Smith 1948a, b; Akher et al. 1952; Aspinall and Whitehead 1970a, b). Mesquite gum from *P. velutina* yields L–Ara and D–Gal as main carbohydrate residues, and traces of D-Glc, D-Man, and D-Xyl after acid hydrolysis were detected (López-Franco et al. 2008). Small amount of uronic acids (4 %), mainly glucuronic acid, was also detected. A notable composition difference with gum arabic is that *P. velutina* mesquite gum does not contain L-rhamnose (Rinaudo et al. 2008; López-Franco et al. 2013).

Mesquite gum contains protein (3–7 %) that is formed mainly by hydroxyproline, serine, glycine, and valine (Fincher et al. 1983; Goycoolea et al. 1998; Orozco-Villafuerte et al. 2003; López-Franco et al. 2008, 2012, 2013). Several studies have established that protein plays an important role on the emulsification properties of the gum (Goycoolea et al. 1995; Vernon-Carter et al. 1996, 1998).

In addition to the polysaccharide and protein components, mesquite gum has a lipid fraction mainly formed by palmitic (C16: 0), stearic (C18: 0), and behenic (C22: 0) fatty acids. Also, traces of phosphorous (P) were detected in the gum. These components could be related to the presence of glycosylphosphatidylinositol anchor (GPI) (López-Franco et al. 2013).

It has been documented that mesquite gum consists of three main fractions obtained by hydrophobic interaction chromatography and referred to as arabinogalactan (AG), arabinogalactan protein (AGP), and glycoprotein (GP). These fractions differ in molecular weight, protein content, and surface activity (López-Franco et al. 2004). The AGP represents about 2.49 % of the total gum, and it has 11 % of protein and a molar mass of 484,000 g mol^{-1} (López-Franco et al. 2004, 2008) and can be classified into classical AGPs due to its composition of carbohydrate (Ara and Gal) and amino acids (abundant quantities of Hyp, Ser, Gly) and because it reacts to the β-glucosyl Yariv reagent (López-Franco et al. 2013).

Unlike gum arabic, there are no models that explain the tertiary structure of AGP of mesquite gum. However, by structural parameters (R_g, R_h, $P_{(\theta)}$) obtained by light scattering, measurements have allowed to conclude that mesquite gum is more extended than gum arabic and that it can be considered as a polydisperse macrocoil (López-Franco et al. 2004) in agreement with the "twisted hairy rope" proposal AGP for gum arabic (Qi et al. 1991).

3.5 Physicochemical Properties

Physicochemical characteristics derived from various studies on the gums from *P. velutina* are compiled in Table 3.

Solubility: Mesquite gum has high solubility in aqueous medium, which can yield solutions above 50 % (w/w) concentration (Goycoolea et al. 1995). It is soluble in aqueous ethanol, with up to 70 % ethanol. In organic solvents mesquite gum has either limited solubility, e.g., in glycerol or ethylene glycol, or it is insoluble, e.g., in alkanes or oils (Vernon-Carter et al. 2000). The solutions of

Table 3 Physicochemical characteristics of mesquite gum from *Prosopis velutina*

Characteristics	Mesquite gum
Appearance[a]	Vitreous nodules
Nodule size (cm)[a]	>2
Nodule color[a]	Light red amber
Moisture (%)[a]	9.45
Total ash (%)[a]	2.6
Tannin (%)[a]	0.35
Protein (%)[d,b]	3.73
pH[a]	4.5–4.6
Specific rotation $[\alpha]_D^{25b}$	+55.10
AEW (g mol^{-1})[a]	1,282
Viscosity 5 % (mL g^{-1})[e]	10.9
Total sugars (%)[c]	96
Arabinose (mol %)[c]	71
Galactose (mol %)[c]	26
Rhamnose (mol %)[c]	nd
Glucuronic acid (mol %)[c]	3
Structural parameters[b]	
M_w (g mol^{-1})	3.86×10^5
R_h (nm)	9.48
R_g (nm)	50.47
ratio R_g/R_h	5.32

nd not detected
[a]From López-Franco et al. 2012
[b]From López-Franco et al. 2004
[c]From Rinaudo et al. 2008
[d]Protein = N × 6.53
[e]At 30 °C in 0.1 M NaCl

this type of gum display a range of colors that vary from slight yellow or amber to dark brown depending on concentration and botanical origin.

Viscosity: The viscosity of aqueous gum solutions decreased as the gum concentration increased (1.0–15 %). The solutions show shear thickening as the shear rate was increased up 637.12 s^{-1} (Vernon−Carter and Sherman 1980). Gum solutions at 20% (w/w) in 0.1 M NaCl, measured at 20 °C, display an average viscosity of 8.7 mL g^{-1} and Newtonian behaviour (Goycoolea et al. 1995). At higher concentrations, from 25% (w/v) and over, the mesquite gum solution exhibited a pseudo-plastic behaviour.

The intrinsic viscosity of 5 % (w/w) mesquite gum solutions in 0.1 M NaCl at 30 °C is 10.9 mL g^{-1}. This result was lower than that obtained with gum arabic at the same experimental conditions (21.18 mL g^{-1}) (López-Franco et al. 2013). This is related to the occurrence of larger-size macromolecular species in gum arabic than in mesquite gum.

Molecular Conformation: Static and dynamic light scattering techniques were used to determine structural parameters of native gum and fractions. It was possible

determine the ratio $R_g/R_h > 2$, indicating the possibility of an elongated structure for all samples of mesquite gum. The dependence of form factor with theoretical model allows to observe that mesquite gum and fractions were best fit by a model for a polydispersed coil structure (López-Franco et al. 2004).

Surface Activity: Mesquite gum showed the highest water and oil absorption at temperatures of 23, 35, and 45 °C in comparison with gum arabic. The activation energy for water and oil absorption for gum arabic was 21.98 and 39.57 kJ mol^{-1}, compared to that of mesquite gum having values of 15.79 and 46.16 kJ mol^{-1}, respectively (Beristain et al. 1996).

Mesquite gum and its fractions have been studied by Langmuir monolayers spread at an air–water interface and compared with gum arabic and its corresponding fractions. The most active species at the interface were those containing greater amounts of protein. However, the observed mechanical properties of the monolayer of mesquite gum and its fractions can also be explained by differences in macromolecular dimensions, conformation, and topology of the molecule and the hydrophobic/hydrophilic sites adsorbed at the interface (López-Franco et al. 2004).

The interfacial tension is affected by the gum concentration and the pH of the solution. It decreases when the gum concentration increases. At high concentrations (i.e., over 25 %, w/v) of mesquite gum, the interfacial tension decreases faster. The influence of pH solution has been also observed, and it has been related to molecular configuration changes of the gum (Vernon-Carter et al. 2000).

Emulsification Capacity: Mesquite gum is a surface-active biopolymer, because of its amphiphilic character. Mesquite gum forms and stabilizes oil-in-water emulsions and has the ability to encapsulate orange citrus oil during spray drying (Vernon-Carter et al. 1996; Beristain et al. 1996; Goycoolea et al. 1997). Mesquite gum solutions of 15 % (w/w) concentration are able to form emulsions with *n*-alkanes of varying chain length, D-limonene, and orange oil. The mean diameter of droplets of *n*-decane, *n*-dodecane, *n*-tetradecane, and *n*-hexadecane was of 4–4.5 μm, and with orange oil the average droplet diameter was found to vary in the range 2.5–3.0 μm. The behavior of particle size (R_h) for D-limonene emulsions showed a higher rate of change with time, at difference, than those orange oil droplets that remained almost without change after more than 100 h (Acedo-Carrillo et al. 2006). This behavior of mesquite gum on the orange oil emulsions to stop or control Ostwald ripening is attributed, among other causes, to the fact that orange oil is less water soluble than D-limonene (Rinaudo et al. 2008).

Encapsulation Capacity: The most widely used encapsulation agents are gum arabic and modified or hydrolysed starches. Mesquite gum has been reported as having the ability to encapsulate orange peel oil (Goycoolea et al. 1997) (80.5 % of the starting oil) (Beristain and Vernon-Carter 1994). A blend of gum arabic and mesquite gum (60:40) was able to encapsulate the same amount of orange peel oil compared to pure gum arabic (Beristain and Vernon-Carter 1995). Another blend of maltodextrin 10 DE and mesquite gum (3:2) retained 84.6 % of the starting orange peel oil, providing better encapsulating capacity. In other studies, the orange peel oil encapsulating capacity of native and low-tannin mesquite gums was slightly lower than gum arabic (Goycoolea et al. 1997, 1998).

Film Forming: Mesquite gum-based films have become an important research topic mainly due to their capability to regulate moisture, lipid migration, and gas transport and can be used to improve the quality and extend the shelf life of foodstuffs. Emulsion films based on mesquite gum as structural agent and a blend of candelilla wax with white mineral oil as the lipid phase prolong the shelf life of treated guava fruit (*Psidium guajava* L.) by retarding ethylene emission and enhancing the texture of the fruits (Tomás et al. 2005a). On the other hand, combinations of mesquite gum (*Prosopis* spp.) with whey protein concentrate form films less effective as moisture barriers (Tomás et al. 2005b). Mesquite gum–chitosan complex has also been used to form edible films against water vapor permeability (Ruiz-Ramos et al. 2006).

Compatibility: Mesquite gum has been used in combination with gum arabic, maltodextrins, lipids, vegetal and animal proteins, chitosan, sodium alginate, and κ-carrageenan. In all cases synergistic effects using the mesquite gum have been observed (Vernon-Carter et al. 1996, 1998; Perez-Alonso et al. 2003; Tomás et al. 2004; Pérez-Orozco et al. 2011).

3.6 Food Applications

Mesquite gum has been used in Mexico for centuries mostly in folk medicine and more recently as a substitute for gum arabic in food and drinks. However, the fact that it does not have approval from the FDA has limited its more widespread use in the world. Many studies account for the novel and beneficial uses of mesquite gum in food and other systems, and the main applications have been reviewed (Vernon-Carter et al. 2000).

As mentioned in the previous section, mesquite gum solutions are effective in the preparation and stabilization of oil-in-water emulsions. This has been exploited mostly for the stabilization of orange peel essential oil and oleoresins. Independent studies have demonstrated that mesquite gum exhibits smaller average oil droplet size and better stability than identical emulsions made with gum arabic (Beristain 1996; Acedo-Carrillo et al. 2006). Mesquite gum has been used in trials by the food industry. Soft drinks made from concentrated orange essential oil-in-water emulsions were found to require 70 % less mesquite gum than gum arabic for achieving similar initial particle size and stability, while no significant differences in flavor were detected among both formulations (Vernon-Carter et al. 2000). In other studies, Aztec marigold (*Tagetes erecta*) oleoresin-in-water emulsions have been stabilized against drop coalescence and loss of color. Also, in chilli oleoresin-in-water emulsions, mesquite gum has been found to confer smaller and more uniform initial particle size and greater stability against droplet coalescence and color degradation than gum arabic (Vernon-Carter et al. 1998).

Flavor and color encapsulation by spray drying are industrial applications of mesquite gum. To this end, mesquite gum has been used as the sole encapsulation agent or mixed with maltodextrin (Beristain and Vernon-Carter 1994;

Goycoolea et al. 1997, 1998) to encapsulate orange essential oil during spray drying. In both series of studies, it was found that mesquite gum has the ability to retain more than 80 % of the oil load, though to a slightly lesser extent than gum arabic. In low-tannin ultrafiltrated mesquite gum, it was found that when only species of 10 kDa are removed, the oil encapsulation capacity is not significantly affected when compared with that of gum arabic (Goycoolea et al. 1997, 1998). More recently, mesquite gum has been evaluated as color protection agent of blueberry extracts microencapsulated by spray drying. The sample showed minimal changes in color after 4 weeks, demonstrating that the gum provides an advantage in the conservation of colorants (Jiménez-Aguilar et al. 2011).

3.7 Nonfood Applications

Among the earliest documented uses of mesquite gum in the chemical industry was as a source of L-arabinose (Anderson and Otis 1930; Loeza-Corte et al. 2007), which is an important ingredient in culture media. The use of mesquite gum mixed with other polysaccharides for the microencapsulation of artificial diet for larvae shrimp (*Litopenaeus vannamei*) was found to improve the survival rates and overall quality of the microcapsules (Pedroza-Islas et al. 2000, 2004). Mesquite gum has also been used as a binder in tablet dosage forms and as a suspending agent, where it compared well with gum arabic and was superior to tragacanth gum (Khanna et al. 1997). In Sonora, household applications of mesquite gum include hardening of hats and as paper glue (Balderrama 1998).

The properties of mesquite gum are very similar to that of gum arabic; this suggests that the applications of mesquite gum can be expanded to pharmaceutical industry in the fields of biomedicine, especially in nanomaterials. For instance, mesquite gum has been studied as stabilizer of iron oxide nanoparticles with potential application in biomedicine (Ortega-Ruiz 2013).

4 Conclusions

Gum arabic is, by far, the most important plant exudates in the international market. Although there is the availability of several new alternative industrial hydrocolloids, gum arabic still is the first option in diverse applications in food, pharmaceutical, and chemical industries. However, the instability of the international market and an increasing demand along with social and political volatility in the producing regions are factors that impulse a continuous search for suitable alternative gums. Among other related gums, there are several that have considerable potential to compete with gum arabic. One of them is the mesquite gum produced by trees of the *Prosopis* genus. This gum has diverse traditional uses in food and other applications by the native cultures of America. Many studies account for the chemical structure and functional properties that are comparable with acacia gums.

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Resistant Starch in Food Industry

22

Leyla Khalili and Amir Amini

Contents

Abstract

Dietary starches can make quite specific contributions to health and are important sources of energy for most people. For both its potential health benefits and functional properties, resistant starch has received much attention. It can positively influence the functioning of the digestive tract, the microbial flora, the blood cholesterol level, glycemic index and assist in the control of diabetes. Apart from the potential health benefits of resistant starch, another positive advantage is its lower impact on the sensory properties of food compared with traditional sources of fiber, as whole grains, bran, or other prebiotics. The low water-holding capacity, increased viscosity and gel formation and high water-binding capacity make it useful in a variety of foods. In this chapter, it was intended to discuss about different resistant starch types, food sources, potential health benefits and food applications of resistant starch.

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1 Introduction

The functional features of resistant starch (RS), along with its potential physiological benefits, provide a means to increase total dietary fiber (DF) in the diet through common foods. DFs are carbohydrate polymers with 10 or more glucose units that are not hydrolyzed by the endogenous enzymes (McCleary et al. 2012). Being metabolized by advantageous colonic microorganisms which are called probiotics is the important property of DF, and this may lead to an enhanced bacterial growth and increased fecal bulk (Homayouni et al. 2014). The health benefits of DF for consumers such as reducing glycemic responses to carbohydrate ingestion, decreasing the risk of colorectal cancer, and enhancing mineral absorption make it a valuable prebiotic (Kumar et al. 2012).

Prebiotics have attracted the attention of producers more than ever due to the importance of them in prevention of certain disease. Prebiotics are nondigestible oligosaccharides that offer many helpful effects on the gastrointestinal system which are shown by selectively stimulating the growth or activity of certain indigenous bacteria (Sarkar 2007). Typical prebiotics are DFs that usually consist of inulin, oligosaccharides, and RS (Buttriss and Stokes 2008).

The growth in the use of new technologies and ingredients is because of the increase in consumer request for high-quality food products. Several factors influence changes in consumer requests, including health concerns (cholesterol, cancer, obesity, etc.), changes in demographic characteristics (ethnics, population aging, etc.), the need for convenience, and changes in distribution systems and price. As a result of these changes, attention in new products, mainly convenience-oriented products prepared using novel technologies, high pressures, etc., has intensely increased in recent years (Fuentes-Zaragoza et al. 2010). The food industry offers quality and convenience to a wide variety of consumers including single households, working couples, the aging population, and others (Fagan and Gormley 2005). To develop these types of products, one must evaluate consumer awareness, the most important quality aspects being that they taste good, appear healthy, and have nutritional value (Garcia-Segovia et al. 2007). Any functional food must be safe, healthy, and tasty as well. Actually, considerable importance is given to functional foods, which, in addition to their basic nutritional functions, provide physiological benefits and/or reduce the risk of chronic diseases, as they either contain (or add) a component with a positive health effect or eliminate a component with a negative one. One of the added components could be RS, which is commonly used as a functional ingredient, especially in foods containing high dietary fiber levels (Mikulíková et al. 2008).

There is general agreement among public health authorities and nutritionists that the inclusion of fiber in the human diet provides health benefits (Férnandez-López et al. 2009). That benefit message has reached consumers, and many meat product

companies, for example, have responded by launching products fortified with fiber. Many studies have found that people on diets high in fiber have reduced risks of certain diseases such as cancer, coronary heart disease, obesity, and possibly diabetes (Canovas and Pérez-Alvarez 2006). Some health benefits linked to fiber consumption are well established (e.g., promoting a regular bowel habit), and others are slowly becoming more firmly established (Buttriss and Stokes 2008; Viuda-Martos et al. 2010).

Resistant starch can influence the functioning of the digestive tract, the microbial flora, the blood cholesterol level, and the glycemic index and assist in the control of diabetes (Fuentes-Zaragoza et al. 2010). So it has received much attention for both its potential health benefits and functional properties.

1.1 Resistant Starch

Starch is the storage form carbohydrate of plants; many different types of starch exist depending on the plant source. A proportion of the starch, however, known as RS, is indigestible even after prolonged incubation with amylase. RS is a component of dietary starch that is not absorbed in the small intestine of healthy individuals and thus reaches the colon undigested, similar to other DFs. It has drawn considerable attention over the last two decades due to its demonstrated and putative positive impacts on health (Perera et al. 2010). Several diseases, especially digestive ones, are caused by inadequate or inappropriate diets. It is reasonable to accept an increase consumption of RS (Topping et al. 2008) which is among the important indigestible carbohydrates and has approximately half calorific value (8 kJ/g) compared with digestible starch (15 kJ/g) (Rochfort and Panozzo 2007). Most health effects of RS can be ascribed to its fermentation by the colonic microbiota (Robertson et al. 2000). Therefore, producing short-chain fatty acids (SCFA) by probiotics makes an environment less liable to the development of cancerous tumors (Yao et al. 2009). Other effects of RS consumption are preventing constipation, increasing excretion frequency and fecal bulk, decreasing production of mutagenic compounds, and lowering the colonic pH and ammonia levels (Fuentes-Zaragoza et al. 2011; Toden et al. 2005). RS can prevent diabetes by lowering postprandial insulin response, increasing insulin sensitivity, lowering postprandial glucose response, and delaying onset of insulin resistance (Torres-Zapata et al. 2012). It can reduce total liver cholesterol by causing higher excretion rates of cholesterol and bile acids (Fuentes-Zaragoza et al. 2011; Yao et al. 2009; Topping et al. 2008; Toden et al. 2005; Hashimoto et al. 2006; Wong et al. 2006). Increased absorption of calcium and magnesium by producing SCFA in large bowel is another effect of RS (Younes et al. 2001; Scholz-Ahrens et al. 2007). It can reduce energy intake due to its low caloric value, resulting in less appetite, so it can prevent obesity (Brown 2004; Bodinham et al. 2010). RS prevents diarrhea by shortening of the duration of rotavirus diarrhea by probiotics (Ramakrishna et al. 2000; Raghupathy et al. 2006). Prevention of colorectal cancer is another important benefit of RS. The Production of SCFA by fermentation of RS caused by

anaerobic bacteria and acetic, propionic, and butyric acid can lower the lumen pH, creating an environment less prone to the formation of cancerous tumors (Homayouni et al. 2014).

Apart from the potential health benefits of RS, another positive advantage is its lower impact on the sensory properties of food compared with traditional sources of fiber, as whole grains, fruits, or bran. Among its desirable physicochemical properties are its swelling capacity, viscosity, gel formation, and high water-binding capacity, which make it useful in a variety of foods (Fuentes-Zaragoza et al. 2010).

1.2 Resistant Starch Types

RS has been classified into four categories in almost all studies; however, RS type five was considered lately (Homayouni et al. 2014). RS1 is a physically inaccessible starch that is protected in cell walls and can be found in whole or partly milled seeds, grains, legumes, and pasta. RS2 is a granular native starch, with highly crystalline structure, that is found in raw potato, unripe bananas, and high amylose starches. RS3 is a retrograded nongranular starch that is found in cooked and retrograded starchy crops and foods. RS4 is a chemically modified starch and resistant dextrin that is found in functional foods with modified fiber. Also RS5 is found in crops or foods that contain starch–lipid complex (Noronha et al. 2007).

2 Application in Food Industry

As an insoluble product, resistant starch is especially suitable for grain-based low- and moderate-moisture foods (Yue and Waring 1998). Its physical properties, such as its low water-holding capacity, allow it to be a functional component that provides good handling in processing and crispness, expansion, and improved texture in the final product. Considering the technological features of RS, numerous food products have been enriched with RS including cheese, ice cream, yogurt, milk dessert, Iranian fermented drink (doogh), bread, corn flakes, cakes, muffins, pasta, and battered fried products (Charalampopoulos et al. 2002; Espírito-Santo et al. 2013; Vernaza et al. 2011; Mahadevamma and Tharanathan 2007; Mangala and Tharanathan 1999; Aravind et al. 2013; Mirzaei et al. 2012; Laguna et al. 2012; Yeo and Seib 2009; Tas 2000). Each type of RS has a distinct profile of physiological and technological functionality. For example, firmness and adhesiveness are textural properties which are the most important parameters for cooking quality of baked cereal products. In addition, due to the different stability and sources of RS, the appropriate selection should be considered. According to Faraj et al. in most cases in which high temperature and moisture are included (e.g., frying and high-temperature drying), RS1 and RS2 may be destroyed, but RS3 is able to resist or can be formed after process (Faraj et al. 2004; Ozturk and Koksel 2009). RS has more efficacies in food technology as mentioned in the following. The most studied products among cereals are pasta and bread.

2.1 Pasta Products

Pasta, a basic foodstuff with several nutritional values and important role in diets, can be easily produced, stored for a long time, and prepared for eating. In addition it has a lower glycemic index (GI) in comparison with starchy foods like white bread (Bustos et al. 2011). The compact structure created by extrusion process causes a compressed protein network that reduces the availability of starch granules to be hydrolyzed by a-amylase (Fardet et al. 1999).

According to the consumers' criteria for choosing high cooking quality pasta, manufacturing a good product which includes the optimum cooking time, low water absorption, and proper texture, with additional nutritional value, becomes a state of art. RS2 in pasta formulation enhanced the quality of the final product.

Pasta enriched with RS4 improved textural characteristics; however, pasta enriched with 5 % bran substituted changed cooking properties positively, whereas substitution with RS2 had no negative effect up to 10 % (Bustos et al. 2011). Increasing the amounts of RS2 in pasta resulted in a significant decrease in yellowness, no change in brightness, and minimal change in redness. Pasta enriched with 50 % of RS2 was brighter than all the other pastas. The substitution of RS up to 20 % did not change the sensory properties such as slippery, firmness, chewy, rubbery, and floury mouthfeel compared to the control pasta. Also in vitro digestibility reduced in 20 % RS2 substitution (Aravind et al. 2013). The properties of pasta dough with RS2 and RS3 in two levels (10 % and 20 % substitution with semolina) were investigated by Aravind et al. As more RS was added, the dough would be weakened. This may be explained by low WHC of RS and its competition with the semolina for hydration (Aravind et al. 2013). According to the results of the studies, the appearance of functional pasta may be of the most important parameter for good marketing.

2.2 Bread

The bread fortification with conventional DFs such as wheat bran and barley flour has some disadvantages like reduced volume, dark color, and disguising of flavor that caused a decrease in customers' acceptance (Sajilata et al. 2006). Ozturk et al. investigated the addition of RS2 (Hylon VII) and RS3 (Novelose 330) in bread formulation, each one at 10 %, 20 %, and 30 % w/w, and evaluated their effects on both properties and RS contents of the bread. Results were shown that doughs supplemented with RS absorbed more water (due to the low WHC) and were weaker than those of the base flour. Loaf volume decreased significantly above the 10 % RS3 and above the 20 % RS2. However crust color values decreased at 30 % addition of RS3 and RS2 supplemented breads.

Firmness of breads increased above 10 and 20 % for the addition of RS3 and RS2, respectively, after 7 days of storage, and RS content increased significantly as the addition level of both RS types increased (Ozturk and Koksel 2009). The results showed that up to 4 days of storage in three different conditions (ambient,

refrigeration, and freezing temperature) the RS content in crumb and whole bread increased and then dwindled (Niba 2003). The decrease might be the result of a converse in retrogradation of amylopectin, instability of amylopectin crystals, or unavailability of starch. This was the result of the formation of starch–protein or starch–lipid complexes, which could be caused by formation of RS5 (Rabe and Sievert 1992). The enrichment of bread with RS may result in the preparation of functional bread with healthy effects on consumers.

The most studied foods among dairies were cheese products.

2.3 Cheese

Mozzarella cheese normally contains about 20–27 % fat and is popular for its use in pizzas, so it is a great candidate for fat substitution with RS (Noronha et al. 2007). A study showed that it was possible to reduce up to 50 % of fat content with Novelose 240 (RS2) or Novelose 330 (RS3) in mozzarella cheese. Hardness linearly increased with increasing fiber content which the effect of RS3 was more than RS2. Cohesiveness was increased linearly with increasing RS2, but RS3 did not influence it (Montesinos-Herrero et al. 2006). It was possible to substitute up to 43.2 % of dry matter with RS in cheeses with 52–60 % moisture content while reserving desirable functional properties. The presence of RS showed a smooth and homogeneous texture without noticeable loss of moisture (Noronha et al. 2007). A kind of RS2 which its commercial name is Hi-maize 240 was also used as a crisping agent in imitation heat-expanded cheese. Arimi et al. produced a functional cheese which its fat content was successfully replaced with 15.3 % Hi-maize 240 (Arimi et al. 2008).

It was stated that RS organizes water molecules to be physically entrapped within the cheese matrix. The effects of RS2 content on aw, moisture sorption isotherms, and functional properties of imitation cheese were studied by Duggan et al. Cheese moisture remained constant (58 %), while the RS2 content was increased from 8.9 % to 18.2 %. With increasing RS2 content, hardness and cohesiveness showed no significant increase except at 12.9 %. Increasing starch concentration from 8.9 % to 12.9 % caused the reduction of aw from 0.986 to 0.978; nevertheless, higher concentration had no significant effect on aw. Finally the sorption behavior of the cheese was not affected by increasing the RS2 content (Duggan et al. 2008). It can be concluded that 18 % RS2 not only had no effect on the cheese texture and overall acceptability but also has healthy effects on consumers.

2.4 Microencapsulation of Probiotics

Microencapsulation is a new method which helps to isolate the bacterial cells from the effects of the hostile environment and gastrointestinal tract, thus potentially preventing cell loss and increasing probiotic survival (Homayouni et al. 2008). To some extent, Kebary et al. have shown that *Bifidobacterium* spp. survive in high

Table 1 Functionality of resistant starch types in food technology

Food products	Type	Result	Reference
Pasta	RS2	↑ Lightness, ↓ adhesiveness, ↓ water absorption	Aravind et al. 2013
	RS3	↓ Cooking loss, ↑ firmness, ↓ in vitro digestibility	Sozer et al. 2007
	RS4	↓ Glycemic index, without negative sensory effects	Bustos et al. 2011b
Bread	RS2	↓ Chewiness, without changing color parameters	Almeida et al. 2010
	RS3	↓ Staling, ↓ final viscosity	Ozturk and Koksel 2009
Confectionaries	RS3	Without changing sensory characteristics of biscuit	Laguna et al. 2012
	RS1	↓ Elasticity and ↓ deformability in muffin	Baixauli et al. 2008
Cheese	RS2	Fat replacer in mozzarella, ↓ water activity	Montesinos-Herrero et al. 2006
	RS3	↑ Homogenous and smooth texture	Noronha et al. 2007
Fermented drinks	RS2	↑ Viability of *L. acidophilus*	Nobakhti et al. 2009
		↑ Viscosity and consumer acceptance	Allgeyer et al. 2010
Yogurt	RS2	↑ Hardness and adhesiveness	Gustaw et al. 2011
		↓ Syneresis	Ramirez-Santiago et al. 2010
Ice cream	RS2	↑ Survival rates of *L. acidophilus*	Haynes and Playne 2002
		↑ Number of *L. casei* and *B. lactis*	Homayouni et al. 2008
Microencapsulation	RS2	↑ Delivery of *B. animalis*	Ziar et al. 2012
		↑ The number of *L. casei*	Sultana et al. 2000
	RS4	↓ Protection against oxidation of fish oil	Chung et al. 2010

numbers in frozen ice milk in beads made from alginate than those made from k-carrageenan. Shah and Ravula reported that the survival of probiotic bacteria in fermented frozen desserts improved with encapsulation. Encapsulation thus may enhance the shelf life of probiotic cultures in frozen dairy products. RS2-Calcium Alginate is a type of microencapsulation material that is used in several food products (Zanjani et al. 2014).

In 2000 Sultana et al. investigated the survival of probiotics encapsulated by alginate–starch gel in yogurt over a period of 8 weeks during storage at 4 °C. They stated the addition of RS2 (Hi-maize 260) at 2 % concentration to alginate mix resulted in an increase in number of *Lactobacillus casei* from 4×10^8 to 3.1×10^{11} CFU/g, although adding 4 % RS2 did not improve the encapsulation efficiency. Over 8 weeks of storage, one log decline was observed in both strains of *Lactobacillus acidophilus* and *Bifidobacterium infantis* when presented as free cultures, whereas encapsulated cells showed a 0.5 log decrease which meant the

positive role of using RS2 (Sultana et al. 2000). The same results were achieved in a study by Ziar et al. for evaluating the viability of *Bifidobacterium animalis* and *Lactobacillus rhamnosus*. After 4 weeks of yogurt refrigeration, no significant differences in bacterial counts were detected. Also under simulated gastrointestinal conditions, the number of viable encapsulated cells was significantly higher than those attended with free cells. Moreover, pH values did not change significantly from day 1 to 28 in *Bifidobacterium animalis* microencapsulated sample; conversely, in yogurt with encapsulated *Lactobacillus rhamnosus*, a significant decrease in pH values was obtained from day 7 cells (Ziar et al. 2012).

In 2008, Homayouni et al. studied the survival of *Lactobacillus casei* and *Bifidobacterium lactis* which was monitored during the storage of ice cream for 180 days at −20 °C. They found that microencapsulation of the specified probiotics with a 2 % starch–calcium alginate mixture was significantly increased at a rate of 30 % in comparison with control sample. Moreover, the points that were given out for color, taste, and texture showed that the addition of encapsulated probiotics had no effect on sensory properties of non-fermented synbiotic ice cream (Homayouni et al. 2008). So the best symbiosis model of prebiotic–probiotic is microencapsulation. Encapsulation is often mentioned as a way to protect bacteria against severe environmental factors (Mirzaei et al. 2012). Application of RS in food industry is summarized in Table 1.

3 Conclusion

Many fiber-enriched foods have been developed with the aim of increasing fiber intake in the diet. RS is a recently recognized source of fiber and is classified as a fiber component with partial or complete fermentation in the colon, producing various beneficial effects on health, and is a perfect DF with many nutritional values. As a functional fiber, its fine particles and bland taste make the formulation of a number of food products possible with better consumer acceptability and greater desirability than those made with traditional fibers. RS shows improved crispness and expansion in certain products, which have better mouthfeel, color, and flavor than products produced with traditional insoluble fibers.

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Inulin and Health Benefits

23

Matthias Moser, Anke Sentko, and Hélène Alexiou

Contents

Abstract

Inulin-type fructans are prebiotic dietary fibers, naturally occurring in numerous plants. The chicory root with its high content of inulin-type fructans is the most common basis for gaining inulin from the plant. The process applied is a hot water extraction. This means, contrary to a number of commercially available dietary fibers, inulin is not synthesized but occurs naturally and was eaten by mankind 100,000 years ago. The colon, the main place of action of inulin-type fructans, is a highly active metabolic organ, containing an excessively complex collection of microbes. Inulin-type fructans are nondigestible in the small intestine and fermented in the large intestine, resulting in modifications in the colonic microbiota composition, towards a healthier pattern, and in the production of several metabolites. Thereby, they are among the very few scientifically proven prebiotics. Several health effects are related to this activity in the colon.

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They may range from local effects, starting with improvement of bowel movement, effects related to local immunity, to more systemic benefits for the body, as a result of the metabolic events, like weight and blood sugar management and bone health. This book chapter aims at providing a comprehensive review of the physiology related to inulin-type fructans and the health benefits related to this. Current developments in understanding the role of the microbiota in health and disease will probably also further contribute to understanding these complex influences originating from the gut and the role "colonic nutrients" such as prebiotic dietary fibers may play therein.

Keywords

Inulin • Oligofructose • Synergy1 • Fiber • Prebiotic • Fermentation • Immune • Energy intake • Infant • Calcium absorption • Glycemia • Aging

Abbreviations

BB-12	*Bifidobacterium animalis* subsp. *lactis* BB12
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CHD	Coronary heart disease
DPP-4	Dipeptidyl peptidase-4
EFSA	European food safety authority
EU	European union
FOS	Fructooligosaccharides
GALT	Gut-associated lymphoid tissue
GDP	Gross domestic product
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide 2
GPR41 43	G protein-coupled receptors 41, 43
GR	Glycemic response
HOMA-IR	Homeostasis model assessment of insulin resistance
hs-CRP	High-sensitivity C-reactive protein
IASO	International Association for the Study of Obesity
IBD	Inflammatory bowel disease
IGN	Intestinal gluconeogenesis
IOF	International Osteoporosis Foundation
IoM	Institute of medicine
IOTF	International Obesity Task Force
ISAPP	International Scientific Association for probiotics and prebiotics
Lc	Long chain
LPS	Lipopolysaccharide
MAF	Mucosa associated flora
NASH	Nonalcoholic steatohepatitis
NCDs	Noncommunicable diseases

NDA	Dietetic products nutrition and allergies
NEC	Necrotizing enterocolitis
PYY	Peptide YY
Sc	Short chain
SCFA	Short-chain fatty acid
T_H1 2	T-helper 1, 2
TNF-α	Tumor necrosis factor-alpha
US	United States
WHO	World Health Organization

1 Introduction

The role of the large intestine for health and well-being has been underestimated for a long time. Nutrients that reach the large intestine are characterized by their indigestibility in the small intestine. Dietary fibers were regarded as an unnecessary burden rather than a necessary nutrient in the history of nutrition research. Today, scientists are even discussing whether dietary fibers should be classified as essential in human nutrition, a discussion that took place at the 10th Vahouny Dietary Fiber Symposium 2014 and will continue (Theis 2014). Physiological effects that support health and well-being are the attenuation of the postprandial glycemic/insulinemic response, reduced blood total and/or LDL cholesterol levels, reduced blood pressure, increased fecal bulk/laxation, decreased transit time, increased colonic fermentation/short-chain fatty acid production, positive modulation of the colonic microflora, weight loss/reduction in adiposity, and increased satiety as discussed at the 9th Vahouny Fiber Symposium (Howlett et al. 2010).

Prebiotic fermentation by the colonic microbiota plays a key role in a number of these health benefits. Inulin-type fructans are naturally derived plant extracts; they are completely fermented in the large intestine and therefore are the ideal source for short-chain fatty acid production (SCFA), contrary to a number of other nondigestible carbohydrates. They are proven prebiotics, i.e., the microbiota is stimulated selectively with numerous positive effects. Intensive research on the role of prebiotics and the selective changes to the microflora and their metabolites is ongoing. This is reflected in the fact that over the last 5 years, more than 1,000 research articles on the prebiotic topic have been published (Theis 2014).

Recent developments in the direction of research go beyond the large intestine as the place of action. Research is related to the gut-brain axis that is influenced by fermentation processes associated with, e.g., prebiotic inulin. A decrease in caloric uptake on a long term leads to a role of inulin-type fructans in healthy nutrition that can prevent the development of overweight and support weight management (Parnell and Reimer 2009; Kellow et al. 2014). A further direction of research is the significance of a lower blood glucose response and the related insulin response

which is discussed to support healthy metabolism in various ways (EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) 2014; De Vadder et al. 2014).

2 Sources, Preparation, and Usage

Inulin-type fructans are naturally occurring dietary fibers. A number of plants build their energy storage by inulin production, i.e., chicory, Jerusalem artichoke, onions, leeks, garlic, bananas, wheat, rye, and barley (Van Loo et al. 1995). All over the world, the chicory root and, to a less extent, the Jerusalem artichoke (tuber) and the Agave (leaf) are used for the commercial production of inulin.

Scientists investigating ancient cooking and eating habits discovered that in the diet of hunters and gatherers 10,000 years ago, the plant-based diet delivered about 135 g of prebiotic inulin-type fructans per man and day (Leach and Sobolik 2010). Today, intake of inulin-type fructans from fruits and vegetables is estimated to be around 5 g per day in Western-style diets in the United States and 3.2–11.3 g in Europe (Mishfegh et al. 1999; Van Loo et al. 1995).

The chicory root contains about 15–20 % of inulin on a fresh weight basis, which allows for large-scale extraction. In Europe, the farming of chicory as a crop has a long tradition as it was used in former times as a substitute for coffee when chicory roots were chopped-up and roasted. The process can be divided into two major phases. Firstly, the chicory roots are washed to remove stones, weeds, sand, and dirt. In the second phase, the roots are sliced and inulin is gained as raw juice by hot water extraction, which is then purified and dried. Inulin extraction is done by hot water (Moser et al. 2014).

Inulin-type fructans are linear polydisperse carbohydrates, mainly composed of fructose units joined by a series of β 2-1 fructosyl-fructose linkages. The chemical formula of inulin is $C_6H_{11}O_6(C_6H_{10}O_5)_nOH$ and the systemic name for all fructans is α-D-glucopyranoside-(1-2)-β-D-fructofuranosyl-[(1-2)-β-D-fructofuranosyl]n (notably, the α-D-glucopyranoside-(1-2) is not always present). The "n," which represents the number of fructose units, ranges mainly from 2 to more than 60. All fructan products contain molecules with a variety of chain lengths. Inulin-type fructans are referred to by various terms, including inulin, oligofructose, fructooligosaccharide (FOS), and short-chain (sc) or long-chain (lc) fructooligosaccharide, sc or lc inulin, or, more consumer-friendly, chicory fiber or chicory root extract. The structure of inulin and oligofructose is visualized in Fig. 1.

In addition to the naturally derived inulin-type fructans from chicory, short-chain fructooligosaccharides are on the market as well. These are fructans that are synthesized from sucrose.

Inulin-type fructans from chicory are soluble dietary fibers that have been used in food applications since the late 1980s. The uniqueness of these dietary fibers is the combination of an impressive scientific basis of nutrition and physiology, excellent organoleptic properties, and parameters relevant for technical applications providing all options for a top quality, tasty, and healthy food product.

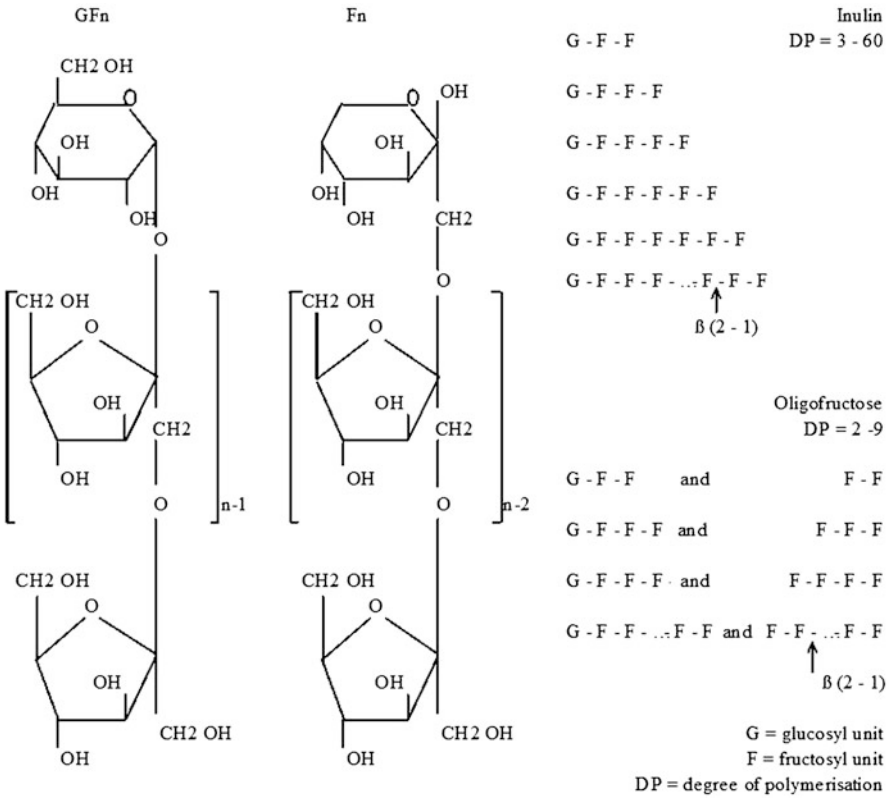

Fig. 1 Chemical structure of inulin and oligofructose from chicory

The contributions of inulin-type fibers to product development are manifold and all product categories are possible. These are, e.g.,

- Fiber enrichment (e.g., for a "high fiber" or "source of fiber" claim).
- Sugar reduction by replacement (also combined application "sugar out, fiber in").
- Fat out – fiber in: inulin has a unique property as a fat replacer. Inulin simulates the mouth-feeling and mouth-coating properties of fat. This enables also lower caloric, light versions of, e.g., dairy products.

  Calorie reduction (fibers often replace available carbohydrates or fat in a recipe; dietary fibers provide 2 kcal/g which is half of an available carbohydrate and less than one fourth of fat) (Moser and Wouters 2014).

Taste is always the key to consumer acceptance. Physiological benefits and support of a health-oriented diet are a cornerstone to success. Awareness of the need to change from a public health perspective having the burden of noncommunicable diseases (NCDs) in mind and the "Silver tsunami" approaching is also increasing.

3 Biological and Physiochemical Properties

3.1 Physiological Properties

3.1.1 Nondigestibility and Fermentability: Inulin-Type Fructans Are Dietary Fibers

Inulin-type fructans from the chicory root consist of a linear fructose polymer in a β-(2→1) fructosyl-fructose configuration, with a possible end-standing glucose unit.

The human enzymes that digest carbohydrates cannot hydrolyze the β-linkages of inulin-type fructans. As a result, inulin including its short-chain fraction, also called oligofructose or fructooligosaccharides (FOS), passes through the mouth, the stomach, and the small intestine without undergoing any significant change and without being metabolized. The nondigestibility of inulin and oligofructose has been confirmed in human studies with ileostomized volunteers. Eighty-eight percent and eighty-nine percent, respectively, of an ingested amount of inulin or oligofructose were recovered in ileostomy effluents, which clearly indicates indigestibility in the small intestine (Ellegård et al. 1997). The small loss of about 10 % can be explained by the difficulties in recovering and analyzing ileal effluents and by the fermentation of fructans by bacteria that colonize the distal part of the small intestine in these patients.

Inulin and oligofructose thus enter the large intestine almost intact where they are totally fermented by the microbiota. Thanks to these properties, inulin and oligofructose are dietary fibers, this is the case not only from the physiological angle but also from the food legislatives perspective. They are recognized as such in most countries worldwide.

In the colon, inulin and oligofructose are completely and selectively fermented by saccharolytic bacteria (mainly *Bifidobacterium* and *Lactobacilli*). This complete fermentation is substantiated by the complete disappearance of inulin-type fructans in vitro (Wang and Gibson 1993) as well as in human studies (Castiglia-Delavaud et al. 1998).

Inulin-type fructans are consequently converted into bacterial biomass, short-chain fatty acids (SCFA), lactic acid, and gases (mainly CO_2 and H_2). Gases are utilized by bacteria, or excreted in stools or after absorption excreted in breath. The principal SCFA resulting from their fermentation are acetate, propionate, and butyrate. Lactic acid is an intermediate that accumulates or that is further metabolized to SCFA by a cross-feeding process. Acetate and propionate are absorbed and systematically metabolized. Butyrate is largely metabolized by the colonic epithelium, where it serves as the preferred energy substrate as well as a regulator of cell growth and differentiation. Butyrate plays an important role in the maintenance of a normal epithelium as well as a reduction in symptoms of inflammatory bowel disease (Roberfroid et al. 2010). SCFA and their effects in the colon and beyond are the subject of intensive research that will be addressed further. As described above, inulin-type fructans can be produced with different chain-length distributions. Fructans with shorter-chains have been shown to be fermented faster than longer

chains (Stewart et al. 2008). Long-chain inulin reaches more distal parts of the colon. Specific chain-length compositions (e.g., Orafti®Synergy1) are produced to take physiological advantage of these properties.

Inulin-type fructans belong to the nutrient category of dietary fibers. Dietary fibers are well known for providing several positive physiological effects, and epidemiological evidence shows that a high consumption of dietary fibers has clearly been associated with a lower risk of cardiovascular disease, type 2 diabetes, hypertension, obesity, and certain large intestine disorders (Anderson et al. 2009).

These epidemiological data serve as a basis for the recommendations in dietary fiber intake made by various government agencies worldwide. Different endpoints might be at the basis for these recommendations.

The US Food and Nutrition Board has, for instance, used data from cohort studies related to coronary heart disease (CHD) for setting its adequate intake recommendation for total dietary fiber intake (Institute of Medicine (IoM) 2005). In Europe, EFSA considered the effect on bowel function as the basis for the adequate intake of dietary fiber and set a level of 25 g per day for adults (adequate for normal laxation) (EFSA 2010). Most public health advisory groups thus provide guidance for achieving the recommended levels of fiber consumption. This is mostly done through traditional food-based dietary advice, recommending the increased intake of whole grains, fruits, and vegetables. However, when comparing actual fiber intake assessments (derived from national nutrition surveys) to the recommended levels in the respective countries, it appears that the dietary fiber intake does not meet the recommendations in most countries around the world. This observation is striking, despite the cultural diversity in eating habits by the various populations (Fig. 2). The fiber gap is thus impressive, despite the widespread knowledge that fibers are "good for you" and the willingness of consumers to follow a healthy diet. This indicates a need for the consumption of more fiber-containing foods and foods enriched with fibers, such as inulin or oligofructose.

The colonic fermentation of inulin-type fructans will result in subsequent benefits. Those will be described in detail in Sect. 3.2.

3.2 Health Benefits

3.2.1 Gut Health

Modulation of the Microbiota Composition: The Prebiotic Effect

The Human Microbiome

Humans are composed, to a large extent, of microbes. This complex ensemble of microorganisms is referred to as the human microbiome. It plays an essential role in our development, immunity, and nutrition and has a profound effect on human health (Greenblum et al. 2012). The gut microbiome plays an essential role in many processes, including vitamin and amino acid biosynthesis, immune development,

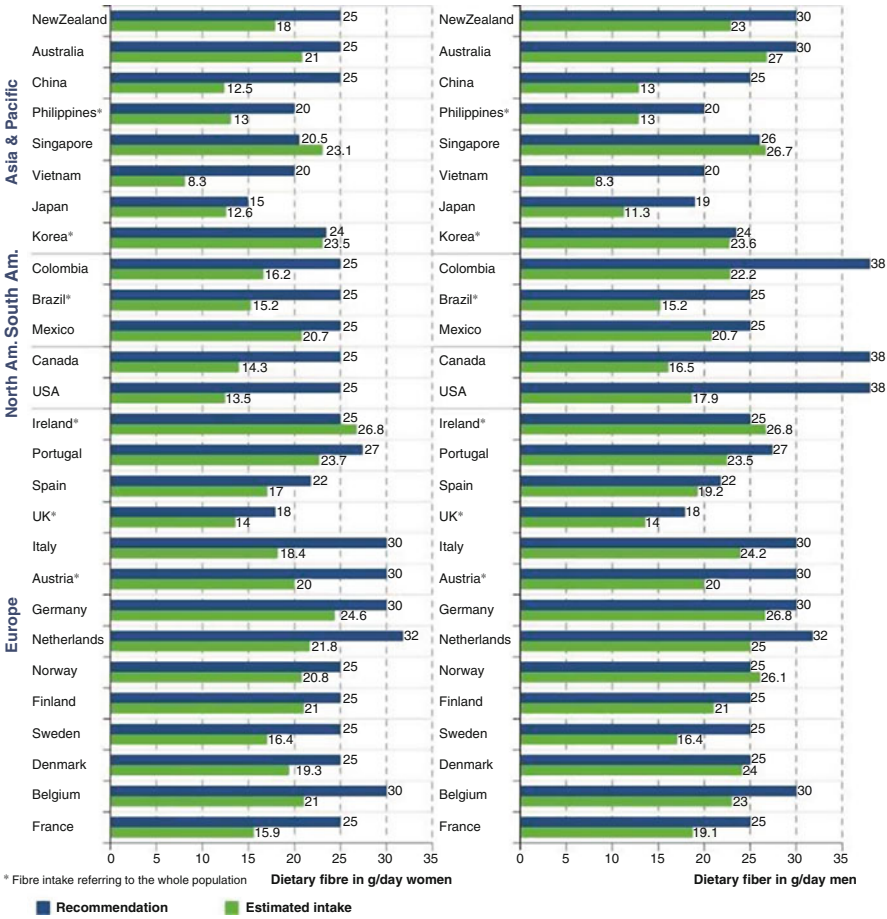

Fig. 2 Dietary fiber "gap" in several countries around the world (Window to Science 1/2011)

and enhanced resistance to colonization by pathogenic microorganisms. Humans harbor over 100 trillion microbial cells (Ley et al. 2006a).

Among the various organ habitats of microbes, the colon is by far the most densely colonized part of the gastrointestinal tract. The colon has been considered for a long time to be the place where digestion ends. Nowadays, with the intestinal microbiota it harbors, it has been elevated almost to the status of an active organ, playing a role in a wide range of host physiological processes (Latulippe et al. 2013).

The colon is indeed a very favorable organ for bacterial growth, reaching a high microbial density of 10^{11} to 10^{12} cells/g content because of its slow transit time, readily available nutrients, as well as favorable pH (Cummings and Macfarlane 1991). The physiology of the colon is controlled to a large extent by the gut microbiota, through the fermentation of indigestible compounds, the production

of secondary metabolites, and their interaction with the host epithelium or with the GALT (gut-associated lymphoid tissue). The immense collection of microbes composing the gut microbiota interacts with the host in a very intricate manner and is involved in the regulation of several metabolic pathways, with immune-inflammatory physiologically connecting the gut, the liver, muscles, and the brain (Nicholson et al. 2013).

Recently, intense research initiatives have been conducted to improve understanding of the complexity of the gut microbiota. Projects, such as the Human Microbiome Consortium, the European Commission's Metagenomics of the Human Intestinal Tract Project, among others, are conducted with the aim of generating resources to enable comprehensive characterization of the human microbiota and allow for an analysis of its role in human health and disease. They are focused on the identity, genetic potential, and metabolic activities of microbes (Bäckhed et al. 2012).

Despite significant progress in understanding the human microbiome, the composition and functional characteristics of a "healthy microbiome" remain to be precisely defined. Although several patterns of microbial colonization associated with various disease states have been documented, a health-associated pattern is more challenging to establish and therefore is less clear at the moment (Bäckhed et al. 2012). A healthy microbiome could be described in terms of stability of a microbial community, or its ability to resist structural changes under stress, or to return to its initial composition after a stress-related change (Bäckhed et al. 2012). Other parameters could be an idealized (presumably health-associated) composition or a desirable functional (metabolic) profile, although this remains to be defined. The concept of a "healthy microbiome" is based on the probability that stabilization of a balanced microbiota and the maintenance of "normobiosis" keep the organisms healthy for longer or in other words provide less harmful loads to the body. A shift in the composition of the microbiota or a loss in its balance such that it may become deleterious to host health is termed "dysbiosis." Certain microbial distributions, for instance, may favor the susceptibility to infections or diseases.

Several disease states have been associated with changes in the composition of fecal and intestinal mucosal communities, including inflammatory bowel diseases, obesity, the metabolic syndrome, type 1 diabetes, allergy, celiac disease, and even autism (Honda and Littman 2012; Million et al. 2013; Manichanh et al. 2012). However, a causal relationship is difficult to establish.

Certain species and/or their metabolic activities are acknowledged as being beneficial to the host when they are present in increased amounts, and bacterial groups such as bifidobacteria and lactobacilli are among the most cited bacterial groups of this category and are part of the potentially health beneficial microbiota. It has been hypothesized that *Bifidobacterium* spp. might improve health status and reduce disease risk (Roberfroid et al. 2010).

Bifidobacterium was first acknowledged as a beneficial bacterial group because it is predominantly present in the feces of breastfed infants, where it is thought to play an important role in the improved health and the development of these infants compared to formula-fed babies. It is recognized that formula-fed infants

are more susceptible to gastrointestinal infections such as diarrhea and other disturbances such as acute respiratory infections (Benno et al. 1984; Heavey and Rowland 1999).

Several other situations of dysbiosis (less stable or unstable gut microbiota) point a decrease in bifidobacteria.

An aging ailing population, subject to infections or antibiotic treatment, is, for instance, more prone to developing microbiota dysbiosis, which also provides an indication of the difference between a balanced and an unbalanced microbiota. Observations have notably shown a reduction in species diversity in anaerobic bacterial groups such as bifidobacteria and prevotella in elderly versus younger people (Woodmansey et al. 2004). These authors also noted lower bifidobacteria compared to healthy elderly, when the elderly were hospitalized and received antibiotics. Bartosch et al. (2004) reported reductions in bacteroides, prevotella, bifidobacterium, and *Faecalibacterium prausnitzii* in hospitalized versus healthy elderly. Under antibiotic treatment, a marked increase in enterobacteria was noted.

In subjects with inflammatory bowel disease (IBD), compositional changes have been evidenced compared to a healthy population. In adults, despite study heterogeneity, lower numbers of lactobacilli, bifidobacteria, and more recently *Faecalibacterium prausnitzii* counts in feces or biopsies have been frequently reported (Favier et al. 1997; Frank et al. 2007; Sokol et al. 2008). Increases in numbers of bacteria that are considered less desirable in the gut have been reported as well, such as higher numbers of Escherichia and enterobacteriaceae in subjects with IBD (Kotlowski et al. 2007; Martinez -Medina et al. 2006; Seksik et al. 2003). Schwiertz et al. (2010a) found a lower number of *Faecalibacterium prausnitzii* and bifidobacteria in children with active and inactive Crohn's disease compared to healthy children. These data, as well as others, demonstrate that there is a difference in the microbial composition of subjects with IBD compared to healthy individuals.

Regarding desirable functional (metabolic activity) of a healthy microbiota, one can look at the end products that result from the microbial activity. Microbes considered beneficial display a carbohydrate or saccharolytic fermentation as the preferred mode of energy generation, resulting in the production of short-chain fatty acids. The fermentation process by a healthy microbiota should not give rise to the formation of toxic metabolites. Microbes considered to be beneficial do not display pathogenic or toxigenic traits and are not associated with diseases. They further may have a range of potential benefits for the host, involving interactions with the immune system and competitive inhibition of pathogens (Binns 2013).

Based on current knowledge, bifidobacteria (and lactobacilli) clearly fulfill all the characteristics that can currently be defined of a "healthy microbiota" and low levels are reported in impaired gut state such as in several cases of dysbiosis.

It is important to realize however that "healthy microbiota concept" does not mean that an individual with a "less beneficial" microbiota signature would show obligatory impairments in body function. A more beneficial microbial pattern cannot be seen in a medical sense, as is the case, for instance, for the link between cholesterol levels and cardiovascular disease.

Inulin-Type Fructans: Prebiotics

In view of the above, the microbiota composition and maintenance and ways that modulate it has become a new target to study for potential impact on health. The diet is one factor which can modulate the composition of the microbiota once established; other factors include the intake of antibiotics, the environment, and disease.

Bifidobacteria and lactobacilli are among the best-known bacteria recognized as having a metabolic activity that is beneficial for the host. Their selective stimulation by specific nutrients (proven for inulin-type fructans and only a few other nondigestible carbohydrates) within the large intestine is referred to as the "prebiotic effect."

The concept of supporting the "good" bacteria selectively within the natural microbiota of an individual by a specific choice of food was developed in Japan within the FOSHU framework legislation in the 1980s. The observations of stimulating bifidobacteria were expanded by Gibson and Roberfroid who already realized the importance of a healthy gut flora for the well-being of the host in 1995 and developed the scientific concept for human gut microbiota modulation or the "prebiotic" concept (Gibson and Roberfroid 1995). Since then, the prebiotic concept has been the topic of intensive research. It is now defined as a "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2010).

Although a number of ingredients that are fermented in the colon claim to be prebiotics, scientists from ISAPP (International Scientific Association for Probiotics and Prebiotics) concluded that only a few ingredients satisfy the criteria for classification of a given food ingredient as prebiotic (criteria defined in Gibson et al. 2004). Inulin and oligofructose are part of this category. Main proof that is lacking for most ingredients is the human evidence.

Individuals' responses to dietary interventions aimed to test effects on the microbiota are highly variable and poorly predictable by in vitro assays. In addition, in vitro models alone cannot take into account the complexity of the ecosystem of the colon and of the numerous bacterial interactions that take place therein. Human intervention trials are thus required. Appropriate techniques for the quantification of bacteria are also an important consideration, and today's molecular techniques are considered to be more reliable as they encompass a much wider diversity of the gut microflora than the plate count techniques. To ascertain consistency and exclude incidental findings, more than one human intervention trial should be performed.

Much of the early research has focused on increasing the relative populations of bifidobacteria and lactobacilli. In that regard, inulin-type fructans have demonstrated their prebiotic effect in a vast amount of human intervention studies. More than 20 studies have shown a consistent and significant increase in bifidobacteria (and sometimes lactobacilli) counts, while having no measurable or significant effect on most other bacterial groups (Gibson and Roberfoid 2009; Roberfroid et al. 2010). The effect on *Bifidobacterium* is selective. The prebiotic effect was

demonstrated with a wide range of daily dosages, starting at 5 g/day (Rao 2001; Bouhnik et al. 2007). Significant compositional changes have been demonstrated after only 1 week of intake (Tuohy et al. 2001; Kleessen et al. 2007). Logically, several studies report that when the prebiotic intake was stopped, the levels of bifidobacteria decreased to their original level. Fructans of all chain lengths have been shown to have this unique effect on the composition of the microbiota in common.

As feces constitute the most readily accessible material to study the effects of prebiotics on the microbiota in a noninvasive manner, most microbiological assessments have been done on stools and have thus assessed the effects of bacteria that are living free in the gut lumen. Bacteria inhabiting the colon might also be attached to the mucosa, and they are thus called "mucosa associated flora" (MAF). Because of their location, it is thought that these bacteria could play a critical role in protecting the mucosal epithelium, notably due to their ability to exclude adherence of pathogens (Langlands et al. 2004). In volunteers who underwent a colonoscopy, Langlands et al. (2004) collected biopsy samples from the mucosa and were able to show that a combination of oligofructose and long-chain inulin (similar to Orafti®Synergy1) increased (mucosal) bifidobacteria and lactobacilli. This increase occurred both in the proximal and distal part of the colon, indicating that the prebiotic fermentation (and the resulting beneficial environment) took place over the full length of the colon.

This selective stimulation most probably originates from the capacity of bifidobacteria to utilize them specifically. Several bifidobacteria strains possess cellular uptake systems and express various β-fructofuranosidase genes, allowing them to hydrolyze the β (2–1) link between fructose moieties and thus to efficiently use inulin and oligofructose as growth substrates in a competitive environment, such as the colon (De Vuyst and Leroy 2011).

In general, most other genera are not affected in a consistent way by inulin or oligofructose intake. Recent data, however, have shown that some other bacteria that could also be considered as members of a healthy gut microbiota may be affected by inulin and oligofructose intake. Studies notably show that *Faecalibacterium prausnitzii* counts are increased upon inulin intake (Ramirez-Farias et al. 2009; Dewulf et al. 2013). Further research is needed to confirm these data and may unravel further interesting findings.

Direct Physiological Outcomes of Fermentation

Benefits from prebiotic fermentation can be examined from the metabolites produced by the colonic microbiota and the impact of these metabolites on health, which is currently a topic of research.

Main substrates available for the colonic microbes are constituted by nondigestible carbohydrates and proteins, which result in saccharolytic and proteolytic fermentation, respectively. Dietary carbohydrates reaching the human large intestine mainly constitute of structural polysaccharides and oligosaccharides of plant origin, as well as resistant starch, synthesized by chemical denaturation of available starch.

As outlined above, the fermentation of inulin and oligofructose by the saccharolytic microbiota results in the production of SCFA. Butyrate, in particular, represents the major intestinal energy substrate of human colonocytes and can be regarded as a supportive marker for physiological gut function in this context. A small proportion of SCFA reaches the general circulation, which could contribute to some of the systematic effects of inulin-type fructans. The process of reabsorption of SCFA explains the low amount that is found back in the feces. SCFA seem also to be involved in many other metabolic processes with systemic effects, such as glucose and energy homeostasis (effects described further on in the chapter). SCFA are indeed also signaling molecules, acting as endogenous ligands for the G protein-coupled receptors FFAR3 and FFAR2 (Brown et al. 2003).

Results from in vitro as well as in vivo studies show that the intake of inulin-type fructans increases the production of SCFA and/or notably the amount of molar ratio of butyrate. A concomitant decrease in cecal pH is also shown. Since bifidobacteria mainly produce acetate and lactate, the question of the origin of the "butyrogenic effect" of inulin and oligofructose has long remained unclear. In vitro studies have revealed that cross-feeding mechanisms between bifidobacteria and butyrate-producing bacteria most probably explain the butyrate production following inulin and oligofructose intake. Certain bacterial strains such as *Eubacterium hallii*, *Retortamonas intestinalis,* or *Anaerostipes caccae* were shown to use lactate or acetate produced by bifidobacteria to convert them into butyrate. In this way, cross-feeding between these microorganisms and inulin degraders (bifidobacteria) may contribute to the butyrogenic effect of inulin and oligofructose (Falony et al. 2006; Belenguer et al. 2006). Butyrate could also directly come from butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, also shown to be stimulated upon inulin intake (Ramirez-Farias et al. 2009; Dewulf et al. 2013).

As the major part of produced SCFA is absorbed and only a small proportion is recovered in stools, the measure of residual SCFA in feces thus does not accurately reflect their rate of production by the colonic microbiota, and no pertinent conclusion can be drawn from measurement of fecal SCFA. SCFA arising from fructans' fermentation are thought to contribute to the integrity and functioning of the epithelial cell lining. The results of a recent randomized, double-blind, crossover study conducted in healthy young volunteers showed that the administration of inulin decreased small intestine permeability as evidenced by lower levels of circulating zonulin and a significantly higher basal glucagon-like peptide 2 (GLP-2) level compared to the control group (Russo et al. 2012). The protein zonulin is involved in mucosal barrier function and the disassembling of the tight intercellular junctions characterizing the early inflammatory states. GLP-2, a peptide secreted from enteroendocrine L-cells present in the distal intestine, enhances mucosal barrier function among other properties. This study indicates a potential effect in regulating intestinal barrier function by inulin. In studies performed in obese rodents as well, the intake of oligofructose was shown to improve gut barrier function by decreasing intestinal permeability and improving tight-junction integrity. These effects were related to higher levels of GLP-2 (Cani et al. 2009a; Everard et al. 2011).

This might have implications that are not limited to the direct environment of the gut, as, for instance, obesity is associated with an increase in gut barrier permeability, thus allowing an atypical translocation of gut bacteria or gut bacteria components into the circulation and tissues (see section on weight management for further information on the topic).

In contrast to the benefits associated with saccharolytic fermentation, proteolytic fermentation that takes place in more distal parts of the colon results in the formation of more toxic metabolites, such as primary and secondary amines, ammonia, aromatic compounds (phenols and indoles), mercaptans, as well as linear and branched short-chain fatty acids (Macfarlane and Macfarlane 1995). Amines may be absorbed by the host and systemically metabolized or further converted by the colonic bacteria. Secondary amines may be precursors of N-nitroso compounds (Calmels et al. 1985). Non-protonated ammonia may be absorbed and may lead to intoxication in subjects with liver impairment. Phenols and indoles are also associated with various adverse effects in the host.

An important amount of proteins in the colon has been linked with chronic colonic diseases, and extended saccharolytic fermentation is thought to contribute to the reduction of the production and impact of products of amino acid metabolism.

Some studies have investigated the effects of inulin-type fructans on some potentially toxic metabolites characteristic of a proteolytic fermentation.

De Preter et al. (2007) studied the influence of Orafti®Synergy1 on the outcome of two biomarkers of proteolytic fermentation, lactose[^{15}N, ^{15}N]ureide and *p*-cresol. Lactose[^{15}N, ^{15}N]ureide is a biomarker allowing the investigation of the metabolic fate of ammonia. It is expected that higher amounts of ammonia are incorporated into colonic bacteria during fermentation, thus reducing its excretion in urine. *p*-cresol is a bacterial metabolite produced in the colon and excreted in urine. Its reduced presence in urine thus reflects a lower proteolytic activity in the colon. In healthy volunteers receiving Orafti®Synergy1 during 4 weeks, the urinary excretion of ^{15}N and *p*-cresol was significantly reduced, thus showing positive effects on colonic ammonia and proteolytic activity. These results correlated as well with an increased bifidobacteria level in the feces. The same research team conducted an in vitro study (anaerobic fermentation with fecal slurries), in which they assessed the impact of Orafti®Synergy1 on the production of a total of 107 different volatile organic compounds. Orafti®Synergy1 promoted a pattern of saccharolytic fermentation and in parallel inhibited the generation of S-compounds, as well as the production of metabolites of proteolytic fermentation, such as phenolic compounds (De Preter et al. 2010).

The effect of fructans on various metabolic activities in the large intestine with toxic potential was also investigated. The activity of fecal β-glucosidase and β-glucuronidase was studied in humans, with most studies showing reduced activity of these enzymes (De Preter et al. 2008; Bouhnik et al. 2007; van Dokkum et al. 1999). These glycosidases are known for their carcinogenic potential. Results do not, however, all point in the same direction, with other investigations reporting no effect on the level of β-glucosidase and β-glucuronidase activities (Kleessen et al. 1997; Gråsten et al. 2003).

In conclusion, the prebiotic concept is now well confirmed by scientists worldwide, as recently reviewed by an expert panel under the umbrella of the International Life Science Institute (Roberfroid et al. 2010). Among its conclusions, the expert panel confirmed that "*the prebiotic effect is now a well established scientific fact. The more data are accumulating, the more it will be recognized that such changes in the microbiota's composition, especially increase in bifidobacteria, can be regarded as a marker of intestinal health.*"

Bowel Regularity

A normal defecation pattern is considered to be an important indicator of gut health and well-being. As dietary fibers, inulin and oligofructose improve bowel function.

The fermentation of inulin-type fructans by the colonic microbiota results in an increased bacterial cell mass and SCFA. Due to the high water content of intestinal bacteria, the moisture content of feces is increased after inulin-type fructan supplementation. As a consequence, stools become softer and excretion is facilitated (Cherbut 2002).

The SCFA that are produced are also susceptible to affect intestinal motility and to lead to faster intestinal transit (Grider and Piland 2007). This results in facilitated fecal excretion as well as enhanced propulsion of colonic contents via chemical and mechanical stimulation of peristaltic reflex. The end result is an increase in frequency of bowel movements.

The intake of inulin and oligofructose has been demonstrated to result in an increase in stool frequency and/or in stool weight in humans in several human intervention studies starting with an intake of 8 g/day (Gibson et al. 1995; Van Dokkum et al. 1999; Menne et al. 2000; Cummings et al. 2001; Kleessen et al. 2007).

A positive impact on stool frequency was not only demonstrated in healthy subjects but also in slightly constipated subjects where 15–20 g/day of inulin was shown to relieve constipation (Kleessen et al. 1997; Den Hond et al. 2000). Oligofructose has also been shown to increase stool frequency and to reduce constipation in adults with irritable bowel syndrome (Isakov et al. 2013).

Data are also supportive of more regular and/or softer stools in infants and young children. In infants aged 4–12 months, the intake of oligofructose included in a cereal resulted in a more regular and softer stools, as well as a lower reported frequency of symptoms associated with constipation (Moore et al. 2003). Positive effects on higher stool frequency and softening of the stools are also reported in neonates receiving Orafti®Synergy1 in an infant formula (Veereman-Wauters et al. 2011; Closa-Monasterolo et al. 2013; for more details, see section on infant nutrition).

3.2.2 Prebiotic Effect and Immune Influence

The intestinal microbiota is a source of antigens and potentially harmful compounds. Most of the time, a good balance exists between the large number of bacteria we cohabit with and our immune cells. With respect to their gut and the interactions with the immune system, humans can be considered to exist in a state of

natural balance with their microbial inhabitants. The immune structure of the gut (the largest of the human body in fact) allows the protection of the body against pathogens and prevents non-virulent microbes and opportunistic pathogens from translocation into the internal milieu (Trebichavsky et al. 2009). Gut bacteria play an important role in immunity, and the intestinal ecosystem is established to avoid colonization by pathogenic bacteria and potentially harmful indigenous microorganisms, known as the "barrier effect."

Several mechanisms play a role in this protective effect, including a direct competition for limited nutrients and the modulation of the host immune response (Kamada et al. 2013).

The prebiotic effect may influence the immune system directly or indirectly as a consequence of intestinal fermentation and the resulting changes in bacterial composition and metabolites present in the colon. The presence of increased numbers of certain bacterial species (including mainly bifidobacteria) may induce an effective competition for nutrients and ecological niches. Bifidobacteria synthesize antimicrobial peptides that inhibit the growth of several pathogens (Collado et al. 2005). In addition, changes in the composition of the colonic microbiota (including increase of certain microbial genus or species and decrease of others) may change the collective immunoreactive profile of the microbiota (Roberfroid et al. 2010).

In addition, the changes in metabolites, i.e., increase in SCFA production, are accompanied by a decrease in the luminal pH, and the totality of acids and in particular the intermediate lactic acid is considered important to discourage obligate and facultative pathogens in the lower gut. These SCFA may further interact with immune cells and enterocytes and modify their activity. G protein-coupled receptors (GPR)41 and GPR43 have been shown to be receptors for SCFA and are expressed on leukocytes as well as enterocytes and enteroendocrine cells in the human colon (Roberfroid et al. 2010).

Few human studies have investigated potential positive influences of inulin-type fructans on indirect parameters related to immunity or, in general, to better resistance or outcome towards infectious diseases. In patients who developed *C. difficile* infection due to an antibiotic therapy, oligofructose supplementation (12 g/day) significantly decreased relapses of diarrhea at the end of the treatment period. This was correlated with significantly higher numbers of total anaerobes and bifidobacteria in the oligofructose group compared with the placebo. Patients who relapsed stayed in the hospital longer than those who did not (Lewis et al. 2005). A trend towards less episodes of diarrhea was also noted by Cummings et al. (2001) in a study involving 244 healthy people receiving oligofructose (10 g/day) traveling to destinations at risk of traveler's diarrhea. In a randomized, double-blind, placebo-controlled trial involving 123 young children attending day-care centers, the addition of oligofructose to a weaning cereal (0.55 g per 15 g of cereal) resulted in significantly fewer episodes of fever and missed days of day care because of diarrhea, as well as a lower use of antibiotics for respiratory illnesses (Saavedra and Tschernia 2002). These data constitute preliminary, yet promising findings that support potential beneficial effects of prebiotics with respect to immune-related status. Additional data from clinical studies are needed to support these effects.

3.2.3 Inulin-Type Fructans in Infant Nutrition

The gastrointestinal tract of the newborn is (almost) sterile at birth, and microorganisms quickly colonize it during and after birth. Essential factors that influence bacterial colonization are the mode of delivery, prematurity, the excessive use of antibiotics during the perinatal period, the mother's microbiota, the infant's genetic signature, and the type of feeding. Breastfeeding is of primordial importance in the initial colonization process that takes place at the beginning of an infant's life. The microbial contact in utero and during the neonatal period (which is influenced by the mode of delivery and breastfeeding) has a long-term impact on the intestinal colonization pattern and plays a crucial role on the immune and metabolic programming of the fetus and infant and consequently on the risk of disease later in life (Rautava et al. 2012). These interactions with colonizing microbes and the consequent establishment of a balanced microbiota are necessary for the development of an appropriate innate and adaptive immune response and of oral tolerance. As a consequence, the disruption of the normal colonization process leads to alterations in the establishment of this immune homeostasis (Rautava et al. 2012; Walker 2013). The state of dysbiosis (e.g., premature delivery, excessive use of antibiotics) consecutive to inadequate colonization is related to aberrant immune activation and to higher risk of immune-related diseases, notably allergic and autoimmune disease (Renz et al. 2011). Several diseases, associated with excessive or aberrant T-helper (T_H)1 or T_H2 immune responsiveness (allergic diseases, type 1 diabetes, celiac disease), have been associated with deviations in the compositional development of the gut microbiota, i.e., a less diverse microbiota lacking, among others, bifidobacterial species, with simultaneous higher levels of specific clostridia (Rautava et al. 2012). Necrotizing enterocolitis (NEC), a condition present in premature infants, is another example of disease characterized by dysbiosis and lower diversity of bacterial species (Torrazza et al. 2013).

At weaning, the microbiota composition changes in numbers and diversity, to begin to resemble that of the future adult. The "adult microbiota" is established around the age of 2 years and stays relatively stable during the adult life (Binns 2013).

Breastfeeding, which is by far the unequivocal preferred mode of feeding infants, results in a more stable microbial stool pattern characterized by higher levels of bifidobacteria in breastfed babies compared to formula-fed babies (Harmsen et al. 2000; Fanaro et al. 2003; Bezirtzoglou et al. 2011). This is notably due to the presence in breast milk of a variety of nondigestible oligosaccharides which are nonabsorbed and stimulate the growth of *Bifidobacteria* and *Lactobacilli*. Human milk oligosaccharides are present in human (but not in other mammalian's milk) at concentrations ranging from 10 to 20 g/L and represent the third most abundant group of milk compounds. They are nondigestible and fermented by the infant's colonic microbiota, therefore being one important factor contributing to the specific microbial composition of breastfed babies. In contrast, formula-fed babies harbor a wider range of bacteria, including bifidobacteria, bacteroidetes, clostridia, enterobacteria, and streptococci. Human milk oligosaccharides comprise a highly complex mixture of oligosaccharides that are attached in various ways through

different linkages. The quantity of these compounds depends on the lactation stage but also on the expression of specific glycosyltransferases in the mammary gland (Rudolff and Kunz 2012). In total, about 200 different molecular species have been detected (Ninonuevo et al. 2006).

Breastfeeding has been shown, among other things, to be protective against infections of the gastrointestinal and respiratory tract and the development of necrotizing enterocolitis in the newborn and decreases the occurrence of allergic and autoimmune diseases or type 1 diabetes later in life (Guaraldi and Salvatori 2012).

Prebiotics in Infants

As infant formulae (as well as breast milk) represent the sole source of nutrients during the first few months of a baby's life, it is crucial that these formulae are of the highest standard possible. Infant formulae must be safe and promote normal growth and development, and their composition, as well as functional and physiological benefits, should come as close as possible to those of human milk.

In the absence of breastfeeding, especially during the first months of life, infant formulae enriched with prebiotics or probiotics may overcome an initial inadequate colonization process and therefore help establish a normal mucosal immune system (Walker 2013). Inulin-type fructans have been evaluated in several studies involving neonates, where they were added to infant formulae.

In 110 healthy neonates, Veereman-Wauters et al. (2011) conducted a randomized, double-blind, placebo-controlled study to assess the effects of Orafti®Synergy1 on the growth, stools, and composition of the microbiota of the babies. Term infants were enrolled within 5 days after birth and were randomly distributed to receive, during 28 days, one of the four infant formulae: standard, enriched with 0.4 or 0.8 g dl^{-1} of Orafti®Synergy1 or 0.8 g dl^{-1} of a commercially used mixture of galacto-oligosaccharides and long-chain inulin in a 90/10 ratio (GOS/FOS). A reference group of breastfed babies was included as well. The bifidogenic effect of Orafti®Synergy1 was confirmed in the babies, as its supplementation in the infant formulae resulted in enhanced *Bifidobacterium* counts after 2 and 4 weeks, reaching levels comparable to the breastfed group. Numbers of lactic acid bacteria, bacteroides, and clostridia were comparable and levels were not modified by the addition of prebiotics. Growth parameters were similar between all groups.

More recently, a study was conducted to confirm the safety and efficacy of Orafti®Synergy1 (Closa-Monasterolo et al. 2013). In a large randomized, double-blind, placebo-controlled study, 252 infants were enrolled within their first 4 weeks of life, for an intervention up to 4 months of age. One hundred thirty-one breastfed babies were also included in the study. The infant formula was supplemented with 0.8 g dl^{-1} of Orafti®Synergy1 (or with maltodextrin as control).

The results of this study showed that Orafti®Synergy1 added to infant formula at a level of 0.8 g dl^{-1} is well tolerated and supports the normal growth of a baby. Water balance as well as blood and urine biochemical parameters were similar in both formula groups and demonstrated safety of the prebiotic formula. Higher

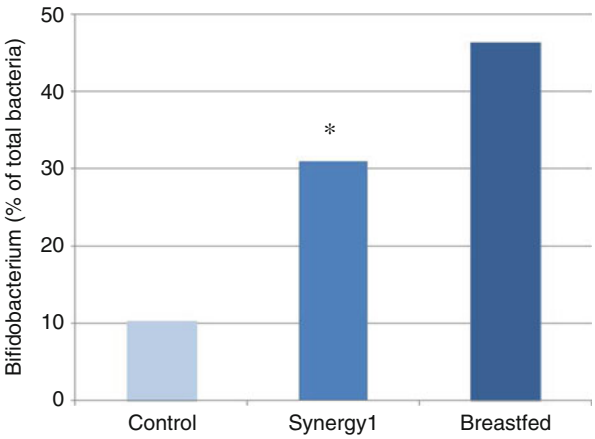

Fig. 3 Percentages of *Bifidobacterium* in the stool samples of infants at 3 months of age. *$p < 0.05$ versus control group (Adapted from Closa-Monasterolo et al. 2013)

bifidobacterial counts were shown following Orafti®Synergy1 intake, resulting in a microbiota composition closer to that of breastfed babies (Fig. 3.).

In addition to the effects on the microbiota, these two studies have shown effects on the stools of the babies. In the study of Closa-Monasterolo et al. (2013), stool frequency increased and stool consistency was softer in the Orafti®Synergy1 supplemented group, compared to the standard formula babies. Similarly, Veereman-Wauters et al. (2011) showed that the infants receiving the prebiotic formula had significantly softer stools, consistency of the stools still being harder than that of the breastfed babies. In a randomized, blinded trial involving 212 term infants assessing and demonstrating the safety of an oligofructose-enriched infant formula (1.5 and 3 g l^{-1}), the babies receiving the formula containing 3 g l^{-1} had less constipation compared to the control group receiving the standard formula (Bettler and Euler 2006).

These effects could be beneficial in preventing the problem of hard stools and of constipation often encountered in formula-fed babies.

Inulin-type fructans have also been evaluated in slightly older infants.

Here also, effects on stools have been shown, by Moore et al. (2003), where 16–46-week-old infants receiving a weaning cereal enriched with oligofructose had slightly more frequent and softer stools and less "hard" stools compared to the group receiving the standard cereal.

Two studies in toddlers between 4 and 24 months of age have shown that the ingestion of oligofructose is associated with a bifidogenic effect, with less events of diarrhea, vomiting, and fever (Waligora-Dupriet et al. 2007) and with a reduced severity of diarrheal or respiratory infectious episodes (decreased antibiotic use and fever) (Saavedra and Tschernia 2002).

Conclusions from studies conducted in infants and young children are that inulin-type fructans are safe, are well tolerated, and support adequate growth when added to infant formulae or to foods for young children. In babies, inulin-type fructans and in particular Orafti®Synergy1 show benefits of modulating the

microbiota of formula-fed babies, by rendering it more similar to that of breastfed babies. As such they have the potential to contribute to both the immediate and long-term health benefits of a balanced microbiota established early in life.

3.2.4 Inulin-Type Fructans and Weight Management

Achieving and sustaining appropriate body weight over a lifespan is vital to maintain good health. Obesity, which is today recognized as a disease, is one of the most important public health problems nowadays, as it affects a large part of the worldwide population. About one billion adults are currently overweight, and a further 475 million are obese (International Association for the Study of Obesity (IASO) 2011).

Overweight and obesity are major risk factors for a number of chronic diseases such as cardiovascular disease, type 2 diabetes, and some types of cancer (World Health Organization (WHO) 2011). Causal factors of overweight and obesity are multiple and their interaction is complex. We can see those as the result of interactions between human biology and environmental factors, being involved in the complex process of regulation of energy balance in order to maintain body weight. Central to the problem however is today's society characterized by excessive energy intake and by minimal energy expenditure, the so-called obesogenic environment.

Strategies to support weight management and to prevent obesity postulate, among others, the development of healthier food choices.

An increase in dietary fiber intake is encouraged, among other things, in an effort to overcome the gap between recommended and actual fiber intake. Apart from their beneficial role in colon health, dietary fibers have received increasing attention with respect to their role in energy intake and weight management.

Epidemiological evidence suggests that a higher dietary fiber intake is associated with lower body weight (Du et al. 2010; Tucker and Thomas 2009).

Foods rich in dietary fiber are usually less energy dense, as dietary fibers have a lower energy value than digestible carbohydrates, fat, and protein. For instance, inulin and oligofructose provide 1–1.5 kcal/g, while fully available carbohydrates provide about 4 kcal/g and fat about 9 kcal/g. Enriching foods with dietary fibers like inulin and oligofructose or replacing ingredients of higher caloric value like sugars and fat can directly reduce the intake of metabolizable energy.

Controlled human intervention studies indicate that individual dietary fibers – apart from their "energy diluting" properties – may specifically influence energy intake and appetite control. Different types of fibers may act differently in this respect.

Recent well-designed human studies, both in healthy, overweight, and obese volunteers, suggest a particular role for Orafti®Synergy1 and oligofructose in promoting a moderate negative energy balance in humans consuming a diet ad libitum.

In experimental animal models, numerous studies have described the effects of inulin-type fructans (5–10 % in feed) and more specifically of Orafti®Synergy1 and oligofructose on lowering energy intake, body weight, and/or fat mass. Several

models were studied, from normal growing rats (Cani et al. 2004), where first evidence of an impact of inulin-type fructans on lower (epididymal) fat mass accumulation and energy intake was shown in several models of obesity.

In Zucker *fa/fa* rats (genetically obese rats), the addition of 10 % Orafti®Synergy1 or oligofructose to the diet of the animals decreased their energy intake and resulted in a lower body weight and lower (epididymal) fat mass development compared to the control group (Daubioul et al. 2000, 2002). These effects were not observed when non-fermentable fibers (e.g., cellulose) were added to the diet, highlighting that it was not a fiber effect per se, but that the effects are linked to the distinct colonic fermentation of prebiotic fibers. These effects on energy intake and lower fat mass and/or body weight were confirmed in several further models, such as diabetic rats (streptozotocin-treated Wistar) (Cani et al. 2005), in a mouse model on a high-fat diet (Cani et al. 2006a, 2007) or a rat model on high-fat diet (Delmée et al. 2006) as well as in a genetically obese mouse model (ob/ob mice) (Cani et al. 2009a; Everard et al. 2011). In a study involving diet-induced obese Sprague-Dawley rats, and receiving either oligofructose (10 %) or a probiotic (*Bifidobacterium animalis* subsp. *lactis* BB12) (BB-12), the prebiotic, but not the probiotic, reduced energy intake, weight gain, and fat mass, which also highlights a probable role for fermentation related to the prebiotic fiber (Bomhof et al. 2014). The observed effects on body weight are persistent over time, as shown by a lifelong intervention study in rats where Orafti®Synergy1 supplementation resulted in a lower body weight evolution during the whole lifespan of healthy animals, and at the same time prolonged their survival rate to 24 months (Rozan et al. 2008).

The specific colonic fermentation is an important aspect to take into account to understand the effects of inulin-type fructans on energy intake and body weight. The observations made in animal studies are accompanied by changes in the levels of gastrointestinal hormones involved in appetite regulation, i.e., mainly of glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin.

GLP-1 is a peptide gut hormone released postprandially in response to nutrient intake. GLP-1 is produced in the intestinal endocrine L-cells, mainly present in the ileum and the colon, and higher circulating levels of this hormone are associated with lower subjective hunger feelings and lower food intake. The gut hormone PYY is a peptide synthesized and released like GLP-1 (from L-cells) present in the distal gastrointestinal tract. PYY levels are low in the fasting state and also rapidly increase in response to food intake. Peripheral infusion of PYY3–36, the major circulating form, reduced appetite and caloric intake in normal-weight and overweight human subjects (Karra et al. 2009). Ghrelin is a hormone primarily released by the stomach but also from the small and large intestine. Its blood levels rise during fasting and rapidly fall postprandially. Higher ghrelin levels are associated with higher appetite and hunger sensations.

In several of the animal studies cited above, the effects on energy intake, body weight, or body fat mass were accompanied by increased intestinal and portal levels of GLP-1 in the fasting state as well as postprandial GLP-1 response in the plasma. Orafti®Synergy1 and oligofructose increased proglucagon mRNA level

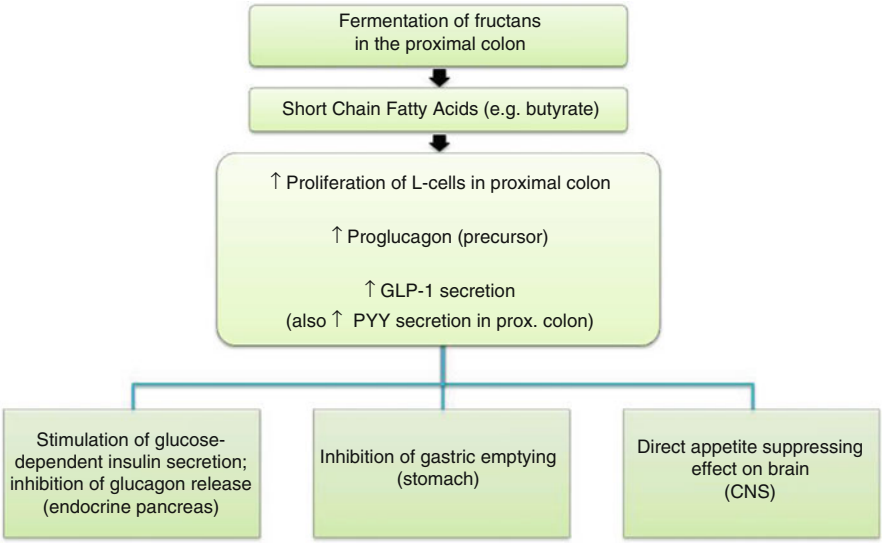

Fig. 4 Proposed mechanism of action of the effects of inulin-type fructans on appetite regulation and food intake (Adapted from Delzenne et al. 2005)

(the precursor of GLP-1) in the proximal colon and cecum in the fasting state and postprandially. Increased postprandial expression of cecal PYY was also shown in lean and obese animals. In mice receiving a GLP-1 receptor antagonist (exendin 9–39) or in GLP-1 receptor knockout mice (i.e., mice lacking a functional GLP-1 receptor), the decrease in energy intake and body weight observed with oligofructose supplementation was abolished, which clearly underlines the role of GLP-1 as mediator of the effects of fructans (Cani et al. 2006a).

The effects of inulin-type fructans on gut peptides and in particular on GLP-1 could be mediated by SCFA, which are also signaling molecules acting as endogenous ligands for the G protein-coupled receptors FFAR3 and FFAR2 (Brown et al. 2003), also named GPR41 and GPR43, respectively. These receptors are expressed in a variety of gastrointestinal cells and signaling through these receptors mediates the synthesis of GLP-1 by L-cells (Tolhurst et al. 2012). Figure 4 outlines the potential mechanism of action of fructans on appetite regulation and food intake.

In humans, several well-designed studies, both in healthy, overweight, and obese volunteers, have confirmed the effects of both Orafti®Synergy1 and oligofructose on reduced ad libitum energy intake in the context of body weight management.

In a randomized, double-blind, placebo-controlled trial, the intake by 36 overweight or obese individuals of 2 × 6 g/day of Orafti®Synergy1 during 3 weeks resulted in a significantly lower energy intake (McCann et al. 2011).

In healthy normal-weight (or normal to slightly overweight) adults as well, two randomized interventions conducted with 2 × 8 g/day of Orafti®Synergy1 or oligofructose (vs. maltodextrin as placebo) have similarly evidenced a reduction

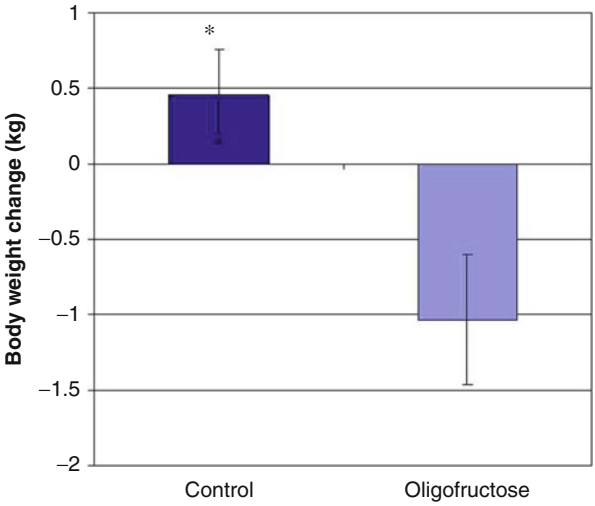

Fig. 5 Effect of 12 weeks supplementation with oligofructose (21 g/day) or placebo on body weight modulation (mean ± SEM) in overweight and obese subjects. *$p < 0.05$ versus placebo (Adapted from Parnell and Reimer 2009)

in energy intake (of 5–6 %) after a 2-week intake period of the fructan. This reduction of energy intake was measured during a test day where participants had ad libitum access to free-choice buffet meals. During the intervention, volunteers were instructed to keep their normal eating habits (Cani et al. 2006b, 2009b). In those studies, the findings on lower energy intake were accompanied by significant effects on appetite sensations (decreased hunger, increased fullness and satiety) as well as significant postprandial higher levels of GLP-1 and PYY (Cani et al. 2009b).

Consequently, benefits on body weight management have further been found in a randomized, double-blind, placebo-controlled trial, involving 48 overweight or obese healthy adults. The subjects receiving oligofructose (21 g/day) experienced a significant reduction in body weight of 1.03 kg, while the control subjects gained 0.45 kg weight, after 12 weeks of intervention (Fig. 5). The weight loss affected mainly body fat mass, in particular trunk fat mass. The weight loss could be explained by the lower energy intake observed in subjects in the oligofructose group. The subjects did not modify their physical activity or dietary habits, thus attributing the observed results concerning body weight, fat mass, and caloric intake to the oligofructose supplementation. An analysis of the blood levels of hormones involved in appetite regulation further revealed lower postprandial ghrelin and higher PYY responses in the subjects consuming oligofructose. GLP-1 levels remained unaffected (Parnell and Reimer 2009). A slight decrease in fat mass was also observed in obese women receiving 16 g/day of Orafti®Synergy1 compared to placebo (Dewulf et al. 2013). An earlier randomized, double-blind intervention trial, initially designed to study the impact of Orafti®Synergy1 on bone metabolism, adds further weight to these more recent observations of favorable effects of Orafti®Synergy1 in the context of body weight management (Abrams et al. 2007a). Ninety-seven healthy adolescents were randomized to receive 8 g/day of Orafti®Synergy1 or maltodextrin during a year. After 1 year, the body mass index (BMI) increase and the total body fat mass were

significantly lower in subjects who received Orafti®Synergy1 compared to maltodextrin. The BMI Z-score (age and sex normalized BMI value) of the adolescents in the maltodextrin group significantly increased, as compared to the Orafti®Synergy1 group where no significant increase was observed during the year. The BMI change in the Orafti®Synergy1 group was consistent with an adequate increase during puberty. The authors concluded that Orafti®Synergy1 supplementation had a significant benefit in the maintenance of an appropriate BMI increase during pubertal growth in primarily non-obese young adolescents which could help avoid undesirable weight increase at this critical stage of life.

On the whole, these well-designed human intervention studies conducted on normal-weight, overweight, and obese subjects have consistently shown a reduction in energy intake with 12 g/day of Orafti®Synergy1 and 16 g/day of oligofructose. Putative mechanisms of action, widely demonstrated in animal models, probably involve the effects of inulin-type fructans on appetite-related hormones, such as GLP-1 and PYY, and subsequent consequences on respective sensations (hunger, satiety...) which are shown in few human studies as well. A recent systematic review of human studies conducted with prebiotics indicates that current evidence supports that prebiotic supplementation increases self-reported feelings of satiety significantly in healthy adults (Kellow et al. 2014).

Weight management is not only a question of losing weight, but also the prevention of unhealthy weight gain. It has been estimated that the increase in obesity rates in the United States (USA) between 1980 and 1994 could be accounted for by an excess of just 4 kcal/day over maintenance energy requirements in women and 13 kcal/day in men (Khan and Bowman 1999). Preventing this small daily calorie gain thus has important repercussions for the prevention of obesity. Inulin-type fructans may help to make such changes.

In addition to the effects described above, another topic of growing interest is the association between obesity and changes in the composition of the gut microbiota. The composition of the gut microbiota has been shown to differ between lean and obese individuals (Ley et al. 2006b). Early studies have reported changes at the phylum level, mainly assessing the Bacteroidetes-to-Firmicutes ratio, with inconsistent associations. Of note, this ratio is a rough measure because such broad classification of bacterial taxa includes pathogens as well as bacteria generally regarded as beneficial to the host (Karlsson et al. 2013). A few human studies examined also possible differences in the gut flora composition below the phyla level, i.e., at the groups or species level and looking at groupings like bifidobacteria, bacteroides, or clostridia. In those studies, in which the bifidobacteria level between normal-weight and overweight or obese individuals was compared, a significantly and/or numerically higher bifidobacteria level related to normal body weight was observed as well (Schwiertz et al. 2010b; Balamurugan et al. 2010).

Determining whether the altered gut microbiota contributes to obesity or the opposite (chicken or egg causality dilemma) requires prospective studies. Kalliomäki et al. (2008) showed that children who had a normal weight at 7 years of age showed a higher Bifidobacterium level during their first year of life than children who became overweight.

The gut microbiota is also suggested to be involved in the development of a low-grade inflammation, that in turn is associated with the metabolic disorders related to obesity (Delzenne et al. 2013). Increased levels of serum lipopolysaccharide (LPS) are shown in obese or high-fat diet-fed individuals. LPS is the main compound of Gram-negative bacteria and is among the most potent inducers of inflammation (Cani et al. 2012). This LPS increase might be the consequence of an increase in chylomicron formation, a decrease in gut barrier integrity and/or a decrease in alkaline phosphatase activity (an enzyme responsible for the cleavage of LPS in the intestine) (Delzenne et al. 2013). Diet-induced obesity in rodents resulted in altered gut microbiota composition (notably with lower levels of Bifidobacterium spp.) and induced a low-grade inflammation state (higher LPS levels) (Cani et al. 2012). In these models inulin-type fructans were able to counteract the increase in LPS levels, notably through an improvement of the expression and activity of proteins involved in gut barrier function (GLP-2) (Delzenne et al. 2013), in addition to the modulations in microbiota composition. Gut microbiota modulations following prebiotic intake (context of obesity and type 2 diabetes animal models) not only abolished gut permeability, endotoxemia, and inflammation but also decreased body weight and fat mass accumulation as consistently observed in several studies (Everard and Cani 2013). In addition, improved glucose homeostasis was also shown.

3.2.5 Inulin-Type Fructans and Blood Sugar Management

Carbohydrates represent the main energy source of the body via the glucose they supply in a direct or an indirect way. Not all carbohydrates or carbohydrate foods are however alike with respect to physiological effects and elevation of postprandial glycemia. Carbohydrates that have a lower glycemic response (GR) may be beneficial for individuals with impaired glucose tolerance. As recently reviewed by leading experts in the field of carbohydrate metabolism, there is convincing evidence from meta-analysis of controlled dietary trials that low glycemic diets improve glycemic control in people with type 2 diabetes and type 1 diabetes (reviewed in ICQC 2013). There is further convincing evidence from meta-analysis of prospective cohort studies that low glycemic diets reduce the risk of type 2 diabetes and of coronary heart disease (reviewed in ICQC 2013). Based on a large body of scientific evidence, these experts concluded that reducing postprandial glycemic response is recognized as a beneficial physiological effect (ICQC 2013; EFSA 2011; Ceriello and Colagiuri 2008; Levitan et al. 2004; Coutinho et al. 1999).

Inulin and Oligofructose Reduce Postprandial Glycemic Responses

Since inulin and oligofructose are nondigestible carbohydrates, they do not contribute to postprandial glycemia. Replacing digestible and glycemic carbohydrates (e.g., sugars or maltodextrin) partially or completely by inulin or oligofructose on a weight-by-weight basis in a food product reduces the amount of available carbohydrates and consequently the postprandial blood glucose response of the food. Depending on the level of refinement, commercially available forms of inulin and

oligofructose may contain varying amounts of accompanying mono- and disaccharides that can contribute to the blood glucose response. A recent systematic review of human studies related to the metabolic benefits of prebiotics concluded that prebiotic supplementation significantly reduces postprandial glucose and insulin concentrations (Kellow et al. 2014).

If fructans (oligofructose) are present in relevant amounts, for instance, in a commercial product with about 15 % sugars, they show only about 20 % of a blood glucose response of glucose, but still provide sweetness to the product (Meyer 2007). Other types of commercial products contain very few or no residual sugars at all. A well-considered choice of oligofructose or inulin with respect to its blood glucose response and other functional properties like pleasant taste makes an optimization of product applications possible.

Further studies (including unpublished data) show significant reductions in the postprandial glycemic response for different foods in which sugars have been replaced by oligofructose at levels of 20 % or more. A linear relationship between the extent of sugar replacement and a reduction in the resulting blood glucose response shows that higher fructan levels will result in greater effects, respectively.

A reduction of postprandial glycemic responses (as long as postprandial insulinemic responses are not disproportionally increased) might be a beneficial physiological effect according to the European Food Safety Authority (EFSA). Based on the data available including a recent unpublished study conducted by BENEO together with other inulin producers, a scientific dossier was compiled by BENEO and submitted to EFSA. It received a positive opinion (EFSA 2014) and is currently subject to the final Regulation that will be adopted by the EU Commission. Food products where sugar has been replaced by inulin-type fructans will be able to bear a claim stating that the "Consumption of a food product containing oligofructose (or inulin) instead of sugars, raises blood glucose concentrations less after consumption, compared to the sugar-containing regular food product" (or similar wording).

Long-Term Blood Glucose Control

Some studies show that a higher intake of inulin or oligofructose with a balanced diet can positively influence markers of blood glucose control and insulin sensitivity.

For instance, in the study by Russo et al. (2010) conducted in a 2-arm double-blind, randomized crossover design, the daily consumption of inulin-enriched pasta with 11 % inulin as part of a controlled diet for 5 weeks was associated with significant beneficial effects on fasting glucose and protein glycation (fructosamine, HbA1c) as well as insulin resistance (Homeostasis Model Assessment of Insulin Resistance, HOMA-IR) in 15 healthy male adults, whereas no significant changes were seen with regular pasta. Significant beneficial effects of inulin-enriched pasta were also seen on HDL cholesterol and triglyceride levels, with no significant changes in the control intervention.

Daubioul et al. (2005) found in their 8-week intervention study conducted in 2-arm double-blind, randomized, placebo-controlled crossover design that the

intake of daily 16 g oligofructose at breakfast and dinner as part of the otherwise normal diet was associated with significantly lower levels of fasting insulin and C-peptide in seven patients with nonalcoholic steatohepatitis (NASH) after 4 weeks in comparison to maltodextrin.

In a randomized intervention study involving women with type 2 diabetes, the effects of inulin supplementation (10 g/day) versus placebo (maltodextrin) were studied with respect to diabetes control and inflammatory status notably. The results of this trial show that inulin significantly reduces fasting plasma glucose, HbA1c levels, fasting insulin, and HOMA-IR in comparison with the control group. In addition, inflammatory markers, such as high-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor-alpha (TNF-α), and LPS, were also significantly reduced upon inulin intake (Dehghan et al. 2013, 2014a, b; Pourghassem Gargari et al. 2013). Subclinical inflammation is linked to the development of insulin resistance, and the results of the above study show positive outcomes with respect to biomarkers of inflammation, in addition to the improvement of the glycemic status of diabetic women.

With respect to mechanisms of action, several data in animals support an effect of inulin-type fructans on enhanced GLP-1 production in the colon (see section on weight management for more details). GLP-1 is secreted in response to the presence of a meal in the gut and stimulates insulin secretion in a glucose-dependent way, thereby contributing to the so-called incretin effect. GLP-1 also inhibits gastric emptying. GLP-1 has become a target for research into antidiabetic therapies: GLP-1 agonists and dipeptidyl peptidase (DPP)-4 inhibitors (the enzyme responsible for the inactivation of GLP-1) are nowadays used for the treatment of type 2 diabetes.

This GLP-1 production might thus be part of the beneficial effects of fructans shown with respect to glycemic control (Roberfroid et al. 2010):

- GLP-1 has been shown to increase β-cell differentiation.
- In streptozotocin rats receiving inulin-type fructans, improvement in glucose tolerance and pancreatic β-cell mass is not reproduced by only pair-feeding restrictions, indicating that the beneficial effects of inulin-type fructans are not due to their effect on appetite alone.
- Improved insulin sensitivity was shown in rodents fed oligofructose, and this effect was mediated by GLP-1 (Cani et al. 2006a).
- Other mechanisms, like the effects on lowering inflammatory tone, could contribute to the positive effects of fructans observed in glucose metabolism.

A recent publication has also suggested a role for SCFA produced by the microbial fermentation of fructans in their metabolic benefits. In an experimental design where rats were fed oligofructose or SCFA (propionate or butyrate), it was shown that oligofructose and SCFA improved glucose tolerance and insulin sensitivity. SCFA were shown to activate intestinal gluconeogenesis (IGN). Intestinal gluconeogenesis has been shown to induce beneficial effects on glucose and energy homeostasis. The metabolic benefits (on glucose control) induced by oligofructose

or SCFA being absent in mice deficient of IGN, this experience pointed to a specific causal role of IGN regulation in the metabolic benefits associated with oligofructose (De Vadder et al. 2014).

We can conclude, from the long-term studies above, that enrichment of the diet with inulin or oligofructose has beneficial effects on biomarkers of long-term blood glucose control such as fasting glucose, HbA1c, and insulin functionality. These data encourage the use of inulin and/or oligofructose as an approach to reduce blood glucose and insulin profiles in the diet of people aiming to improve blood glucose control in the prevention and management of glucose tolerance impairments, beyond their beneficial effects on colon health.

3.2.6 Inulin-Type Fructans and Bone Health

Osteoporosis is a major widespread health-care problem, with around one in three women and one in five men being at risk of an osteoporotic fracture (International Osteoporosis Foundation (IOF) 2013). Osteoporosis is a chronic and progressive disease characterized by low bone mass and micro-architectural impairment of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Bone mass increases during childhood and adolescence, reaching its peak value during young adulthood (Prentice et al. 2003). It is estimated that a 10 % increase of peak bone mass in children reduces the risk of osteoporotic fracture during adult life by 50 % (IOF 2013). Therefore, prevention of osteoporosis starts early on by maximizing peak bone mass during adolescence. Calcium, as a structural component of the bone, plays an essential role here, as it represents 40 % by weight of the mineral in the skeleton (Prentice et al. 2003). Adequate calcium intake is thus clearly an important factor in maintaining bone mass throughout life. In addition to dietary intake (often inadequate), intestinal absorption is a key factor that controls the retention of calcium in the body and thus the mineralization of the bones.

The fractional absorption of calcium depends on a number of aspects, including the calcium load and source. The absorption of calcium from cow's milk is around 30 %, but the efficiency of this process declines as we get older. Two main mechanisms are involved in calcium absorption: an active transcellular transport, which takes place mainly in the small intestine, is vitamin D mediated, and becomes saturated with an increased dietary calcium load, and a passive, paracellular, non-saturable process, which takes place throughout the whole intestine and which can be influenced by inulin-type fructans.

Colonic fermentation of inulin-type fructans is thought to be the main mechanism contributing to their effect on the stimulation of intestinal calcium absorption. The SCFA and lactic acid produced as a consequence of fermentation contribute to lower the luminal pH in the colon, which brings some of the insoluble calcium complexes arriving in the colon into solution, thus making it available for absorption (Scholz-Ahrens and Schrezenmeir 2002; Raschka and Daniel 2005). As a consequence, inulin-type fructans influence the passive absorption contribution in the colon. This was confirmed in studies with animal models showing that the solubility of dietary calcium in the large intestine was enhanced during supplementation with different inulin-type fructans (Younes et al. 2001; Coudray et al. 2003).

Other mechanisms may include a direct exchange of these SCFA on transcellular calcium absorption by increasing the exchange of cellular H^+ for luminal Ca^{2+}, increased butyrate production which stimulates colonic mucosal proliferation thus increasing the absorptive surface area of the gut, and/or an increase in mucosal calbindin D_{9K} and vitamin D receptor in the large intestine (Roberfroid et al. 2002; Cashman 2006).

In more than 20 animal studies, the beneficial effects of inulin-type fructans related to higher intestinal calcium absorption, bone turnover, and bone mineralization have been demonstrated. The positive effects on calcium absorption of inulin-type fructans did not affect the absorption of other nutrients. Several studies further demonstrated favorable effects by showing an increase in bone mineral density and/or content (BMD/BMC) (Roberfroid et al. 2002; Scholz-Ahrens et al. 2002; Kruger et al. 2003; Nzeusseu et al. 2006; Weaver et al. 2010) and could even lead to improved biomechanical properties of the bones (Lobo et al. 2006). Positive outcomes were also confirmed in ovariectomized rats, a model mimicking the estrogen-deficient postmenopausal status of a woman (Scholz-Ahrens et al. 2002; Zafar et al. 2004; Legette et al. 2012). It was shown that inulin-type fructan supplementation could prevent the ovariectomy-induced loss of bone structure, compared to rats in the control group (Scholz-Ahrens et al. 2002).

Studies comparing Orafti®Synergy1 with oligofructose and/or inulin in parallel showed that the positive effects on calcium absorption were more pronounced with Orafti®Synergy1 (Coudray et al. 2003; Legette et al. 2012). This can be explained by the fact that Orafti®Synergy1 may have a more favorable chain-length distribution, which results in a more evenly distributed fermentation pattern, which includes both the proximal and distal part of the colon. Hence the surface of the full length of the colon can be used to increase calcium absorption efficacy.

This observation was confirmed in a randomized, double-blind human study, where 8 g/day of Orafti®Synergy1 significantly increased true calcium absorption (by almost 20 %) in adolescent girls compared to placebo, while oligofructose did not show a similar effect at the same dose (Griffin et al. 2002). In contrast, a previous study had shown a positive outcome on calcium absorption with 15 g/day of oligofructose (van den Heuvel et al. 1999). Both studies recruited young adolescents because of the importance of a high calcium absorption during that life stage. In a multicenter extension of their first study, Griffin et al. (2003) demonstrated that volunteers with a low calcium absorption efficiency under basal conditions seemed to benefit the most from Orafti®Synergy1 intake.

Beneficial effects were also demonstrated in a placebo-controlled, randomized, double-blind crossover study in postmenopausal women (Holloway et al. 2007). A 10 g/day ingestion of Orafti®Synergy1 significantly increased true calcium absorption, while the control group showed a slight decrease in calcium absorption. In addition, certain biomarkers related to bone formation (serum osteocalcin) or to bone resorption (urinary deoxypyridinoline cross-links) were positively modulated.

To demonstrate that the effects of Orafti®Synergy1 on calcium absorption occur mainly in the colon, Abrams et al. (2007b) included kinetic measurements to

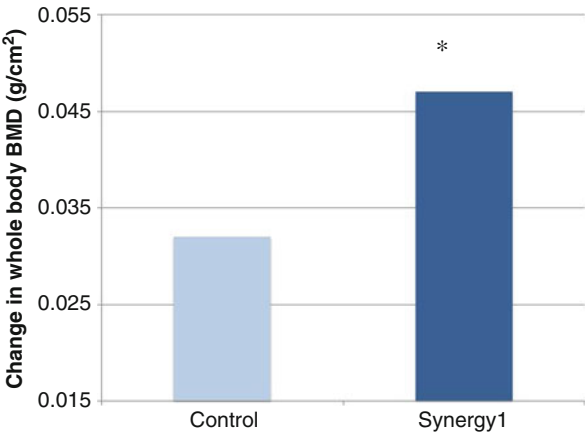

Fig. 6 Changes in whole-body bone mineral density after supplementation with Orafti®Synergy1 in children. *$p = 0.01$ versus control (Adapted from Abrams et al. 2005)

evaluate the time course of calcium absorption. Based upon literature showing that calcium absorption takes place in the colon around 7 h after oral intake, this research team not only confirmed the increased calcium absorption with Orafti®Synergy1 in young adults, but they also showed, by following the kinetic of calcium absorption in the blood, that the relative increase in calcium absorption after Orafti®Synergy1 intake was mainly related to the colonic part of the gastrointestinal tract.

One human intervention study has examined the effects of Orafti®Synergy1 on calcium absorption and on bone mineralization on a long term. A total of 100 adolescents (from 9 to 13 years old) were involved in a 1-year randomized, double-blinded intervention study and were supplemented with 8 g/day of either Orafti®Synergy1 or placebo (maltodextrin). True calcium absorption was significantly enhanced after 8 weeks in the Synergy1 group, and this beneficial effect was maintained during the whole intervention year. At the end of the experimental period, adolescents supplemented with Orafti®Synergy1 had a significantly greater increment in both whole-body bone mineral density (BMD) (Fig. 6) and whole-body bone mineral content (BMC). The authors noted that the net benefit associated with intake of Orafti®Synergy1 was an average increase in calcium accretion to the skeleton of approximately 30 mg per day (Abrams et al. 2005).

In conclusion, the totality of the data shows that the ingestion of inulin-type fructans, in general, and Orafti®Synergy1, more specifically, results in an increased calcium absorption, and the study of Abrams et al. (2005) further shows that this increase in calcium absorption persists over the long term and that the additionally absorbed calcium is reaching the bones and used there (increased bone mineralization).

3.3 Healthy Aging and Noncommunicable Diseases

Human aging is inevitable and has an impact on morbidity, quality of life, as well as health-care costs. Our societies are aging, and both the proportion of older people

and the life expectancy are increasing throughout the world. In Europe, the proportion of people aged 65 years and over is expected to be 24 % of the population in 2030, and in the United States (USA), this group may number 72 million (or 19 % of the population) by 2030 (US Department of Health & Human Services). Similar increases in the elderly population have been reported in Asia and in South America. These demographic changes have implications for health, as well as for pensions, social care, and housing. Healthy aging, i.e., an absence of chronic disease and slower decline in cognitive and physical function, is the goal of everyone. However, disease statistics tell a different story and confirm that a significant proportion of older people experience chronic disease, poor mobility, and frailty in their final years. The demographic figures and concern have prompted the World Health Organization (WHO) to develop and publish a 2012–2020 strategy for healthy aging in Europe focused on prevention of falls and promotion of physical activity among other policy interventions (WHO 2012). Life expectancy continues to rise in Europe, but healthy life expectancy is not improving, measured as the number of years spent free of activity limitation at a particular age. Thus, while a man from France, Spain, or Italy could expect to live to around 78 years, he will spend the last decade of his life with a chronic illness. The gap between actual and healthy life expectancy is greater in countries with a lower gross domestic product (GDP) suggesting that poverty has a detrimental impact on wellness.

Getting older healthier is strongly influenced by the development of noncommunicable diseases (NCD), the leading cause of death in the world (WHO 2011). NCDs include cardiovascular disease, obesity, cancer, type 2 diabetes, osteoporosis, chronic respiratory, and age-related macular degeneration. According to the same WHO report on NCDs, raised blood pressure, tobacco use, raised blood glucose, physical inactivity, and overweight and obesity are the risk factors underlying most NCDs. The development of NCDs is a long-term silent process, caused by the additive effects of genetic susceptibility, age-related physical and metabolic changes, as well as years of lifestyle deviations.

In this global context, it is clear that prevention of risk factors is a much more efficient approach than the treatment of established medical consequences. Prevention is achieved through changes and adaptations in lifestyle, including a balanced energy intake and a balanced food choice. These, for instance, include small changes to modifiable risk factors, such as lowering total and/or LDL cholesterol, aiming for lower blood glucose and insulin profiles, maintaining a healthy body weight, increasing dietary fiber intake, and taking regular physical exercise.

In view of all health-related aspects that have been reviewed in the present book chapter, it appears that inulin-type fructans, through their impact in the large intestine and globally in several aspects of the functioning of our body, may support healthy nutrition, through different windows of opportunity and at different ages.

During the first year(s) of life, nutrition is capital for a normal development of the infant and child and strongly influences health later in life. Early-life programming is notably becoming an accepted scientific concept with nutrition having an impact on sensitive time periods of a fetus and infant development that have long-term consequences. Adaptations during these sensitive periods may predispose

individuals to health problems such as metabolic syndrome or related diseases, including glucose intolerance, insulin resistance, or obesity (Nauta et al. 2013). Large-scale research programs, e.g., in the European Union, are investigating various aspects in infant nutrition. Getting closer to breast milk feeding conditions is the goal, if the "gold standard" breastfeeding is not the option chosen by the mother. In infants and young children, inulin-type fructans from chicory promote the growth of naturally occurring bifidobacteria selectively so that the composition of the intestinal microbiota of a bottle-fed baby gets closer to that of a breastfed baby. Stools are also a target of prebiotics in infants as there is less constipation and less features related to constipation when added to infant formulae.

Childhood and adolescence are critical periods of life for building up adequate bone mass density and reducing therefore the risk of osteoporosis later in life. In this context, a specific inulin-type fructan (Orafti®Synergy1) was shown to increase calcium absorption with resulting effects on bone mineralization. Effects on better calcium absorption have been shown in postmenopausal women as well. The mode of action is linked to fermentation and the resulting environment in the colon that favors the absorption of additional calcium.

During adulthood, the promotion of bacteria considered as representative of a healthy microbiota is regarded as something important for overall digestive health balance. Weight management is a nutrition challenge that an important part of the adult population is facing. Related to overweight and obesity, diabetes mellitus is of key concern as both diet-related challenges lead to health political avalanches. The increase in weight during this period is very often around 1 kg per year or higher leading to significant overweight and metabolic consequences. Obviously, there is a multifactorial logic behind this. Ingredients such as inulin-type fructans that influence energy intake naturally and have an impact on blood glucose response are new approaches in a healthy diet supporting proper weight management and blood glucose management.

In elderly, as cited already, on top of all other health concerns, osteoporosis prevention is highly relevant. In all age groups, digestive health is certainly in the focus. The concerns related to cancer and other gut-related diseases are increasing, constipation affects an important proportion of the population (increasing with age), and digestive health plays an important role in overall well-being. Most people know about fiber and are interested in bridging "the fiber gap" and more and more receptive as well to learning about positive influences of prebiotics of their gut microbiota.

4 Conclusion

The present book chapter highlighted the broad spectrum of effects of inulin-type fructans. As dietary fibers, they easily allow the enrichment of traditional foods and help consumers to meet their fiber needs. As fermentable fibers, inulin-type fructans show their efficacy firstly in the large intestine, where their selective fermentation by the microbiota induces favorable changes in its composition, with most human

studies showing a consistent and selective stimulation of *Bifidobacterium* ssp. Further research focuses on changes in the metabolites produced, resulting from the saccharolytic fermentation. Because of the central role of the large intestine in health, and the intricate relationships that exist between microbes in the colon, their metabolites, and human metabolism, the effects of inulin-type fructans are not restricted to the colon. Metabolic effects radiate towards other parts of the body and are mediated by several metabolites from fermentation. Experimental research shows that these metabolites find receptors to convey their messages and explain the changes observed in terms of metabolism with the actual resulting health benefits. Recent human data support benefits of inulin-type fructans on energy homeostasis, controlling the level of calories ingested. These events probably also arise from the colon, through modulations of gut peptides involved in appetite regulation. The colon is also a center of inner protection, and several plausible mechanisms exist by which inulin-type fructans may influence immune functions starting in the gut. Research is ongoing in this area as well.

Positive effects on bone metabolism, with higher calcium absorption and resulting higher bone mineralization, have been demonstrated. To date, hundreds of studies have been conducted with inulin-type fructans, and all point, in one way or another, to benefits, from babies, where the microbiota development is central, to an aging population.

Knowledge about the microbiota composition and its health effects is probably still only the tip of the iceberg. Understanding the effects of the microbiome further on health and on the pathogenesis of several common diseases will allow further health developments. In that context, colonic nutrients that have an impact on these processes and favor them in a beneficial way may play an important role in supporting health. Further exciting research is likely to contribute to understanding the impact of prebiotic inulin-type fructans on health.

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Active Edible and Biodegradable Starch Films

24

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Contents

Abstract

Starch appears as a good alternative to petro-based plastics. This biopolymer exhibits a greater potential to obtain biodegradable films, thanks to its easy availability and low cost. Starch has been used to develop edible, biodegradable, active films using different strategies: blending with antimicrobial polymers, active nanoparticles, and other antioxidant or antimicrobial compounds, such as essential oils of phenolic extracts. The incorporation of these compounds to starch or starch blend films has an impact on their physical properties which can affect their functionality as a packaging material.

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1 Introduction

In the last few years, a great number of studies have focused on the development of environmentally friendly materials to substitute the petro-based plastics as a means of solving the environmental problems that they cause and to reduce the petroleum dependence, whose reserves are limited. Substituting synthetic nonbiodegradable plastics requires the development of low-cost materials with similar properties which can compete in the packaging market (Psomiadou et al. 1996; Mali et al. 2002; Mali et al. 2006; Famá et al. 2007; Jiménez et al. 2012d). Likewise, the biodegradable polymeric films should be obtained from renewable sources (Lu et al. 2005; Tharanathan 2003; Yu and Chen 2009). Of the polymeric materials which are biodegradable or compostable (bioplastics), obtained from renewable sources, starch occupies a significant position. Starch constitutes more than 60 % of cereal kernels and is relatively easy to separate from the other chemical components (Arvanitoyannis and Kassaveti 2009). The film-forming ability of starch and its easy availability and low cost make it one of the raw materials with greater potential to obtain biodegradable films.

Starch granules are not soluble in cold water due to the strong interchain hydrogen bonds which hold the starch chains together. However, when starch is heated in water, the crystalline structure is disrupted and water molecules interact with the hydroxyl groups of amylose and amylopectin, producing the partial solubilization of starch (Hoover 2001). Heating starch suspensions in an excess of water or of another solvent able to form hydrogen bonding (e.g., liquid ammonia, formamide, formic acid, chloroacetic acid, and dimethyl sulfoxide) at high temperatures (between 65 °C and 100 °C approximately depending on the type of starch) provokes an irreversible gelatinization (destructuration) process. This process is greatly affected by the kind of solvent and the starch–solvent ratio. Starch gelatinization or grain destructuration is required for the purposes of obtaining starch films. This process introduces irreversible changes in starch granules, such as the loss of crystallinity, water absorption, and the swelling of the granules (Zhong et al. 2009; Carvalho 2008). Gelatinization involves two steps: the hydration and diffusion of the solvent into the starch granules and the melting of starch crystals (Donovan 1979; Liu et al. 1991; Jenkins and Donald 1998). The gelatinization process and its relevance in the production of starch-based films are reviewed in the next section.

Starch films exhibit some very good properties, since they are tasteless, odorless, transparent, and with very low oxygen permeability, which is highly appropriate for food preservation. However, they also have some drawbacks: highly sensitive to water (hygroscopic character), which provokes changes in mechanical response depending on the relative humidity; high values of water vapor permeability; and retrogradation phenomena, associated to the progressive chain aggregation/crystallization, depending

on the molecular mobility in the system. To mitigate these aspects, different strategies have been considered such as starch blending with other components – lipids (Jiménez et al. 2012a; Ortega-Toro et al. 2014a), organic (Curvelo et al. 2001; Müller et al. 2009; Cano et al. 2014) or inorganic fillers (Carvalho et al. 2001; Avella et al. 2005), and other hydrocolloids (Jiménez et al. 2012b, c; Ortega-Toro et al. 2014b). The use of different polymer compatibilizers (Ortega-Toro et al. 2014b) or starch modifications (Kaur et al. 2012) has also been tested in order to increase the polymer's compatibility and to improve film properties.

The incorporation of bioactive compounds, with antimicrobial or antioxidant properties, in order to obtain active films has also been studied in starch or starch blend films (Pyla et al. 2010; Zhong et al. 2011; Bonilla et al. 2013; Jiménez et al. 2013, 2014; Fabra et al. 2014). Active films represent a great hope for the food industry and consumers. They make it possible to prolong the product shelf life, thus increasing benefits for producers and consumers through waste reduction, while food quality can be better assured. Nevertheless, the incorporation of these compounds to starch or starch blend films has an impact on their physical properties which can affect their functionality as a packaging material. In this chapter, the effect of starch blending with antimicrobials or antioxidant compounds on the film's active and physical properties is analyzed.

2 Starch in the Development of Biodegradable Films

2.1 Starch: Structure, Properties, and Sources

It is well known that starch is composed of two types of polysaccharides, amylose and amylopectin, polymers of α-D-glucose connected by (1→4) linkages with very different properties (Table 1). Amylopectin is the major component of most starches (Table 2) and consists of a number of chains bounded together by a (1→6) linkage, leading to an extensively branched structure of high molecular weight. On the other hand, amylose is considered to have an essentially linear structure with lower molecular weight than amylopectin. Amylose content usually ranges around 20 % in most cases, except for waxy starch, in which the amylose content is almost zero.

Even if the chemical composition of starches is very simple, every starch has its own characteristic shape and size distribution. The different shapes and sizes of most common starches are detailed in Table 2, which range from the typical large spherical granules (up to 100 μm) found in potato to the small polygonal particles (2–10 μm) found in rice. In general, the amylose content of starch is proportional to the granule size and maturity of starch (Pan and Jane 2000).

Starch granules are composed of layers of both amylose and amylopectin around a nucleation center called the hilum. Often, these layers in many starch grains are visible when viewed under the light microscope. The granule grows alternating radial amorphous and semicrystalline rings from the hilum, forming a lamellar structure. The amylopectin, which comprises around 75 % of the granule, is mainly responsible for the granule crystallinity and exhibits birefringence under

Table 1 Characteristics of amylose and amylopectin

Property	Amylose	Amylopectin
Shape	Mainly linear	Branched
Molecular weight	<2,6 million	50–500 million
Linkage	Mainly α-1,4	α-1,4 and α-1,6
Color with iodine	Blue	Reddish brown

Table 2 Amylose and amylopectin contents (wt. percentage) of some starches

Starch type	Source	Shape	Size (μm)	Amylose content	Amylopectin content
Tapioca	Root	Oval	3–28	17	83
Potato	Tuber	Oval, spherical	10–100	20–30	80–70
High-amylose corn	Cereal	Polygonal		55–70	45–30
Dent corn	Cereal	Polygonal	5–30	25	75
Waxy corn	Cereal	Polygonal	5–25	<1	>99
Wheat	Cereal	Round, lenticular	5–35	25	75
Rice	Cereal	Polygonal	2–10	19	81
Pea	Legume	Oval	–	25–40	75–60
Cassava	Root	Round	–	9–22	91–78

cross-polarized light. Thus, the starch grains appear bright against the dark field and each starch grain shows a Maltese cross. The crossing point of the Maltese cross is located at the hilum (Gallant et al. 1997).

The presence of crystalline structure in the starch granules has also been analyzed by X-ray diffraction. Three types of crystalline structure have been identified as A, B, and C types, the latter consisting of a mixture of A and B types. As commented above, the granule crystallinity is mainly due to amylopectin, whose side-chain branches intertwine to form the double helices, whose association gives rise to the crystalline zones, whereas amylose remains mainly amorphous in the granule (Donald 2004). Due to this fact, the larger amylose content of the B-granule starch is likely to result in a lower percentage of the crystallinity in the B-granule starch than the A-granule starch (Zeng et al. 2011). As an example, the degree of crystallinity in the native cassava starch was found to be 38 %. The X-ray diffraction pattern of each starch sample is the "fingerprint" of the crystalline structure within starch grains.

Crystalline structures are also formed in starch products from amylose and amylopectin fractions. According to the characteristic X-ray diffraction lines, the crystalline structure of starch products can be divided into four types: A, B, C, and V. Of these, A, B, and C types are similar to those observed in native starch, while V type is typical of the amylose–lipid complexes in which the helical conformation of amylose chains is involved.

During the gelatinization process, starch granules undergo an irreversible order–disorder transition. If the granules become fully swollen, high temperature and shear cause granule disruptions with an increase in viscosity and translucency. This is due to the dissolution and leakage of the amylose from the granules and to

the loss of initial native crystallinity of starch. Starch gelatinization is an endothermic process which can be analyzed by DSC. The gelatinization temperature depends on the starch–water ratio. With a large excess of water, only one endotherm can be observed by DSC, whereas with no excess of water, the only endotherm appears at higher temperatures. With intermediate water content, two endothermic transitions are observed, corresponding to the hydration of the granule and to the melting of the crystallites, respectively (Carvalho 2008).

Once the starch has been gelatinized, a recrystallization process takes place due to the rearrangement of amylose and amylopectin molecules into an ordered structure during the cooling process. Composed of linear chains, the crystallization rate of amylose is much greater than amylopectin, which remains semicrystalline (Gidley and Bulpin 1989).

Starch, as a semicrystalline structure, also exhibits a glass transition. The glass transition temperature (Tg) of the starch amorphous regions at low moisture content is around 90–180 °C, depending on the variety of starch and the water content (García et al. 2000; Delville et al. 2003; Zeleznak and Hoseney 1987).

2.2 Film Preparation

Starch films can be obtained from the native starch or its components, amylose and amylopectin, by two main techniques: solution casting and subsequent drying (wet method) and thermoplastic processing (dry method). Modified and soluble or pre-gelatinized starches have also been used to obtain starch films (Jiménez et al. 2012d). In the wet method, films are formed from a film-forming dispersion or emulsion, which contains a high percentage of water. A great number of studies have been carried out using this technique to obtain films based on starch from different sources (Jiménez et al. 2012d). Gelatinization of starch in the aqueous dispersion is required prior to film formation. In the thermoplastic processing (dry method) of starch, the water content is much lower and it can only be used with thermoplastic starch (TPS). Thermoplastic materials become soft (melted or rubbery), and so, they can be molded into a determined shape when submitted to a thermal/mechanical process. Starch does not present this characteristic in its native state, but it is capable of becoming a thermoplastic material if it is adequately treated.

2.2.1 Wet Process

The most commonly used laboratory-scale method for obtaining edible films is the drying of the cast film-forming dispersion. The complete process for the starch films could be divided into several steps: starch gelatinization, homogenization of the mixture, casting, and drying.

In the gelatinization step, granules are disrupted in an excess of water. The optimal process conditions depend on the origin of the starch, since the granule structure depends on the starch source. The type of starch and the plasticizer content affect the gelatinization temperatures. A wide range of gelatinization temperatures and times have been reported. The gelatinization step is avoided if pre-gelatinized

starch is used to obtain films. Pre-gelatinized starches have been precooked and drum-dried to yield products which disperse quickly in cool water to form stable suspensions.

Gelatinization conditions (temperature, time) change when other components are present in the starch aqueous dispersion. For instance, the gelatinization temperature increased as the glycerol content rose (Tan et al. 2004), but it did not change when triglycerides (Chiotelli and Le Meste 2003) or fatty acids (Zhou et al. 2007) were added to various types of starch in different conditions. In order not to modify gelatinization conditions, additives of the films may be mixed with starch after gelatinization, as Petersson and Stading (2005) have described in films with native potato starch and commercial monoglyceride.

Alkaline medium can also be used to provoke starch gelatinization. The use of an alkali produces starch hydrolysis, which leads to granule disruption. This method is called cold gelatinization and can be used in combination with a low gelatinization temperature step (Bertuzzi et al. 2007). However, some negative effects of cold gelatinization on the final properties of starch films have been described by different authors. These are mainly due to the partial hydrolysis and reduction of the polymer molecular weight which leads to a decrease in film mechanical resistance and poor barrier properties. SEM observations showed a homogeneous matrix when hot gelatinization was carried out, whereas cold gelatinization led to a cracked structure.

Starch formulations with lipids or other nonmiscible components require a homogenization step in order to obtain a stable emulsion and an adequate dispersion of all the components. In general, this step is carried out by means of different equipments, such as a rotor-stator Ultra-Turrax homogenizer (Jiménez et al. 2012a), a Fischer Scientific high-speed homogenizer (Han et al. 2006), or a Brabender Viscograph (Petersson and Stading 2005).

In formulations containing different hydrocolloids, a mixing step is also required to obtain film-forming dispersions. Different studies prepared dispersions by means of a step mixing starch with other polymers. As a consequence of the homogenization step, bubbles are frequently incorporated into the film-forming dispersions. If this occurs, vacuum devices are used (Famá et al. 2006) to remove these bubbles, which can provoke microholes in the final films.

After the gelatinization and homogenization steps, film-forming dispersions must be poured or cast on dishes and allowed to dry in controlled conditions. Casting has been carried out either immediately after the homogenization step or after cooling to a determined temperature, and afterwards, films are dried in controlled conditions in order to eliminate the excess water and to obtain an easily manageable film. Leveled dishes of different materials have been used for film formation, such as Petri dishes of polystyrene, polyethylene, polytetrafluoroethylene (Teflon®), stainless steel, or glass (Jiménez et al. 2012d).

Different film-drying conditions have been applied, without a standard method. Nevertheless drying conditions seriously affect both the film functionality and its physical properties, which make it enormously difficult to compare the properties of the different films. Table 3 summarizes the different conditions used to obtain

Table 3 Conditions of gelatinization and drying for different types of starch films

Starch source	Gelatinization conditions	Drying conditions	References
Sago	85 °C, 30 min	40 °C, 20 h	Abdorreza et al. (2011)
Cassava	70 °C, 1 min	30 °C, 18, 24 h	Bergo et al. (2008)
Corn	Alkaline medium (10 g/L NaOH)	60 °C, 8 h	García et al. (2000)
Pea	Boiling temp, 15 min	Room temp, at least 40 h	Han et al. (2006)
Potato, maize, banana	98 °C, 30 min	40 °C, 48 h	Hernández et al. (2008)
Quinoa	97 °C, 30 min	34–50 °C, 55 % RH, for different times	Araujo-Farro et al. (2010)

starch films from distinct sources. Most of the studies used glycerol as plasticizer, while some films were obtained with sorbitol and polyethylene glycol (Abdorreza et al. 2011). It is necessary to add plasticizers so as to adequate the film mechanical properties, thus reducing its brittleness. The kind of starch and plasticizer and the process conditions greatly affect the film properties.

2.2.2 Dry Process

The casting method, described above, involves conditions that do not permit large-scale manufacturing. The production of starch films by means of thermoplastic treatment is more appropriate. As native starch is not a thermoplastic material, it must be processed in order to obtain this characteristic. Thermoplastic starch (TPS) is generally produced by processing a starch–plasticizer(s) mixture in an extruder (or other melt-blending equipment) at temperatures between 140 °C and 160 °C, under high-pressure and high-shear conditions. The result of the process is the disruption of starch granules and the mixture with plasticizers, in order to obtain a rubbery material, without brittleness (Forsell et al. 1997). Carvalho (2008) described TPS as an amorphous or semicrystalline material composed of gelatinized or destructurized starch containing one plasticizer or a mixture of them.

According to Liu et al., starch can be thermally processed by means of different techniques (2009), such as sheet/film extrusion, foaming extrusion, injection molding, compression molding, and reactive extrusion (a special type of extrusion in which chemical reaction and typical extrusion take place). These techniques can be used with starch in combination with both natural and petroleum-derived polymers (Frost et al. 2011).

The thermal process of starch usually includes two steps. First, the starch is mixed with plasticizers and extruded in order to disrupt the starch granules, thus obtaining TPS, followed by a final step in which the obtained paste (or pellets) is thermo-molded to form films. Once the starch is in an amorphous state, it can be injection molded, extruded with a film-blowing die, or thermopressed (Jiménez et al. 2012d).

The thermal processing of starch biopolymers also involves several chemical and physical changes (water diffusion, granule expansion, gelatinization,

decomposition, melting, and crystallization) in the starch granules in which gelatinization plays an important role in the conversion of starch to a thermoplastic (Liu et al. 2009). In this case, gelatinization is achieved at low moisture content due to the high-shear and high-pressure conditions used which tear down the starch granules, allowing faster water transfer into the starch molecules. The mechanical disruption caused by the intense shear conditions provokes a loss of crystallinity in the starch samples.

Different studies analyzed the influence of the extruder's temperature on the properties of the final product and found a significant effect (Li et al. 2011).

3 Active Films Based on Starch

3.1 Antimicrobial Activity of Starch Films

The antimicrobial activity of starch films can be achieved by blending this polysaccharide with another polymer exhibiting antimicrobial activity or by adding natural antimicrobial compounds or nanoparticles. Examples of innovative bioactive starch films are shown in Table 4.

Chitosan is a nontoxic, biodegradable, biocompatible, and antimicrobial material and can be used both as a matrix or an additive for packaging materials. Lopez et al. (2014) obtained thermoplastic corn starch films with chitosan by melt-mixing and thermo-compression. These films exhibit antimicrobial properties against Gram-positive and Gram-negative bacteria (*Staphylococcus aureus*, *Escherichia coli*). In addition, the starch–chitosan association improves the mechanical and barrier properties of the starch matrix. Authors reported an increase in both the tensile strength and elastic modulus. Moreover, by adding 10 g of chitosan or chitin to 100 g of starch, water vapor permeability decreases by 35 % and 56 %, respectively. Others studies showed more complex polymer associations. Bie et al. (2013) developed an antimicrobial material based on poly(lactic acid)/starch–chitosan blends, with slow-release properties, in which chitosan acted as an antimicrobial agent while PLA and starch together were used as a slow-releasing device. The release of chitosan was observed to occur in two stages, initially with a very fast release stage and afterwards with a slow, but durable, release stage. These two stages exhibited the effectiveness and long residual action of the antimicrobial property of the blends against two strains (*E. coli*, *S. aureus*).

Natural plant extracts (mainly essential oils or pure substances derived from them) have also been extensively studied as antimicrobial compounds as a result of the increase in consumer demand for a more limited use of chemicals. Several studies reported interesting antimicrobial effectiveness of starch–essential oil composite films (Souza et al. 2013; Ghasemlou et al. 2013; Jiménez et al. 2014). Cinnamon essential oil was successfully incorporated into starch matrix, the films exhibiting significant antimicrobial activity against several fungi (*Penicillium commune* and *Eurotium amstelodami*) (Souza et al. 2013). In this study, the addition of cinnamon essential oil significantly modifies the film mechanical properties.

Table 4 Examples of starch films with antimicrobial properties

Film composition	Microorganisms	References
Corn starch/lysozyme	*Listeria monocytogenes*	Fabra et al. (2014)
Corn starch/chitosan–chitin	*Escherichia coli*	Lopez et al. (2014)
Corn starch/essential oils	*Escherichia coli* *Staphylococcus aureus*	Ghasemlou et al. (2013)
Corn starch/tannic acid	*Listeria monocytogenes* *Escherichia coli*	Pyla et al. (2010)
Corn starch/buttermilk	*Listeria innocua*	Moreno et al. (2014)
Corn starch/ε-poly-L-lysine	*Escherichia coli* *Bacillus subtilis* *Aspergillus niger*	Zhang et al. (2015)
Corn starch/sodium caseinate/ nanoliposomes –essential oil	*Listeria monocytogenes*	Jiménez et al. (2014)
Corn starch/Na–montmorillonite	*Escherichia coli* *Staphylococcus aureus*	Heydari et al. (2014)
Cassava starch/cinnamon oil	*Penicillium commune* *Eurotium amstelodami*	Souza et al. (2013)
Sweet potato starch/chitosan	*Staphylococcus aureus*	Shen et al. (2010)
Maize starch/poly(lactic acid)/chitosan	*Escherichia coli* *Staphylococcus aureus*	Bie et al. (2013)
Rice starch/chitosan/Ag nanoparticle	*Escherichia coli* *Staphylococcus aureus* *Bacillus cereus*	Yoksan and Chirachanchai (2010)
Potato starch/clay nanocomposite/ potassium sorbate	*Aspergillus niger*	Barzegar et al. (2014)
Wheat starch/chitosan/lauric acid	*Escherichia coli* *Bacillus subtilis*	Salleh et al. (2014)
Tapioca starch/natamycin/nisin	*Listeria innocua* *Saccharomyces cerevisiae*	Olle Resa et al. (2014)

The tensile strength (TS) and elongation at break (E) of films with incorporated cinnamon essential oil varied from 2.3 ± 0.4 to 1.05 ± 0.16 MPa and from 260 ± 35 to 190 ± 20 %, respectively. Therefore, an increase in the content of cinnamon essential oil, glycerol, and emulsifier lowered the TS and the E of the films. Of the natural compounds, enzymes with antimicrobial activity can be added to the starch matrix. A recent study reported that the incorporation of lysozyme (0, 50, 75, and 100 mg lysozyme/g hydrocolloid) to starch films was effective at reducing *Listeria monocytogenes* growth in vitro at 10 °C (Fabra et al. 2014). Antimicrobial peptides can also be incorporated into the starch matrix to obtain active films. In this sense, ε-poly-L-lysine was added to corn starch films (Zhang et al. 2015). This peptide is a homo-poly(amino acid) characterized by a peptide bond between the α-carboxyl and the ε-amino groups, which is industrially produced by *Streptomyces albulus*. ε-Poly-L-lysine molecules are cationic, surface-active agents due to their positively charged amino groups in water, and they have been shown to have a wide antimicrobial spectrum by growth inhibition studies on

yeast, fungi, and Gram-positive or Gram-negative bacteria. In this study, the antimicrobial activity of starch films with ε-poly-L-lysine against bacteria and molds (*E. coli*, *Bacillus subtilis*, *Aspergillus niger*) was tested. Authors concluded that composite films exhibited poor antifungal activity but effective inhibition against *E. coli* and *B. subtilis*. Films containing 2 % (w/w) antimicrobial peptide effectively inhibited the growth of the tested bacteria ($P < 0.05$).

Other common antimicrobial compounds, used in edible films, are nanoparticles. The nanocomposites obtained by the incorporation of the low percentage of clay to starch films exhibit interesting barrier, thermal, and oxidative properties in comparison with the pure film. Barzegar et al. (2014) showed that the addition of potassium sorbate to the starch–nanoclay films leads to an increase in water vapor permeability and elongation at break and a decrease in tensile strength. A minimum concentration of 7.5 % of sorbate inhibits the growth of *A. niger* in vitro. In this study, the authors conclude that these active starch–clay nanocomposite films could be successfully applied to preserve foods, especially bakery products. Yoksan and Chirachanchai (2010) developed silver nanoparticle-loaded chitosan–starch films. The incorporation of silver nanoparticles led to a slight improvement in the tensile and oxygen gas barrier properties of the films, with diminished water vapor/moisture barrier properties. In addition, silver nanoparticle-loaded films exhibited enhanced antimicrobial activity against *E. coli*, *S. aureus*, and *B. cereus*.

The control of the release of antimicrobial compounds is a complex phenomenon. The diffusion of the antimicrobials is dependent on numerous factors, such as the film structure, the food characteristics, and the storage conditions (Cagri et al. 2004). The degree of interactions between the active compound and the polymer matrix could also affect the release of the antimicrobial. Fabra et al. (2014) studied the effect of the incorporation of lysozyme on the microbial properties of starch films against a foodborne pathogen, *L. monocytogenes*. The authors reported that the structure of the film and the possible interactions existing between the polymer and enzyme must be considered to understand the antimicrobial effectiveness of the starch films.

3.2 Antioxidant Activity of Starch Films

Starch films do not exhibit antioxidant activity naturally, but the addition of antioxidant compounds, such as α-tocopherol, was evaluated in recent studies (Table 5). α-Tocopherol is a lipid-soluble antioxidant whose antioxidant activity has been clearly documented (Ruperez et al. 2001; Manzanarez-Lopez et al. 2011). Like other tocols (tocopherols and tocotrienols), α-tocopherol acts as a free radical scavenger specifically within cell membranes, by preventing the oxidation of polyunsaturated lipids by free radicals, especially hydroxyl radical OH (Hejtmánková et al. 2010). Jiménez et al. (2013) reported that the incorporation of α-tocopherol greatly increased the antioxidant capacity of starch–sodium caseinate films (1.37 ± 0.06 instead of 0.249 ± 0.002 mM Trolox equivalent antioxidant capacity). Polyphenol, such as tannic acid, could be equally added to the starch matrix to

Table 5 Examples of starch films with antioxidant properties

Film composition	Method	References
Corn starch/sodium caseinate/oleic acid/α-tocopherol	*Trolox* (*ABTS*)	Jiménez et al. (2013)
Corn starch/buttermilk	*Trolox* (*ABTS*)	Moreno et al. (2014)
Wheat starch/chitosan/essential oils (basil, thyme)/ citric acid/α-tocopherol	*Trolox* (*ABTS*)	Bonilla et al. (2013)
Cassava starch/essential oils (cinnamon, fennel)	*DPPH*/*FRAP*	Oriani et al. (2014)

confer antioxidant properties (Pyla et al. 2010). Tannic acid, present in a variety of plants and fruits, is considered as a "generally recognized as safe" (GRAS) food additive. In this study, the antioxidant activity of starch films containing tannic acid on soybean oil, doubling the induction time of oil oxidation, was shown.

4 Physical Properties of Active Starch Films

4.1 Barrier Properties

One of the main functions of food packaging is to avoid or minimize moisture transfer between the food and the surrounding atmosphere. Water vapor permeability (WVP) should, therefore, be as low as possible in order to optimize the food package environment and potentially increase the shelf life of the food product (Hosseini et al. 2013).

The moisture barrier properties of edible films have been studied by many authors. Edible films and coatings made from carbohydrates and proteins generally exhibit lower moisture barriers due to their hydrophilicity (Hiemenz and Rajagopalan 1997). To improve the moisture barrier, hydrophobic compounds, such as lipids, have been added to hydrophilic materials. Surfactants have also been used to reduce the surface tension of the film-forming dispersion, improving the wettability and adhesion of the coating (Ribeiro et al. 2007; Rodríguez et al. 2006). Several authors studied the effect of active molecules on water permeability. As lipids are known to improve water barrier properties, the incorporation of essential oils into edible coatings was an attempt to improve the water vapor barrier. Lipids that are solid form coatings with a better water vapor barrier than liquid lipids (Olivas and Barbosa-Cánovas 2005). Essential oils are liquid, which could justify the slight reduction of the water vapor transmission rate (WVTR) when they are incorporated in the film formulation. No differences in the WVTR of the coatings based on cassava starch (CS) were observed when essential oils were added at low concentrations. Oriani et al. (2014) analyzed the effect of different concentrations of cinnamon bark and fennel essential oils in edible cassava starch (CS) coatings applied to apple samples. Edible coatings prepared with 2 % and 3 % CS and 0.30 % (v/v) essential oils (fennel or cinnamon bark) exhibited similar WVTR values, although they were significantly different to that obtained for uncoated fruit.

Ghasemlou et al. (2013) developed an antimicrobial film based on corn starch and two essential oils from *Zataria multiflora* Boiss (ZEO) or *Mentha pulegium* (MEO) at three concentration levels (1 %, 2 %, and 3 % (v/v)). The obtained results indicate that there was a significant difference ($P < 0.05$) in the WVP values between films containing ZEO or MEO and the control films. The addition of ZEO or MEO improved the barrier properties of starch films, decreasing the WVP by up to 50 % relative to the control sample. The lower WVP of the starch films containing either ZEO or MEO was attributed to the hydrogen and covalent interactions between the starch network and these polyphenolic compounds.

The oxygen and carbon dioxide permeabilities of biodegradable films or coatings are also an important characteristic to take into account when respiration or oxidation reactions could affect product quality. Edible films with antioxidants could provide continuous protection for foods against oxidation during storage or after opening, while also providing a recyclable packaging material (Min and Krochta 2007; Kleen et al. 2002; Herald et al. 1996). Edible films and coatings can prevent the deterioration of many food products because they often possess excellent oxygen barrier properties. Thus, the relationship between antioxidant addition and any oxygen permeability change in the films is of immediate concern. According to Ghasemlou et al. (2013), the presence of essential oils from *Zataria multiflora* Boiss (ZEO) or *Mentha pulegium* (MEO) modifies the oxygen permeability (OP) of composite corn starch films. The OP value of the pure starch film was 12.11 $cm^3 \mu m m^{-2} day^{-1} kPa^{-1}$, indicating that this film is a very good oxygen barrier. The incorporation of 3 % (v/v) ZEO to the starch film increased the permeability value to 16 $cm^3 \mu m m^{-2} day^{-1} kPa^{-1}$.

4.2 Mechanical Properties

The mechanical properties of edible films and coatings depend on the type of film-forming material and especially on its structural cohesion. Cohesion is the result of a polymer's ability to form strong and/or numerous molecular bonds between polymeric chains, thus hindering their separation. This ability depends on the polymer structure and especially molecular strength, geometry, molecular weight distribution, and position of its lateral groups (Guilbert et al. 1996). The mechanical properties of edible coatings are greatly affected by the physical, chemical, and temperature conditions, which influence the film stability and flexibility. Moreover, it is well known that the environmental conditions during the production, storage, and use of these materials affect their mechanical properties (Garcia et al. 2009).

Perazzo et al. (2014) have developed an innovative active food packaging from cassava starch containing green tea and palm oil carotenoid extracts, as active natural compounds to be used as packaging for fatty products, thus adding value to different agro-industrial chains. They showed that the addition of colorant and green tea extract significantly improved the functional properties of the resulting films. These changes were attributed to the interactions between functional groups

of starch, colorant, and polyphenols of green tea extract which give rise to a cohesive molecular reorganization, thus providing greater rigidity to the material compared to the controls.

Moreno et al. (2014) studied the effect of buttermilk (BM) incorporation on the properties of the film-forming dispersions and the mechanical characteristics of corn starch films. The impact that heat treatment has on films containing buttermilk was analyzed. The incorporation of BM to starch films provoked a significant decrease in film stiffness and resistance to break without any notable changes in film stretchability, except for 60 % of non-heated BM, when films become more extensible but very soft.

Bonilla et al. (2013) showed the effect of adding different antioxidants – essential oils (basil and thyme), citric acid, and α- tocopherol – on the mechanical behavior of wheat starch–chitosan films. The incorporation of active compounds had different effects on the mechanical parameters, depending on their specific nature. Citric acid promoted an increase in the elastic modulus but a decrease in the tensile strength and deformation at break, probably due to cross-linking with starch chains.

4.3 Optical Properties

The functionalization of a biodegradable film, such as starch, as an active packaging or coating can either improve the visual presentation of the packaging or the food product or, on the contrary, decrease their attractiveness. This is one of the most important limits to the use of biodegradable films on an industrial scale. The objective of most coating producers is to reach transparency or at least to reach the same color as the product surface so as to make it very discreet. The main case where color is not a problem is chocolate, for which the visible brown color is an attractive part of the food product. This particular attractiveness offers new developments for food coatings, because young consumers are always attracted by a new product with unexpected colors.

Moreno et al. (2014) show the spectral distribution of the internal transmittance (Ti) of the films, as an indicator of the translucency level. The internal transmittance decreased as the buttermilk content rose in the starch films, mainly at low wavelengths, which indicates a rise in the film opacity. This was explained by the formation of a more heterogeneous structure with changes in the refraction index through the film structure, which promotes light dispersion. As the ratio of the dispersed phase (BM components) becomes higher, a loss in transparency occurs.

Ghasemlou et al. (2013) show the color parameters, **L**, **a**, and **b**, and the total color difference (ΔE) values for pure starch-based films and those containing essential oils. There were no differences observed between the **a** values of any film studied. Generally, starch films with *Zataria multiflora* Boiss (ZEO) exhibited higher L values but lower b values than those with *Mentha pulegium* (MEO), although films with MEO were darker in appearance. According to Bonilla et al. (2013), the functionalization of wheat starch–chitosan films with essential

oils (basil and thyme), citric acid, and α- tocopherol modified the visual aspect. All the antioxidants provoked a loss in film gloss but a gain in transparency, while α-tocopherol induced yellowness and a reduction in the whiteness index.

5 Conclusion

Starch has been used to develop edible, biodegradable, active films using different strategies: blending with antimicrobial polymers, active nanoparticles, and other antioxidant or antimicrobial compounds, such as essential oils of phenolic extracts. In most of the cases, films or coatings were obtained by casting the film-forming dispersions containing the active compounds. However, the industrial production of films requires the use of thermo-processing applied to conventional plastics. Starch can be processed in this way, but a great number of the active compounds are thermolabile and cannot be submitted to the high temperatures used in these processes. Likewise, the incorporation of active compounds to the polymer matrix provokes positive or negative changes in their functional properties that must be evaluated, together with the release kinetics of antimicrobials/antioxidants into the food system. The extension of active polymer layers on thermo-processed films can be a good strategy with which to obtain active films, avoiding the thermostability problems of active compounds. The properties of both the bilayer films and the bioactive release kinetics must be studied so as to offer adequate starch active packaging materials for food preservation

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Dextran and Food Application

25

Damini Kothari, Deeplina Das, Seema Patel, and Arun Goyal

Contents

Abstract

An increasing consumer trend towards healthy and additive-free food has made dextran from food grade lactic acid bacteria (LAB) an attractive solution. Dextrans are homopolysaccharides of D-glucose produced by extracellular dextransucrase released from LAB of the genera, viz., *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Weissella*, and *Pediococcus*. Dextrans have been known for their viscosifying, emulsifying, texturizing, stabilizing attributes in food

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applications. Dextran has the potential to be recruited as a novel ingredient replacing the commercial hydrocolloids in bakery and other food industries. Prebiotic oligosaccharide production by hydrolysis of dextran is a rather new field, garnering research and industrial attention. The applications, available sources, preparation, and characterization of dextran and problems associated with its use have been discussed. This chapter also highlights the key developments in recent times and discusses the importance of bio-prospecting novel dextran-producing isolates from biodiversity.

Keywords
Dextran • Dextransucrase • Exopolysaccharide • Food • Lactic acid bacteria

1 Introduction

The progressively increasing demand of natural polymers for various industrial applications has led to the exploration of microbial exopolysaccharide (EPS) in recent years. Among several EPS, dextran has gained worldwide recognition due to its biodegradability and biocompatibility properties (Patel et al. 2010; Aman et al. 2012; Varshosaz 2012). The EPS dextran was first discovered by Louis Pasteur (1861) as a microbial product in wine. Scheilber in 1874 confirmed that this microbial polysaccharide has a positive (dextrorotatory) optical rotation with the empirical formula $(C_6H_{10}O_6)_n$ and therefore named as "dextran." The physiological roles of EPS in the microbial host are not yet completely understood, but they are involved in protection against dehydration, pathogenicity, biofilm formation, and quorum sensing. The presence of a dextran layer around the bacterial cell may have paramount effects on the cellular diffusion properties. Dextran has gained importance owing to its applications in the food, pharmaceutical, biomaterial, photo film manufacturing, and fine chemical industries. A large number of lactic acid bacteria (LAB) are known to produce dextran.

Hehre in 1941 reported the first cell-free synthesis of dextran using sucrose as the substrate from enzyme dextransucrase. Dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase) is the key enzyme that catalyzes the synthesis of dextran from sucrose. Dextrans generally vary in their molecular weight, spatial arrangement, type and degree of branching, and length of branched chains, depending on the source of strains and also on the cultivation conditions. A survey of dextrans from 96 strains (primarily *Leuconostoc mesenteroides*) demonstrated that the amount of α-(1 → 6) linkages in a specific dextran can vary from 50 % to 97 % of the total glycosidic linkages (Jeanes et al. 1954). Apart from α-(1 → 6) linkages in main chain, dextrans also contain α-(1 → 2)-, α-(1 → 3)-, and α-(1 → 4)-branched linkages. Branched dextrans have also been reported to possess prebiotic (Das et al. 2014) and anticancer potentials (Shukla and Goyal 2013).

Dextrans are extracellular bacterial homopolysaccharides with a linear backbone of α-linked D-glucopyranosyl repeating units. Dextrans belong to α-D-glucans

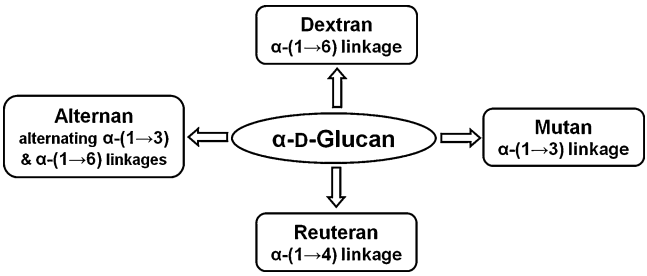

Fig. 1 Classification of α-D-glucan depending upon the type of linkages

containing α-(1 → 6) linkage in the main chain and variable amounts of α-(1 → 2)-, α-(1 → 3)-, or α-(1 → 4)-branched linkages. There are four distinct types of α-D-glucan produced by LAB (Fig. 1): (i) mutan contains a majority of α-(1 → 3) linkages in main chain and is produced by *Streptococcus* species; (ii) alternan containing alternating α-(1 → 3) and α-(1 → 6) linkages, reported only in *L. mesenteroides*; (iii) reuteran containing α-(1 → 4) linkages found only in *Lactobacillus reuteri* (Monchois et al. 1999; van Leeuwen et al. 2008); and (iv) dextran containing α-(1 → 6) linkages in main chain and α-(1 → 2)-, α-(1 → 3)-, or α-(1 → 4)-branched linkages (Jeanes et al. 1954; Naessens et al. 2005; Kothari and Goyal 2013). The production of dextran has been extensively studied in LAB of genera, viz., *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Weissella*, and *Pediococcus*.

2 Sources of Dextran

Dextran occurs naturally in small amounts in foods, such as refined crystalline sugar, maple syrup, sauerkraut juice, and honey, and also as a component of dental plaque. Dextran is synthesized by the action of bacterial enzyme, dextransucrase, on sucrose. Dextransucrase is the sole industrial enzyme used in the commercial production of dextran and is produced by LAB of genera, viz., *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, and *Weissella*. The structure of each type of dextran depends on the microbial strain and hence on the specific dextransucrase. To date, commercial dextran is produced from *Leuconostoc mesenteroides* NRRL B-512F and serves as a model in studying the structure of dextran and the mechanism of its biosynthesis by dextransucrase (Robyt et al. 2008; Siddiqui et al. 2014).

The amount of dextran produced however is practically insufficient to meet the dextran requirements of the various industries; hence, there is the need for the isolation and characterization of hyper dextran-producing LAB. Several examples of dextran with their linkage pattern from LAB isolated from various food sources are mentioned in Table 1.

Table 1 Dextrans with their linkage pattern from different LAB isolated from various food sources

Microorganism	Source	Linkage	References
Leuconostoc mesenteroides CMG713	Grape	α-(1 → 6) linkages only	Sarwat et al. (2008)
Leuconostoc mesenteroides AA1	Fermented cabbage	α-(1 → 6) linkages only	Aman et al. (2012)
Lactobacillus satsumensis NRRL B-59839	Water kefir grains	44 % α-(1 → 3) and 37 % α-(1 → 6)	Cote et al. (2012)
Lactobacillus plantarum DM5	Marcha, fermented beverage	86.5 % α-(1 → 6) and 13.5 % α-(1 → 3)	Das and Goyal (2014)
Pediococcus pentosaceus CRAG3	Fermented cucumber	75 % α-(1 → 6) and 25 % α-(1 → 3)	Shukla and Goyal (2013)
Weissella cibaria CMGDEX3	Cabbage	96.6 % α-(1 → 6) and 3.4 % α-(1 → 3)	Ahmed et al. (2012)
Weissella confusa Cab3	Fermented cabbage	97 % α-(1 → 6) and 3 % α-(1 → 3)	Shukla et al. (2014)
Weissella cibaria JAG8	Apple peel	93 % α-(1 → 6) and 7 % α-(1 → 3)	Rao and Goyal (2013)

3 Dextransucrase

Dextransucrases (EC. 2.4.1.5) are the sole industrial enzymes used in the commercial production of dextran (Parlak et al. 2013). Dextransucrase is classified in the family of glucansucrase, and most of the enzymes classified in this family use sucrose as the D-glucopyranosyl donor to synthesize α-D-glucans of high molecular mass with the concomitant release of D-fructose. They are also referred to as glucosyltransferases (GTF) because they synthesize α-glucan polymers using the glucose unit of sucrose (Leemhuis et al. 2013). Glucansucrases have been listed within family 70 glycoside hydrolase (GH70) in carbohydrate-active enzyme database (http://www.cazy.org/Glycoside-Hydrolases.html) based on sequence similarity (Cantarel et al. 2009; Vujicic-Zagar et al. 2010). They are evolutionarily closely related to the enzymes such as amylosucrase, cyclodextrin glucanotransferase, amylomaltase, and α-amylase from families GH13 and GH77 (Cantarel et al. 2009). Together with the families GH13 and GH77 enzymes, they form the clan GH-H (Vujicic-Zagar et al. 2010; Leemhuis et al. 2013). However, glucansucrases are much larger enzymes (~1,600–1,800 amino acid residues) than GH13 and GH77 (~500–600 amino acids), and they contain an N-terminal domain of variable region of unknown function (Vujicic-Zagar et al. 2010). Glucansucrases usually display a $(\beta/\alpha)_8$ barrel-shaped protein folding pattern and the acid–base-assisted substrate catalysis via a double-displacement (retaining) mechanism. GH70 enzymes are transglucosylases produced by LAB of genera, viz., *Streptococcus*, *Leuconostoc*, *Weissella*, and *Lactobacillus* (Monchois et al. 1999;

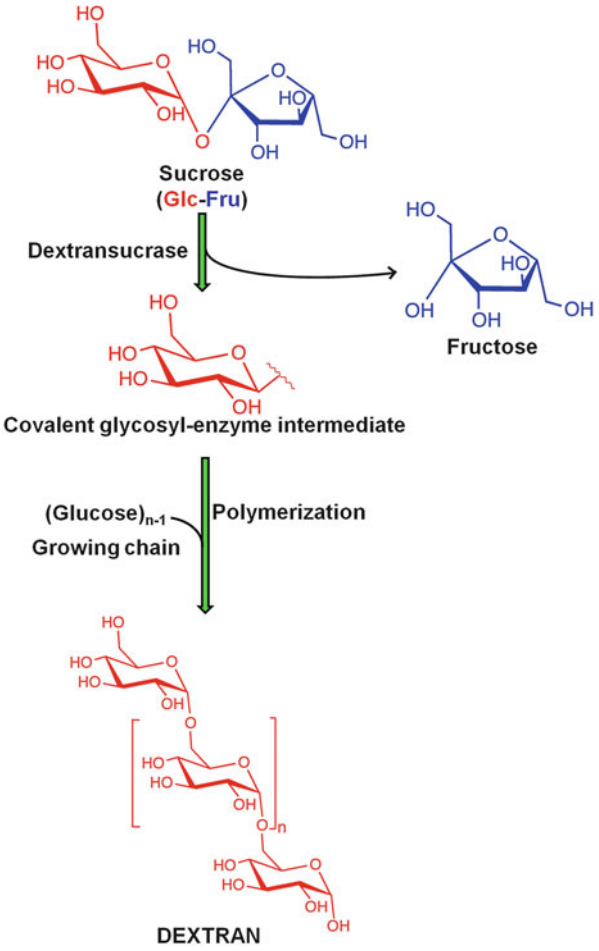

Fig. 2 Double-displacement mechanism of dextransucrase reaction (Adapted from Leemhuis et al. 2013)

Ito et al. 2011; Leemhuis et al. 2013). Four distinct types of GH70 glucansucrases have been identified based on the polysaccharides produced by them (Andre et al. 2010).

- Dextransucrase (E.C. 2.4.1.5): dextran, containing above 50 % α-(1 → 6) linkages
- Mutansucrase (E.C. 2.4.1.5): mutan, containing above 50 % α-(1 → 3) linkages
- Reuteransucrase (E.C. 2.4.1.5): reuteran, containing above 50 % α-(1 → 4) linkages
- Alternansucrase (E.C. 2.4.1.140): alternan, containing alternating α-(1 → 3) and α-(1 → 6) linkages

The enzyme dextransucrase synthesizes dextran from sucrose with concomitant release of fructose by double-displacement mechanism (Fig. 2). In the first stage of

double-displacement reaction, α-(1 → 2) glycosidic linkage of sucrose is cleaved with the release of fructose, and a glucosylenzyme intermediate is formed, in which the glucosyl unit is covalently attached to the catalytic nucleophile via a β-glycosidic linkage. In the second stage of reaction, the covalently bound glucosyl moiety is transferred to the accepting nonreducing end of sugar of a growing glucan chain, with reformation of the α-glycosidic bond (Leemhuis et al. 2013).

4 Preparation of Dextran

Dextran is produced commercially by cultivating *L. mesenteroides* strains in situ in growth medium supplemented with sucrose and in vitro by using purified dextransucrase with sucrose as a substrate (Leemhuis et al. 2013). The dextran of desired molecular weight can be achieved by the direct enzymatic synthesis using purified dextransucrase, which allows more control over the reaction conditions as compared with the fermentative synthesis (Falconer et al. 2011). The production of dextran by dextransucrase from LAB is affected by factors like temperature, aeration, and concentration and type of medium components (Tsuchiya et al. 1952; Lazic et al. 1993; Purama and Goyal 2005; Bejar et al. 2013). Dextran production is also influenced by solubility, viscosity, nitrogen, phosphorus, and ash content of the medium (Jeanes et al. 1954). The molecular weight of dextran is inversely proportional to the concentration of enzyme and directly proportional to the concentration of sucrose. Moreover, the molecular weight of dextran increases as the temperature increases from 20 °C to 30 °C (Falconer et al. 2011). Several physical and chemical techniques such as UV irradiation (Patel and Goyal 2010; Agrawal et al. 2011; Siddiqui et al. 2013), ethyl methanesulfonate (Kim and Robyt 1994) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Kitaoka and Robyt 1998), and site-directed mutagenesis (Funane et al. 2005) have been used for the enhancement of dextransucrase and dextran production from various LAB.

A single dextransucrase can catalyze the synthesis of several types of dextran linkages, thereby permitting the formation of a branched polymer (Neely and Nott 1962; Smith et al. 1994). Certain bacterial strains have been shown to produce dextrans of different structures due to the elaboration of different dextransucrases (Cote and Robyt 1982; Zahnley and Smith 1995). Thus, the structure of each dextran is a characteristic of the specific dextransucrase produced by a specific microbial strain (Jeanes et al. 1954; Vettori et al. 2012; Kothari and Goyal 2013).

5 Properties of Dextran

5.1 Physicochemical Properties

Dextran polymers have a remarkable diversity in chain length and in physicochemical properties due to the variation in degree of branching in their glucose backbone. In general, dextran is readily soluble in water, dimethyl sulfoxide, formamide,

ethylene glycol, and glycerol but insoluble in monohydric alcohols, e.g., methanol, ethanol, and isopropanol, and also most ketones, e.g., acetone and 2-propanone. However, the water solubility of dextrans depends upon the branched linkage pattern. Linear dextrans have high water solubility, and the aqueous solutions behave as Newtonian fluids. However, some branched dextrans showed shear rate thinning effect, exhibiting non-Newtonian pseudoplastic behavior (Das and Goyal 2014). Viscosity of dextran solution depends on its concentration, temperature, and molecular weight. As dextran is a neutral polysaccharide, the viscosity is not significantly influenced by changes in pH or salt concentration. Dextrans with >43 % branching through α-(1 → 3) linkages are water insoluble. Dextrans have molecular weight in the range of 3–500,000 kDa. Dextrans with a molecular weight of 2,000–10,000 kDa exhibit the properties of an expandable coil, and at lower molecular weights (<2,000 kDa), dextran is more rodlike. Low molecular weight dextrans (40, 60, and 70 kDa) are generally preferred in clinical applications (Naessens et al. 2005). High molecular weight dextrans with few branched linkages are required for the application in sourdough (Lacaze et al. 2007). The surface morphological studies of dextran revealed a porous structure (Shukla and Goyal 2013; Das and Goyal 2014). The dextran has excellent thermal stability with degradation temperature ~300 °C (Das et al. 2014; Rao et al. 2014).

5.2 Structural Properties

The specificity of the synthesized linkages in the dextran is strain dependent. The most studied dextran is produced by *Leuconostoc mesenteroides* NRRL B-512 F. It contains 95 % α-(1 → 6) linkages and 5 % α-(1 → 3)-branched linkages (Naessens et al. 2005; Vettori et al. 2012). *L. mesenteroides* NRRL B-742 produces a dextran with 87 % α-(1 → 6) linear linkages and 13 % α-(1 → 4)-branched linkage. The strain *L. mesenteroides* NRRL B-1299 produces a rare kind of dextran with 63 % α-(1 → 6), 27 % of α-(1 → 2), and 8 % of α-(1 → 3) linkages (Remaud-Simeon et al. 2000). The molecular structure of dextran is shown in Fig. 3.

6 Characterization of Dextran

The structural characterization of dextran is an important factor for its utilization. A general strategy for dextran characterization is shown in Fig. 4.

6.1 Isolation of Pure Dextran

The structural analysis of dextran starts with its isolation in pure form in such a way that the chemical and physical properties are not affected (Leemhuis et al. 2013). The recovery or purification from culture medium or enzymatic reaction mixture generally involves the following steps: (i) cell removal by centrifugation or filtration

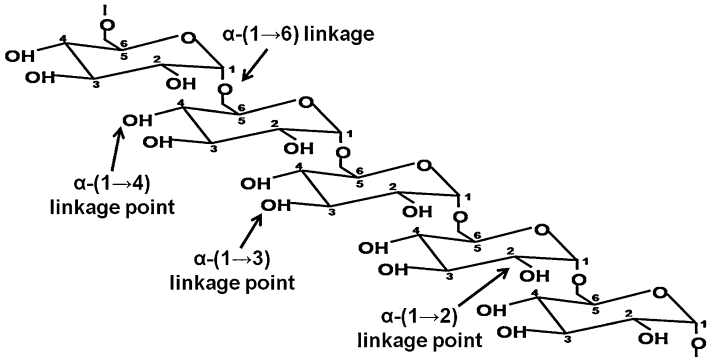

Fig. 3 Structure of dextran showing α-(1 → 6) glycosidic bonds in main chain and possible branches of smaller chains with α-(1 → 2), α-(1 → 3), or α-(1 → 4) linkages

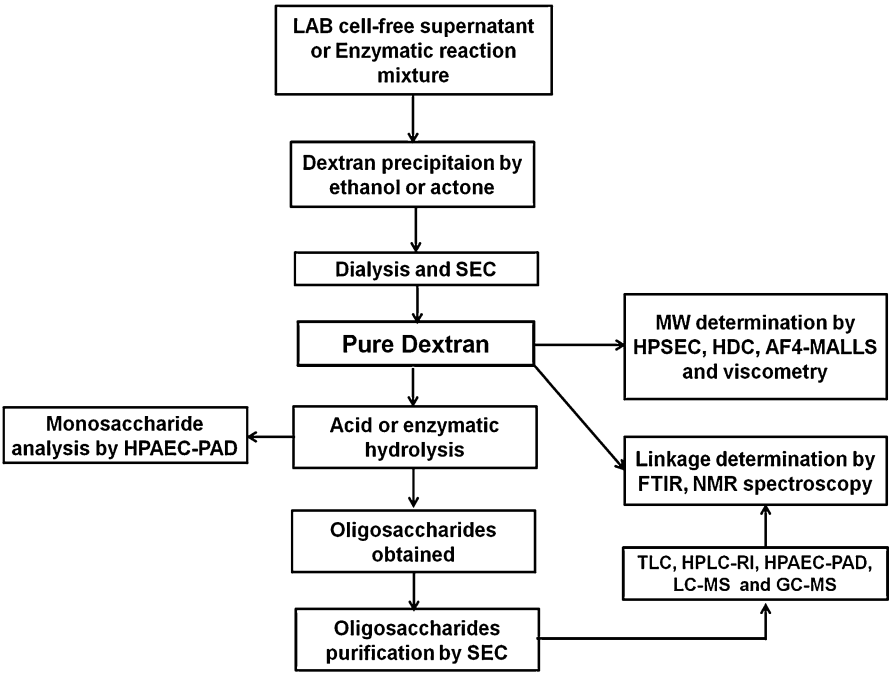

Fig. 4 A general strategy for the characterization of dextran

in case of culture medium, (ii) dextran precipitation from the cell-free supernatant or enzymatic reaction mixture by the addition of water-miscible organic solvents (e.g., ethanol, acetone, etc.), (iii) re-precipitation and dialysis of dextran, and (iv) size-exclusion chromatography (SEC) of dextran (Vettori et al. 2012; Shukla et al. 2014; Das et al. 2014). The high molecular weight dextran can be purified by SEC; however, low molecular weight dextran can be purified by ultrafiltration.

6.2 Molecular Weight Analysis

The molecular weight (MW) of dextran can be determined by colorimetric determination of reducing sugar, viscometry, hydrodynamic chromatography (HDC), high performance size-exclusion chromatography coupled with refractive index detector (HPSEC-RI), or with multi-angle laser light scattering (HPSEC-MALLS) (Leemhuis et al. 2013). However, higher branched polysaccharides are not well fractionated by means of classical SEC due to their shear scission, low exclusion limit (Cave et al. 2009), and limited resolution (Vilaplana and Gilbert 2010). Avoiding these problems, asymmetrical flow field flow fractionation (AF4) has emerged as a powerful technique for determination of the macromolecular structure of high molar mass branched biopolymers up to 10^8 Da (Rolland-Sabate et al. 2011). Recently, AF4 coupled with MALLS have been used to determine the MW of hyperbranched α-glucans (2×10^6 to 4.3×10^7 Da) (Rolland-Sabate et al. 2014).

6.3 Structural Unit and Glycosidic Linkage Analyses

The monosaccharide composition of dextran can be determined by acid hydrolysis followed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (Mopper et al. 1992; Kothari and Goyal 2013; Shukla et al. 2014). The structural characterization of dextran can also be accomplished by well-known techniques such as one-dimensional (^{1}H and ^{13}C) or two-dimensional (TOCSY, NOESY, ROESY, and HMQC) nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM) (Maina et al. 2008; Bounaix et al. 2010; Vettori et al. 2012; Shukla et al. 2014). To elucidate the structure of larger dextrans, it is often necessary to prepare oligosaccharides from the native polysaccharide by mild acid or enzymatic hydrolysis (Leemhuis et al. 2013). These oligosaccharides are then identified by thin layer chromatography (TLC), high performance liquid chromatography with refractive index detector (HPLC–RI), HPAEC–PAD, mass spectrometry coupled with liquid or gas chromatography (LC–MS or GC–MS), and FTIR and NMR spectroscopy (Naessens et al. 2005). Finally, by combining the dextran analysis data as well as structural information of the oligosaccharides produced will unveil all the structural features of the dextran (van Leeuwen et al. 2008).

7 Food Applications of Dextran

Dextran has been studied as a food ingredient since the 1950s. The US Food and Drug Administration (US FDA) currently lists dextran as GRAS (generally recognized as safe) additive for food and feed applications. In general, dextran is used as gelling, viscosifying, texturing, and emulsifying agent in various food products (Leemhuis et al. 2013). Commercial applications of dextran from LAB are

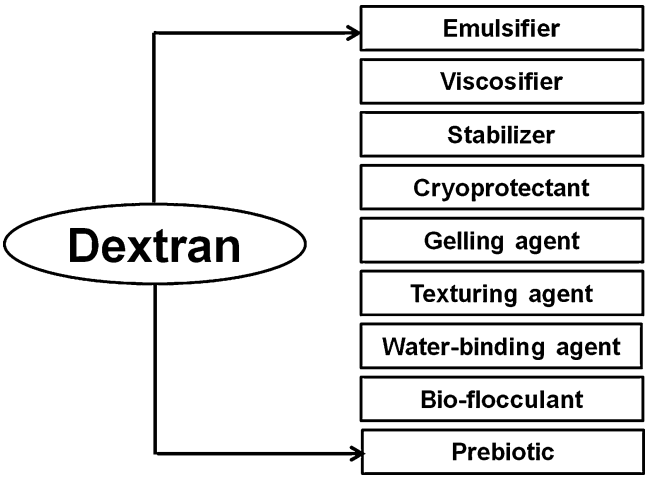

Fig. 5 Properties of dextran used in food industry

generally found in food and pharmaceutical industry; however, dextran also has several potential applications in photo film manufacturing, fine chemical, cosmetic, paper, petroleum, and textile industries (Naessens et al. 2005; Leemhuis et al. 2013). Due to the heterogeneity of dextran produced by various LAB, their application may depend on well-defined chemical and physicochemical properties. The properties of dextran that are applied in food industry are shown in Fig. 5.

Long-chain, high molecular weight polysaccharides that dissolve or disperse in water to give improved rheological (gelling, thickening) or physicochemical (emulsion stabilization, particle suspension, etc.) properties are important for food product formulation. A current consumer trend towards healthy and additive-free food has made the dextran an attractive food ingredient (Table 2). The microorganisms such as *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Lactobacillus sanfrancisco* are used for the production of dextran for its application in food processing without any restriction.

7.1 Bakery

The incorporation of dextran in bread for the improvement of rheological properties and quality is gaining interest (Galle et al. 2012; Wolter et al. 2014). The increasing knowledge of sourdough fermentation generates new opportunities for its use in the bakery field. In situ dextran production from *Weissella* sp. and *Leuconostoc mesenteroides* improved the freshness, mouthfeel, texture, loaf volume, softness, and shelf life of sourdough wheat bread (Katina et al. 2009; Galle et al. 2012). It came forth that dextran should have a high molecular weight and few branched linkages for the application in sourdough (Lacaze et al. 2007). The European Commission has approved the use of dextran in baked goods, up to the levels of 5 %.

Table 2 Food applications of dextran

Applications	References
Bakery	Katina et al. (2009)
Improves freshness, mouthfeel, softness, crumb texture, loaf volume, and shelf life	
Confectionary	Maina et al. (2011)
Improves moisture retention and viscosity and inhibits sugar crystallization and as gelling agents in gum and jelly candies	
Fermented dairy products	Mende et al. (2013)
Increases viscosity and creaminess and reduces syneresis	
Ice cream	Naessens et al. (2005)
Cryoprotectant	
Frozen and Dried foods	Bhavani and Nisha (2010)
Protection from oxidation and chemical changes and preservation in texture and flavor	
Cheese making: reduced-fat cheese	Awad et al. (2005)
Improves water binding and increases moisture content in the nonfat substances	
Prebiotics	Olano-Martin et al. (2000), Sarbini et al. (2013), Rao et al. (2014), Das et al. (2014)
Functional food	
Protein–dextran conjugates	Zhang et al. (2012), Chen et al. (2014), Spotti et al. (2014)
Improves emulsifying, foaming, gelling, and solubility attributes of protein by Maillard reaction	

The addition of 2 % native dextran increases the water absorption of flour dough by about 12 %. However, in situ formation of dextran in sourdough was reported to be more effective than external addition (Brandt et al. 2003). High molecular weight dextrans of (1–2) $\times$ 10^6 Da have been approved by the European Union as food ingredients in bakery products (Naessens et al. 2005). The required molecular mass has been reported to be from 2 $\times$ 10^6 to about 4 $\times$ 10^6 Da (Katina et al. 2009).

Celiac disease is an autoimmune, nutrient-induced disorder, triggered in genetically susceptible individuals by ingesting gluten from wheat, rye, barley, and other closely related cereal grains (Goggins and Kelleher 1994). It was reported that celiac disease is a major health problem affecting around 1 % of population in the western world (Mustalahti et al. 2010). Currently, the only available treatment is the complete avoidance of gluten-containing cereals (Arendt et al. 2011). Gluten is an important protein-building structure which contributes to appearance and crumb structure in many bakery products. The replacement of gluten in bread presents a significant technological challenge due to the low nutritional quality, poor sensory characteristics such as dry crumb, poor mouth feel, and off flavors of gluten-free products (Galle et al. 2012; Hager and Arendt 2013). Hydrocolloids are currently used to substitute gluten and to obtain gluten-free bread with acceptable sensory properties (Galle et al. 2010; Hager and Arendt 2013). Incorporation of sourdough

to a gluten-free formula gained interest recently in bread making. Dextran from *Weissella* and *Leuconostoc* species improves dough rheology and bread texture and can be used to replace nonbacterial hydrocolloids such as guar gum and hydroxypropyl methylcellulose for the generation of gluten-free soft bread with good texture and shelf life (Tieking and Ganzle 2005; Galle et al. 2012). Hence, dextran holds potential application in baking industry for the generation of gluten-free food products for patients suffering from celiac disease (Schwab et al. 2008; Galle et al. 2010; Rao and Goyal 2013).

7.2 Confectionery

Dextran is used for maintaining flavor, viscosity, moisture, inhibition of sugar crystallization, and as gelling agent in gum and jelly candies in confectioneries (Maina et al. 2011). It is also used in soft drinks, flavor extract, milk beverages, and icing.

7.3 Ice Cream

Dextran is also used as a cryoprotectant in ice cream (Naessens et al. 2005). Dextran is bland, odorless, tasteless, and nontoxic and is considered to have many advantages over other ice cream stabilizers. Ice cream mixes containing 2–4 % dextran conferred beneficial properties on viscosity (Bhavani and Nisha 2010).

7.4 Fermented Dairy Products

The texture of yogurt and yogurt-like products made from milk by fermentation with LAB can be modified by in situ production of EPS (Cerning 1995; Tamime and Robinson 1999). EPS produced by LAB, particularly dextran, positively affected the rheological properties of acidified milk gels with enhanced viscosity, creaminess, and reduced syneresis because of its water-binding ability (Mende et al. 2013) and hence can replace the commercially used texturizers, viz., xanthan, carrageenan, pectin, guar gum, and β-glucan.

7.5 Frozen Foods

The favorable properties of dextran for stabilizing vacuum, air-dried, and freeze-dried or frozen foods enable the use of dextran in fish products, meat, vegetables, and cheese. A film of dextran could protect food from oxidation and other chemical changes and also help to preserve texture and flavor. The increasing demand for fast food in frozen or dried state creates an opportunity for the use of dextran as a preservative, as well as a texture, flavor, and smell enhancer (Bhavani and Nisha 2010).

7.6 Reduced-Fat Cheese

The fat reduction in cheese results in many textural and functional defects. The high casein content in reduced-fat cheese imparts a firm and rubbery body and texture. Dextran is a good candidate for making reduced-fat cheese for several reasons. Dextran has the ability to bind water and increase the moisture in the non-fat mass (Awad et al. 2005).

7.7 Prebiotics

In recent years, there is a considerable interest in the use of prebiotics as functional foods in order to modulate the composition of the colonic microbiota to provide health benefits to the host (Saad et al. 2013). Foods containing prebiotic have also been associated with the protection against risk of several diseases, viz., bowel cancer, inflammatory bowel disease, diarrhea, coronary heart disease, obesity, osteoporosis, cholesterolemia, and type 2 diabetes. The α-(1 → 6) linkages are known to be resistant to hydrolysis by human intestinal enzymes, which results in the slow digestion of dextran in human. Moreover, α-(1 → 2) linkages are also highly resistant to the attack of digestive enzymes (Remaud-Simeon et al. 2000). Dextran and dextran-derived oligosaccharides have also been reported to increase the fraction of *Bifidobacterium* species in an in vitro model of the fermentation process in the human colon exhibiting prebiotic activity (Olano-Martin et al. 2000). A low molecular weight dextran containing α-(1 → 2)-branched linkages was also reported to act as prebiotic with selective effect on the gut microbiota (Sarbini et al. 2013). This dextran induced the growth of beneficial bacteria such as *Bifidobacterium* sp. and *Lactobacillus* sp. Recently, dextrans from *Weissella cibaria* JAG8 (Rao et al. 2014) and *Lactobacillus plantarum* DM5 (Das et al. 2014) showed promising prebiotic potential with very low gut digestibility and selective stimulation of probiotics.

7.8 Protein: Dextran Conjugates

Proteins are widely used in the food products such as beverages, yogurt, mayonnaise, and ice creams due to their functional properties, viz., emulsifying, foaming, gelling, and solubility (Oliver et al. 2006; Zhang et al. 2012). The functional properties of proteins can be improved by the conjugation of proteins and polysaccharides through Maillard reaction (Spotti et al. 2014). The Maillard reaction or nonenzymatic browning refers to any chemical reaction involving the interaction between amines and carbonyl compounds. Maillard reaction adds to the aroma, taste, and color of coffee and cocoa beans, bread, cakes, cereals, and meat (Martins et al. 2001). Dextran-conjugated proteins have displayed significant improvement in physical and chemical properties of proteins, such as thermal stability, emulsification, and antioxidant properties (Zhu et al. 2010).

The improvement of functional properties of different proteins, such as ovalbumin, lysozyme (Chen et al. 2014), peanut protein (Liu et al. 2012), soy protein (Zhuo et al. 2013), and whey protein (Spotti et al. 2014), after conjugation with dextrans has been studied.

8 Side Effects of Dextran

Dextrans have acquired the GRAS status from the US FDA, which was renewed in 2013. However, several side effects are also associated with dextran polymer. Dextran is involved in the process of dental caries. Pathogenic bacteria of the genus *Streptococcus* produce insoluble dextran, which favors the adhesion of bacteria on the teeth and causes the formation of dental plaque. The orally ingested dextran rapidly converts into glucose, and therefore, it is harmful to the diabetic patients. The swelling or osmotic effect of dextran is also associated with acute renal failure in patients. It has also been reported that the high molecular weight dextran induces anti-dextran antibodies, leading to anaphylactoid reactions in some patients.

The production of dextran has been recognized as a nuisance to sugar industry for decades. Dextran formation creates processing problems in raw sugar factory operations and leads to a decline in sugar recovery, translating into economic loss. Dextran also creates processing difficulties by increasing juice viscosity, poor clarification, and crystal elongation. In addition, dextran has a significant impact on the resulting market on the final processed product. In some cases, dextran formation is also responsible for food spoilage in rum industry and cured meat products.

9 Conclusions

The sources, preparation, characterization, and food applications of dextran have been described. The biocompatibility, high water solubility, and water-holding capacity make dextran an important food ingredient. Therefore, the hunt for new, novel sources of dextran seems to be an interesting quest for food applications. Moreover, the production of dextran using cheaper substrates such as food wastes and agricultural by-products is prerequisite for its economical recovery at industrial level. Dextran conjugation has aided the design of new tailor-made polymers with different molecular weights, shapes, structures, and functional activities. It will be useful from an application viewpoint, if the functional properties of dextran, viz., rheology, molecular weight distribution, degree, and length of branching, and the fine structure of the dextran-conjugated products would be explored in greater details. The identification and characterization of novel dextransucrases with random mutagenesis followed by high-throughput screening will provide the best methods to obtain novel types of dextrans.

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Galactans and Its Applications

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Contents

Abstract

Galactan is a generic term to design a very large family of polysaccharides identified in terrestrial plants and other organisms but above all in red marine macroalgae. They are mainly represented by agars and carrageenans which have been largely investigated and used in industry for their unique rheological properties. The recent emergence of many studies, which report the wide range of biological activities of various galactans, opens the way to a new field of applications. This chapter focuses on the origins, the structural characteristics associated to extraction procedures, the biological activities, and the techno-functional properties of galactans.

Keywords

Galactan • Carrageenan • Agar • Polysaccharide • Application • Rheology • Bioactivity

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1 Introduction

"One chapter more about 'galactans!'" will be probably the first thought of the future readers. We hope this point of view will change after reading this chapter which aims to provide a synthetic and updated document with the main advances on galactans and its applications to the community of students and academics. Galactans (Gs), sulfated galactans (SGs), and galactan-containing polysaccharides are widely distributed in terrestrial plants and macroalgae. SGs from marine macroalgae have no equivalent in terrestrial plants and may constitute up to the 70 % of the dry matter of some red seaweeds. As other polysaccharides, galactans present shared features such as their high density of hydroxyl functions (-OH) leading to a hydrophilic character and a high capacity to establish hydrogen bond network. So, they extensively modify the rheology of aqueous media into which they are introduced, even at low concentrations. This is the basis of their functional properties as thickening and gelling agents. In the case of SGs such as carrageenans and agars, these properties are reinforced by their sulfate contents which give an anionic character able to interact with other compounds. The structure of SGs from marine red macroalgae, despite a simple backbone composed of a disaccharidic unit, is in fact highly complex depending on its substitution by (i) sulfate groups, (ii) non-galactose monosaccharides, (iii) the nature of galactose stereoisomers, and (iv) the presence of 3,6-anhydrogalactose. This structural variability conducts to the existence of numerous families of SGs with specific rheological properties and leads the manufacturer to develop specific processes often closely guarded as closed secrets. Indeed, SGs from red marine macroalgae form an old family of texturing agents which has known a success story in an economic point of view. Nowadays, SGs are currently used in dairy products, pastry, bakery, meat products, spreads, sauces, pet foods, and beverages as they thicken and stabilize food systems. According to some Food and Agriculture Organization (FAO) statistics, the sole world carrageenan seaweed farming production increased from less than 1 million wet tonnes in 2000 to 5.6 million wet tonnes in 2010, with the corresponding farmgate value increasing from USD 72 million to USD 1.4 billion. Moreover, the recent emergence of glycosciences and the improvements in the knowledges of polysaccharidic structures offer a new area of research and applications for these complex carbohydrates. In this context, the description of wide biological activities obtained with SG from natural occurring has increased for therapeutic applications, e.g., anticoagulant, immunomodulatory, antithrombotic, antiviral, and antitumor effects. Even if this new way of innovation has not yet known an economical takeoff, the increase of patents and academic publications allows optimism. Overall, this chapter focuses on the recent developments in the topic of galactan polysaccharides and their applications.

2 Galactan Sources Associated to Their Structures

Sulfated galactans (SGs) are among the most marine polysaccharides studied since the last two decades, as also sulfated fucans or glycosaminoglycans (GAGs). Composed of α-L- and/or β-D-galactopyranosyl (gal*p*) units, the structures of SGs

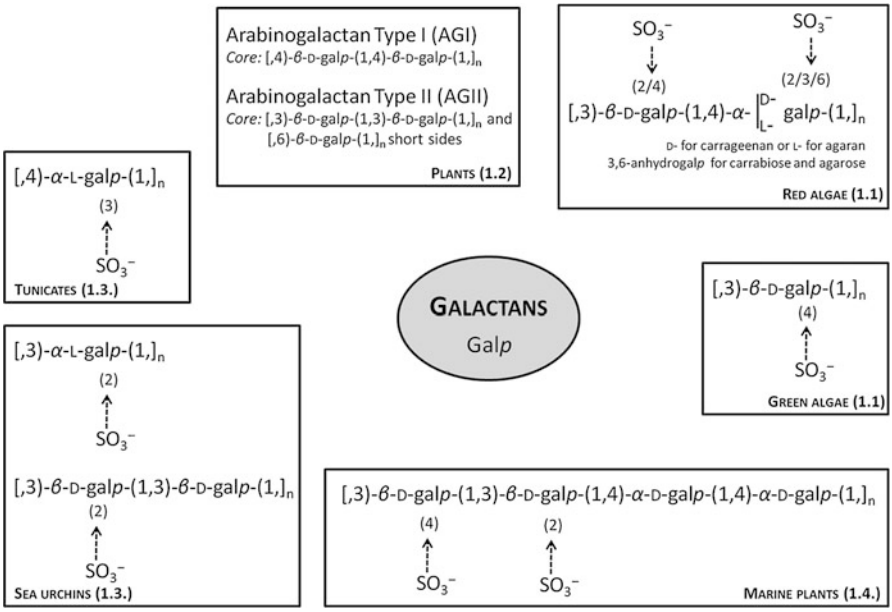

Fig. 1 The occurrence and structural diversity of galactans in nature

are in general complex and heterogeneous (Pomin and Mourão 2008) although their main structural features are conserved among phyla (Pomin 2010). Owing to their sulfate groups, SGs possess a strongly anionic character which increases possible electrostatic interactions with other charged oligo- and/or polysaccharides and/or proteins. In addition to these molecular interactions, SGs usually exhibit high molecular weights (MWs) higher than 100 kDa (Pomin 2010), which contribute to their biological potential and rheological behavior. Depending on the point of view, *stricto* sensu SGs are exclusively synthesized by marine organisms (Pomin and Mourão 2008). However, the occurrence of *largo* sensu SGs is quietly extensive since these GAG mimetics can be extracted from seaweeds, superior plants, marine invertebrates, and also microorganisms and fungi (Fig. 1) (Arifkhodzhaev 2000; Pomin and Mourão 2008; Delattre et al. 2011; Jiao et al. 2011).

2.1 Well-Known Sources of SGs: The Abundant Seaweeds

Marine algae are well known for their content in sulfated polysaccharides (Pereira et al. 1999; Berteau and Mulloy 2003). Marine SGs are widely abundant in red algae (Pomin and Mourão 2008; Delattre et al. 2011) and also found in green algae (Farias et al. 2008). Thus, SGs are currently extracted from *Rhodophyta* (red algae) cell walls (Craigie 1990) and more recently from the genus *Codium* (green algae) (Bilan et al. 2007; Farias et al. 2008). Nowadays, only 70–80 species of *Rhodophyta,* among more than 4,000 species, are used for industrial production

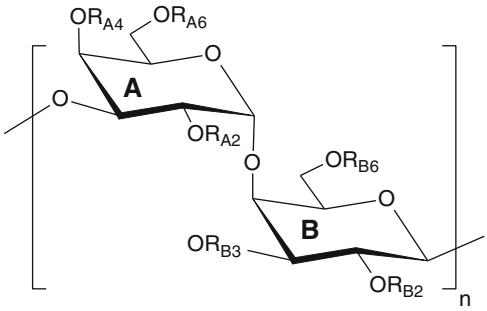

Fig. 2 Schematic representation of SGs from red seaweeds from Delattre et al. (2011). R_{A2}: H or SO_3^-; R_{A4}: H or SO_3^- or $C_3H_4O_3$ (cyclic ketal with O_6), R_{A6}: H or CH_3 or SO_3^- or $C_3H_4O_3$ (cyclic ketal with O_4), R_{B2}: H or CH_3 or SO_3^-, R_{B3}: H, R_{B6}: H, SO_3^-

of galactans (Delattre et al. 2011). As described by Witvrouw and De Clercq (1997), the most important ones belonged to Florideophyceae (2nd subclass of *Rhodophyta* division), orders Gelidiales and Gigartinales, and mainly genera *Ahnfeltia*, *Chondrus*, *Eucheuma*, *Furcellaria*, *Gelidiella*, *Gelidium*, *Gigartina*, *Gracilaria*, and *Pterocladia*. Based on their stereochemistry, the SGs produced by these red algae are classified as agarans, carrageenans, and D/L-hybrids which are also called nonideal SGs or complex SGs (Delattre et al. 2011; Jiao et al. 2011). Note to mention the origin of the name of "agaran" derived from "agar" which means jelly in the Malay language (Knutsen et al. 1994; Lahaye 2001), and "carrageenans" comes from the name of an Irish village (Bixler 1994).

In general, they are constituted of a linear backbone of 3-linked *β*-D-gal*p* residues (A units) and 4-linked *α*-D/L-gal*p* residues (B units) respecting an alternated sequence (AB)*n* (Fig. 2). B units can also be partially or totally replaced by 3,6-anhydro derivatives. Various hydroxyl groups can be substituted by methyl groups, sulfate ester groups, or pyruvic acid (Usov 1998; Delattre et al. 2011). The sulfate pattern is the major structural variation of SGs, and the distribution is quite heterogeneous and specific of red algae species and biotic/abiotic conditions when the samples are collected. Besides, it is noteworthy that *largo* sensu SGs can be ramified by neutral monosaccharides residues such as xylose, glucose, mannose, and/or arabinose.

2.1.1 Agarans

SGs with B units of the L-series are termed agarans (Fig. 3a) (Rees 1969; Knutsen et al. 1994). Agarans can be divided in two groups, i.e., agars and agaroids (Craigie 1990; Delattre et al. 2011), depending on the part of B units in the form of 3-6-anhydro-*α*-L-gal*p* (*α*-L-AnGal*p* units) and the percentage of sulfate groups. Thus, the ratio of *α*-L-AnGal/*α*-L-Gal units is higher in agars than agaroids, on the contrary to the percentage of sulfate groups which is close to 2 % for agars or up to 20 % for agaroids (Lahaye 2001; Delattre et al. 2011). However, this distinction is not always done in the literature, and numerous works just refer to the regular agaran backbone, more or less substituted by various chemical groups (Pomin and Mourão 2008; Jiao et al. 2011).

Agars are essentially extracted from *Gelidium*, *Gracilaria*, *or Pterocladia* species (Table 1) and are made up of a linear polysaccharide named agarose and a mixture of heterogeneous highly charged galactans called agaropectin (Fig. 3b) (Rinaudo 2007; Delattre et al. 2011).

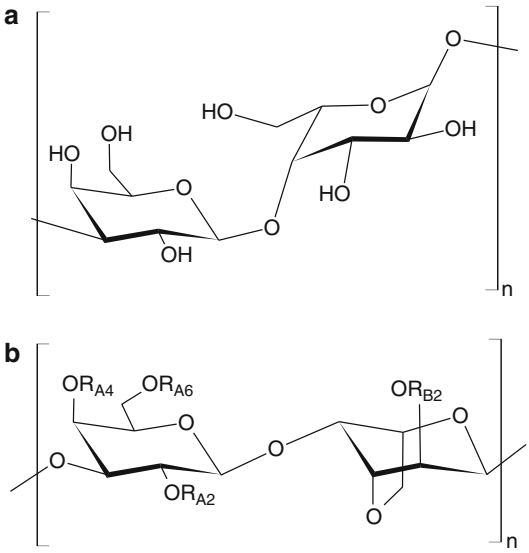

Fig. 3 Schematic representations of **a** generic agarans and **b** the alternating agarobiose sequence constituting agarose and agaropectin. R_{A2}: H or SO_3^-; R_{A4}: H or SO_3^-or $C_3H_4O_3$ (cyclic ketal with O_6), R_{A6}: H or CH_3 or SO_3^-or $C_3H_4O_3$ (cyclic ketal with O_4), R_{B2}: H or CH_3 or SO_3^-

Agarose, with an average molecular weight of 120 kDa, is often highly substituted in R_{A6} position by methyl groups (up to 20 %). On the other hand, agaropectin exhibits a similar backbone to agarose with numerous substituted groups as sulfate, pyruvate, methyl, or glycuronate (Furneaux and Stevenson 1990; Knutsen et al. 1994; Akari et al. 1996; Rinaudo 2007).

The backbone of agaroids is close to agarobiose but the ratio of AnGal units is lower than for agars and the ratio of R_{A6} sulfate groups is greatly higher (until ten times). Agaroids are widely studied (Table 2) and especially porphyrans which are isolated from *Porphyra* species, e.g., *Porphyra capensis* (Zhang et al. 2005), *Porphyra haitanensis* (Zhang et al. 2004, 2009), or *Porphyra umbilicalis* (Delattre et al. 2011). They are constituted of 3-linked 6-*O*-methyl-*β*-D-gal*p* and 4-linked 6-*O*-sulfo-*α*-L-gal*p* (Fig. 4a). Porphyrans-like are also produced by *Polysiphonia* species, such as *Polysiphonia abscissoides* (Miller and Furneaux 1997), *Polysiphonia atterima* (Miller 2003), *Polysiphonia nigrescens* (Prado et al. 2008), or *Polysiphonia strictissima* (Miller and Furneaux 1997). In fact, many species of Ceramiales produce agarans such as *Ceramium*, *Chondria*, *Cladhymenia*, *Delesseria*, *Laurencia*, *Odonthalia*, or *Rhodomela* (Table 2). Note to mention some species belonging to these genera produced polysaccharides containing both agaran- and carrageenan-type backbones, e.g., *Rhodomela larix* (Takano et al. 1999). Besides, the regular agaroid backbone can include *O*-linked methyl, pyruvate, and xylosyl groups as reported for species like *P. nigrescens* or *Acanthophora spicifera* (Gonçalves et al. 2002; Duarte et al. 2004; Prado et al. 2008). These structures are often considered as belonging to the well-known D/L-hybrids or also complex galactans (Delattre et al. 2005). It is the same for funorans, which are extracted from species such as

Table 1 Some red seaweeds producing agars

Species	Sugar units	Substitutions[a]	Linkages	Yields (%)	References
Gelidium crinale	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$	β-(1,3) α-(1,4)	–	Murano et al. 1998
Gelidium floridanum	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $C_3H_4O_3$	β-(1,3) α-(1,4)	–	Bouzon et al. 2005
Gelidium pusillum	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$	β-(1,3) α-(1,4)	–	Murano et al. 1998
Gelidium robustum	D-Gal*p*, L-Gal*p*, L-AnGal*p*	–	β-(1,3) α-(1,4)	25–44	Sousa-Pinto et al. 1996
Gelidium serrulatum	D−Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $C_3H_4O_3$	β-(1,3) α-(1,4)	–	Murano et al. 1998
Gelidium sesquipedale	D-Gal*p*, L-AnGal*p*	$^{(C4,6)}SO_3^-$	β-(1,3) α-(1,4)	–	Guerrero et al. 2014
Gracilaria arcuata	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $^{(C2,6)}CH_3$	β-(1,3) α-(1,4)	33.2	Tako et al. 1999
Gracilaria blodgettii	D-Gal*p*, L-Gal*p*, L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	25–36	Freile-Pelegrín and Murano 2005
Gracilaria cervicornis	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $^{(C4,6)}CH_3$	β-(1,3) α-(1,4)	25–39	Freile-Pelegrín and Murano 2005
Gracilaria crassissima	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $^{(C6)}CH_3$	β-(1,3) α-(1,4)	14–30	Freile-Pelegrín and Murano 2005
Gracilaria cornea	D-Gal*p*, L-Gal*p*, L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	20.1	Freile-Pelegrín 2000
Gracilaria edulis	D-Gal*p*, L-Gal*p*, L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	–	Villanueva and Montano 1999
Gracilaria salicornia	D-Gal*p*, L-Gal*p*, L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	2.9–15.7	Calumpong et al. 1999
Gracilaria sp.	D-Gal*p*, L-Gal*p*, L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	–	Friedlander and Dawes 1984
Gracilariopsis persica	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Glc, Xyl*p*	$^{(C2,4,6)}SO_3^-$, $^{(C6)}CH_3$	β-(1,3) α-(1,4) [β-(1,6)]	29	Salehi et al. 2011
Hydropuntia cornea	D-Gal*p*, L-Gal*p* (?), L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	14–43.3	Pereira-Pacheco et al. 2007
Pterocladia capillacea	D-Gal*p*, L-Gal*p* (?), L-AnGal*p*	SO_3^-	β-(1,3) α-(1,4)	–	Lai and Lii 1998
Pterocladia lucida	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Xyl*p*, Glc*p*	SO_3^-, $C_3H_4O_3$	β-(1,3) α-(1,4) [β-(1,2)]	4–19	Chiovitti et al. 2004

[a]Substitution location and type are given for Gal and AnGal units

Table 2 Some red seaweeds producing agaroids

Species	Sugar units	Substitutions[a]	Linkages	Yields (%)	References
Acanthophora spicifera	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl, D-Glc	$^{(C2,3,6)}SO_3^-$, $^{(C6)}CH_3$, $C_3H_4O_3$ ketal	β-(1,3) α-(1,4)	20–30	Duarte et al. 2004
Ceramium rubrum	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl	$^{(C2,3,6)}SO_3^-$	β-(1,3) α-(1,4)	–	Miller and Blunt 2002
Chondria macrocarpa	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl	$^{(C2,6)}SO_3^-$	β-(1,3) α-(1,4)	–	Furneaux and Stevenson 1990
Cladhymenia oblongifolia	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C2,3,6)}SO_3^-$, $C_3H_4O_3$ ketal	β-(1,3) α-(1,4)	–	Miller and Blunt 2000
Delesseria sanguinea	Gal, AnGal*p*, Xyl, Glc, Man, Fuc, GlcA	SO_3^-	β-(1,3) α-(1,4) [β-(1,2)]	11.6	Grünewald and Alban 2009
Laurencia obtusa	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl	$^{(C2)}SO_3^-$, $^{(C6)}CH_3$	β-(1,3) α-(1,4)	12.8	Canelón et al. 2014
Laurencia filiformis	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl	$^{(C2)}SO_3^-$, $^{(C2)}CH3$, $C_3H_4O_3$ ketal	β-(1,3) α-(1,4) [β-(1,2)]	16.2	Canelón et al. 2014
Palisada flagellifera	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl	$^{(C2,\ 6)}SO_3^-$, $^{(C2,6)}CH_3$; $C_3H_4O_3$ ketal	β-(1,3) α-(1,4)	8–85	Ferreira et al. 2012
Porphyra capensis	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, $^{(C6)}CH_3$	β-(1,3) α-(1,4)	17–25	Zhang et al. 2005
Porphyra haitanensis	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Fuc	$^{(C6)}SO_3^-$, $^{(C2,6)}CH_3$	β-(1,3) α-(1,4)	–	Zhang et al. 2004
Porphyra umbilicalis	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Man, Xyl, Glc	$^{(C6)}SO_3^-$, $^{(C6)}CH_3$	β-(1,3) α-(1,4)	53	Peat et al. 1961
Polysiphonia abscissoides	D-Gal*p*, L-Gal*p*, L-AnGal*p*	$^{(C6)}SO_3^-$, CH_3	β-(1,3) α-(1,4)	–	Miller and Furneaux 1997
Polysiphonia atterima	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Xyl,	$^{(C6)}SO_3^-$, $^{(C2)}CH_3$	β-(1,3) α-(1,4)	–	Miller 2003
Polysiphonia nigrescens	D-Gal*p*, L-Gal*p*, L-AnGal*p*, D-Xyl, Glc	$^{(C2,6)}SO_3^-$, $^{(C3,6)}CH_3$	β-(1,3) α-(1,4) [β-(1,6)]	1–9	Prado et al. 2008
Polysiphonia strictissima	D-Gal*p*, L-Gal*p*, L-AnGal*p*, Xyl	$^{(C6)}SO_3^-$, CH_3	β-(1,3) α-(1,4)	–	Miller and Furneaux 1997

[a]Substitution location and type are given for Gal and AnGal units

Gloiopeltis complanata or *Gloiopeltis furcata* (Takano et al. 1995, 1998) and are composed of AB alternative backbone of agars but whose C2 and C6 hydroxyl groups of 3-β-D-gal*p* and 4-α-L-gal*p* (Fig. 4b) are substituted by sulfates or methyl groups.

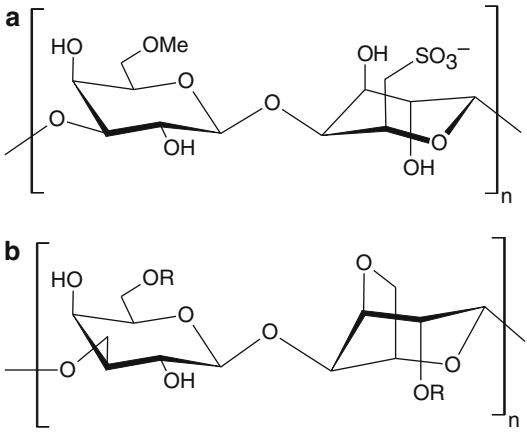

Fig. 4 Schematic representations of **a** porphyran and **b** funoran. R: CH_3 or SO_3^-

2.1.2 Carrageenans

SGs with B units of the D-series are termed carrageenans (Knutsen et al. 1994). D-gal*p* (B residues) of the repeating unit AB, also called carrabiose, can be replaced by 3-6-anhydro-*α*-D-gal*p* (*α*-D-AnGal*p*). Carrageenans can be isolated from various species of red algae, such as *Gigartina*, *Chondrus*, *Eucheuma*, or *Hypnea* (Table 3) (Stanley 1987; Prajapati et al. 2014). Carrageenans are classified by a Greek prefix according to their own structural characteristics such as sulfation and AnGal*p* patterns and their solubility in potassium chloride, which has been largely described in the literature (Smith and Cook 1953; Usov 1998; Lahaye 2001; van de Velde et al. 2004; Delattre et al. 2011; Jiao et al. 2011). Besides, other carbohydrate residues (Xyl, Glc, Uronic acids) or pyruvate or methyl ether groups can also be found (Knutsen et al. 2001). These multiple variations give birth to the well-known large structural heterogeneity of carrageenans (Lahaye 2001). Note to mention carrageenans are also high molecular weight polysaccharides, between 100 and 1,000 kDa (Kloareg and Quatrano 1988; Campo et al. 2009). These polysaccharides are split into various basic groups whose the kappa (κ), iota (ι), or lambda (λ) carrageenans (Campo et al. 2009), but this nomenclature slightly changes depending on authors (Fig. 5). The kappa family includes κ-carrageenans which can be obtained by alkali treatments from the μ precursors. The iota family includes ι-carrageenans which can be obtained by similar treatments from the ν precursors (Anderson et al. 1973). Each member of these families is C4 sulfated (A unit). Yet, ι-carrageenans are C2 sulfated and less composed of 3,6-anhydro-gal*p* (25–30 %) than the subfamily κ-carrageenans (up to 35 %). Thus, κ- and ι-carrabioses have respectively one (20 %) and two (33 %) sulfate ester groups (w/w) (Fig. 5). It is noteworthy that μ and ν precursors are composed of a 6-*O*-sulfated B unit instead of a 3,6-anhydro-gal*p*. The red seaweeds *Kappaphycus alvarezii* (also named *Eucheuma cottonii*) (Anderson et al. 1973; Estevez et al. 2000; Rudolph 2000) and *Hypnea musciformis* (Cosenza et al. 2014) are some examples of κ-carrageenan sources while ι-carrageenans can be extracted from species such as

Table 3 Some red seaweeds producing carrageenans

Species	Main types obtained (w/o treatments)	Other types	Yields (%)	References
Betaphycus gelatinum	κ, β	μ, γ	71	Pereira et al. 2009
Calliblepharis jubata	κ, ι	ν	–	Pereira et al. 2009
Callophyllis hombroniana	θ	–	16–22	Falshaw et al. 2005
Chondracanthus chamissoi	κ, ι	μ, ν	–	Pereira et al. 2009
Chondracanthus teedei	κ, ι	–	–	Pereira et al. 2003
Chondrus crispus	κ, λ, ι	μ	30–60	Matsuhiro and Urzua 1992
Eucheuma denticulatum	ι	ν	–	Campo et al. 2009
Furcellaria lumbricalis	κ, β	γ, μ	–	Yang et al. 2011; Correc et al. 2012
Gigartina skottsbergii	κ, ι, λ, θ	ξ, μ, ν	–	Ciancia et al. 2005; Doyle et al. 2010
Halymenia durvillei	λ	–	15–18	Fenoradosoa et al. 2009
Hypnea musciformis	κ	μ (few amounts)	5.5–39.4	Cosenza et al. 2014
Iridaea cordata	κ, γ	–	59–65	Waaland 1975
Kappaphycus alvarezii	κ	μ	29–31	Estevez et al. 2000; Periyasamy et al. 2014
Mastocarpus stellatus	κ, ι	μ, ν	13–38	Hilliou et al. 2006
Sarconema filiforme	ι, α	–	6–35	Chiovitti et al. 1998
Sarcothalia crispata	κ, ι, λ	μ, ν	–	Falshaw et al. 2001
Tichocarpus crinitus	κ, β	γ, μ	–	Correc et al. 2012

w/o with or without chemical treatments

Eucheuma denticulatum (also called *Eucheuma spinosum*) (Campo et al. 2009), *Eucheuma spinosum* (Funami et al. 2007), or *Sarconema scinaioides* (Kumar et al. 2012) (Table 3). Note that Pereira et al. (2009) have described numerous sources of carrageenans produced by carrageenophytes. On the other side, the lambda family includes carrageenans which are not C4 sulfated (B unit) and not composed of 3-6-anhydro-gal*p* but which possess three (41 %) sulfate ester groups (w/w) per carrabiose. The sulfate groups are located on C2 of A unit and C2 and C6 of B unit (Fig. 5). It is noteworthy that λ-carrageenans are extracted from species of

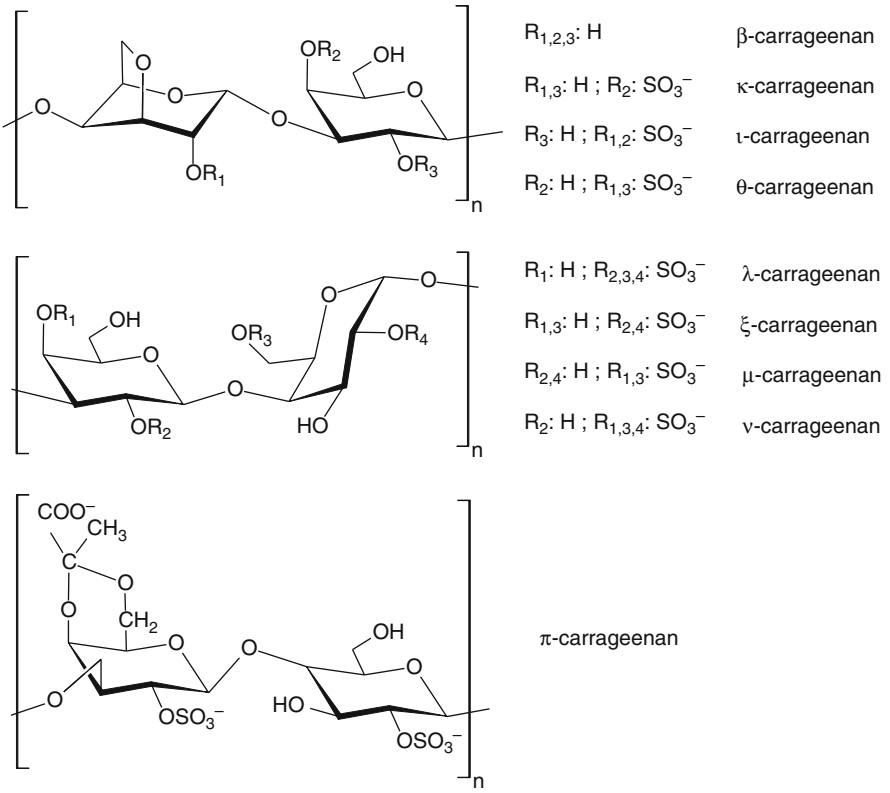

Fig. 5 Schematic representations of main carrageenans

the *Gigartina* and *Chondrus* genera (Zhou et al. 2006). Three subfamilies are categorized in the lambda family, i.e., the theta (θ), xi (ξ), and pi (π) carrageenans, where only the 4-*α*-D-gal*p* unit varies. θ-carrageenans are only one-time C2 sulfated on the B unit and are obtained from chemical treatments (Ciancia et al. 1993) or naturally found in algae, e.g., *Callophyllis hambroniana* (Miller 2003; Falshaw et al. 2005). As reported by Delattre et al. (2011), desulfated carrageenan (Penman and Rees 1973) and pyruvated carrageenan (DiNinno et al. 1979) have already been observed in tetrasporophytes from different species of *Gigartinaceae* (McCandless et al. 1983; Falshaw and Furneaux 1995, 1998).

To date, natural carrageenans often occur as mixtures of different hybrid types (Jiao et al. 2011). Craigie (1990) reported the difficulty to estimate if a carrageenan is constituted of one or two distinct types of carrageenans. Thus, κ/β- (Yang et al. 2011), κ/ι- (Hilliou et al. 2009), κ/μ- (Jouanneau et al. 2010), or ν/ι- (van de Velde 2008) hybrids have already been described in the literature. Otherwise, it has been well established that the nature of carrageenans also depends on the different generations of biological cycle. Sulfate and AnGal patterns can thus be affected.

Falshaw et al. (1995, Falshaw and Furneaux 1998, Falshaw, Bixler and Johndro 2001) clearly highlighted the presence of κ/ι- or ι/κ-hybrids in the gametophytes of *Gigartina* species, such as *Gigartina atropurpurea*, *Gigartina alveata*, *Gigartina pistillata*, or *Sarcothalia crispate*, while tetrasporophytes contained λ-, ξ-, and/or π-types.

2.1.3 Complex Galactans (D/L-hybrids)

Complex galactans, also named D/L-hybrids or nonideal galactans, are SGs from red algae composed of the alternating AB sequence (Fig. 2) which was previously described but ramified by other pentoses and/or hexoses and with other various substitutions such as sulfate groups, pyruvic acid ketals, or methoxyl groups. In this way, xylogalactans can be considered as nonideal SGs and can be extracted as example from *Corallina officinalis*, *Lithothamnion heterocladum*, *Jania rubens*, or *Calliarthron cheilosporioides* (Cases et al. 1994; Navarro and Stortz 2008; Martone et al. 2010; Navarro et al. 2011). Also termed corallinan, they are nonideal agarans with a *β*-D-xyl*p* ramified on *O*-6 position of D-gal*p* units. Other xylogalactan structures were also isolated from red seaweeds such as *Delesseria sanguine* (Grünewald and Alban 2009), *Laurencia obtuse* (Canelón et al. 2014), *Palisada flagellifera* (Ferreira et al. 2012), or *Polysiphonia nigrescens* (Prado et al. 2008). Highly complex structures have also been isolated from *Asparagopsis armata*, *Kappaphycus alvarezii* (*β*-D-xyl and *β*-D-glc substituents), *Pachymenia lusoria* (Xyl substituent), *Cryptonemia crenulata* (*β*-D-xyl and *β*-D-gal substituents), *Grateloupia indica* (Fuc, Glc, Gal substituents), *Gymnogongrus torulosus* (*β*-D-xyl), or *Halymenia durvillei* (Xyl, Ara, Fuc) (Miller et al. 1995; Estevez et al. 2004; Garon-Lardière 2004; Zibetti et al. 2005; Chattopadhyay et al. 2007; Estevez et al. 2008; Fenoradosoa et al. 2009). Note to mention that an in-depth study of literature obviously highlights that numerous complex galactans from red algae are classified by many authors as ideal SGs with minor amounts or other sugar residues. Thus, this complex galactan category is not well documented.

2.1.4 3-β-D-Galp-1 Units from Green Algae

Red algae are often considered as the only source of interesting galactans because of their structures (related to various physicochemical properties) and "the ease" of production/extraction/purification steps. Alternatively, SGs have also been characterized in green seaweed species belonging to the genus *Codium*. It is well described that the cell walls of *Codium* species, such as *Codium isthmocladum* and *Codium yezoense*, are constituted of galactose-composed polysaccharides and other heteropolysaccharides (Matsubara et al. 2001; Bilan et al. 2007; Farias et al. 2008). SGs from green algae exhibit a backbone of 3-linked *β*-D-galp-1 units mainly sulfated in position C4 and also minor amounts of substitutions, e.g., sulfate groups in positions C6 or pyruvic acid groups forming cyclic ketals with Gal units (Fig. 6) (Bilan et al. 2007; Farias et al. 2008). Other sugar residues (Gal, Ara, Glc) can also be found, as it has already been observed for *Codium fragile* or *Codium cylindricum* (Love and Percival 1964; Matsubara et al. 2001;

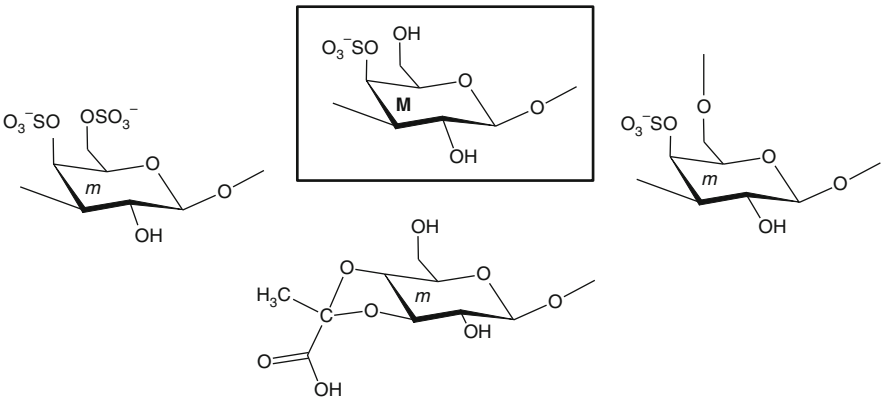

Fig. 6 Schematic representations of green algae SGs from *Codium isthmocladum*. *M* Major residue, *m* minor residue

Estevez et al. 2009; Lee et al. 2010a, b). Overall, the 3-linked β-D-galp-1 units seem to be the dominant of these SG backbones, bearing in mind that the green algal SGs are more complex than those of red algae (Pomin 2010).

2.2 Parallel Sources of SGs: The Superior Plants

Extensive investigations of polysaccharides from higher plants have been performed since the last two decades, and it was well defined that plants can contain a very large number of carbohydrate polymers. Beside starch material and fructans, plants are also composed of cell wall storage polysaccharides (CWSPs), mainly present in seeds (McCann and Roberts 1991; Carpita and Gibeault 1993). CWSPs can be split into three families: mannans, xyloglucans, and galactans. Note to mention that seed cell wall galactans were firstly characterized in 1947 from the seeds of *Lupinus albus* (Hirst et al. 1947). Galactans and galactan-composed polysaccharides are at least distributed in 30 families (Arifkhodzhaev 2000). Arabinogalactans can be categorized depending on their structures into two types, i.e., arabino-4-galactans (arabinogalactans I, AGI) and arabino-3,6-galactans (arabinogalactans II, AGII) (Aspinall 1970).

2.2.1 Arabinogalactan I

AGI are composed of a main backbone of (1-4)-linked β-D-gal*p* with short side chains of (1-5)-linked α-ara*f* attached in *O*-3 position. Other residues such as galactose, arabinose, and ferulic acid could also be found, for example, in some tissues of sugar beet (Ralet et al. 2005). Overall, AGI can be isolated from various primary walls of dicotyledonous plant tissues including numerous fruits (Perez et al. 2000). A non-exhaustive list of AGI plants sources is given in Table 4.

Table 4 Some plants producing galactans, adapted from Arifkhodzhaev (2000)

Species	Monosaccharide composition	MW (u.a.)	Linkages	References
Galactans				
Allium cepa	Gal	–	(1,4)	Sen et al. 1971; Ha et al. 2005
Allium sativum	Gal, Ara	–	(1,4) (1,6)	Das et al. 1977
Dolichos lablab Linn.	Gal, GalA	120 k	(1,2) (1,4) (1,6)	Ghosh and Das 1984
Nicotiana tabacum	Gal	60–80 k	(1,4)	Eda et al. 1986
Salix alba L.	Gal	5,35 k	(1,4) (1,6)	Toman et al. 1972
AGI				
Aegle marmelos	Gal, Ara, Rha, Xyl, Glc	–	(1,2) (1,5)	Basak and Mukherjee 1982
Centrosema plumari	Gal, Ara	–	(1,4) (1,6)	Morimoto et al. 1962
Lycopersicon esculentum	Gal, Ara, Xyl, Glc	–	(1,4)	Iwai et al. 2013
Opuntia dillenii	Gal, Ara	–	(1,3) (1,4)	Srivastava and Pande 1974
Solanum betaceum	Gal, Ara, Rha, Xyl, Man, Glc, UA	40–1300 k	(1,3) (1,4) (1,5)	do Nascimento et al. 2014
Solanum tuberosum	Gal, Ara	–	(1,3) (1,4) (1,6)	Wood and Siddiqui 1972
Strychnos nux-vomica	Gal, Man, Xyl, Ara	–	(1,3) (1,4) (1,6)	Andrews et al. 1954
Symplocos spicata	Gal, Ara	–	(1,3) (1,4) (1,6)	Tiwari and Tripathi 1976
AGII				
Acacia glomerosa	Gal, Ara, GlcA, Rha	–	(1,3) (1,6)	León de Pinto et al. 2001
Acacia tortuosa	Gal, Ara, Xyl, GlcA	–	(1,3)	León de Pinto et al. 1998
Allochrusa gypsophiloides	Gal, Glc	2 k	(1,2) (1,6)	Arifkhodzhaev 1996
Aloe arborescens	Gal, Ara	30 k	(1,2) (1,6)	Yagi et al. 1986
Angelica acutiloba	Gal, Ara, Rha, Glc	83–96 k	(1,3) (1,4)	Zhang et al. 1996
Atractylodes lancea	Gal, Ara, Xyl, Man, Glc	–	(1,3) (1,6)	Yu et al. 2001
Endopleura uchi	Gal, Ara, Rha, Xyl, Man, Glc, UA	109 k	(1,3) (1,6)	Bento et al. 2014
Glycyrrhiza uralensis	Gal, Glc, Ara, Rha	69 k	(1,2) (1,6)	Shimizu et al. 1990
Hericium erinaceus	Gal, Fuc	19 k	(1,2) (1,6)	Zhang et al. 2006

(*continued*)

Table 4 (continued)

Species	Monosaccharide composition	MW (u.a.)	Linkages	References
Impatiens parviflora DC	Gal, Rha, GalA	20–70 k	(1,2) (1,4)	Hromádková et al. 2014
Madhuca indica	Gal, Ara, Rha, Xyl, GlcA	–	(1,2) (1,3) (1,4) (1,6)	Sarkar and Chatterjee 1984
Malva verticillata	Gal, Glc, Ara	77 k	(1,3) (1,6)	Shimizu et al. 1991
Raphanus sativus	Gal, Ara, Glc, Xyl, UA*	52 k	(1,3) (1,6)	Tsumuraya et al. 1987
Salvia officinalis	Gal, Ara, Glc	2–93 k	(1,3) (1,5) (1,6)	Capek 2008
Spondias dulcis	Gal, Ara, GlcA, Man	–	(1,3) (1,6)	Martínez et al. 2003
Vernonia kotschyana	Gal, Rha, GalA	1,150 k	(1,2) (1,3) (1,4) (1,6)	Nergard et al. 2005

MW Molecular weight, *UA* Uronic acid

2.2.2 Arabinogalactan II

AGII are highly complex polysaccharides often associated to proteins and described as well-known arabinogalactan proteins (AGPs). These proteoglycans are widely distributed in plants and are one of the most complex natural macromolecules. AGII can be extracted from various plants (Table 4), along with covalently linked pectic polymers (Vincken et al. 2003). Type II arabinogalactans are constituted of (1,3)- and (1,6)-β-D-gal*p* residues. Short side chains of (1,6)-β-D-gal*p* units including one to three residues in length are also present. Terminal α-L-ara*f* residues are often attached on position *O*-3 and *O*-6 of this backbone. Note to mention that few amounts of Glc*p*A can also be found.

2.3 Animal Sources of SG: The Use of Marine Invertebrates

As previously said concerning the large distribution of galactans in the nature, SGs have been described in some species of invertebrates (Fig. 1). Thus, ascidians (also called tunicates or sea squirts), such as *Herdmania momus* or *Styela plicata*, respectively contain a sulfated galactan composed of 3-sulfated, 4-linked α-L-gal*p* residues, i.e., [,4)-α-L-gal*p*-3(OSO_3^-)-(1,]$_n$ (Santos et al. 1992), and a similar backbone structure with non-sulfated L-gal*p* branched in *O*-2 position of the central core, i.e., [4)-α-L-gal*p*-2[1)-α-L-gal*p*-2(OSO_3^-)-3(OSO_3^-)-(1,]$_n$ (Mourão and Perlin 1987). It is interesting to understand that SGs are components of the extracellular matrix of these invertebrates and so play a similar role to glycosaminoglycans in vertebrates (Mourão and Perlin 1987). On the other hand, sulfated galactans are also found in sea urchins. Few examples are fully documented but *Echinometra lucunter* and *Glyptocidaris crenularis* can be cited as galactan

producers. Thus, *Echinometra lucunter* produces a 2-sulfated 3-linked α-L-galactan, i.e., [,3)-α-L-gal*p*-2(SO_3^-)-(1,]$_n$ (Alves et al. 1997) while *Glyptocidaris crenularis* produces a disaccharide repeating structure of alternating 2- and non-sulfated 3-linked β-D-galactan, which is similar to galactan structures from green algae (Pomin and Mourão 2008).

2.4 Alternative and Original Sources

2.4.1 Potential of Marine Plants

SGs have never been described in vascular flowering plants (angiosperm) until the recent work of Aquino et al. (2005) on *Ruppia maritima*. This marine plant, which grow in highly saline marine environments, produced an SG composed of a regular tetrasaccharide unit of [,3)-β-D-gal*p*-2(OSO_3^-)-(1,4)-α-D-gal*p*-(1,4)-α-D-gal*p*-(1,3)-β-D-gal*p*-4(OSO_3^-)-(1]$_n$. It is interesting to note that these units are not distributed in an alternating sequence as in the case of red seaweeds. Aquino et al. (2005) suggested that this structure could contribute for the structural arrangement of the cell wall but also be involved in the osmotic regulation phenomena. As reported by Pomin (2010), the authors speculated about the role of sulfate groups for salt ion sequestration.

2.4.2 Why Not Investigating Microorganisms or Fungi?

The polysaccharide market is continuously blooming in terms of biotechnological operations and value addition. The specific needs of newer structures (and so properties) and the high competitiveness of the market require perpetual innovative changes. These changes can concern the sources of SGs, the extraction procedures, and the physicochemical modifications of the polysaccharides (eco-friendly if possible). The production of SGs by the bacterial fermentation could be a significant alternative to the seaweed exploitation (algaculture and/or harvesting) and to the use of plants or animals (Delattre et al. 2011). Indeed, the production of bacterial polysaccharides is subjected to fewer constraints and clearly presents advantages in terms of: (i) harvesting steps depending on seasons, (ii) production rates and yields, (iii) extraction procedures and yields, (iv) media (at least water) and energy saving, etc. That is the reason why screening of original microorganisms is becoming one of the newest challenge of this decade. As examples, galactan structures could be produced by *Methylobacterium sp.* (Verhoef et al. 2003), *Lactococcus lactis* subsp. *cremoris* H414 (Gruter et al. 1992), and *Lactococcus lactis* subsp. *cremoris* B891 (van Casteren et al. 2000). It is interesting to note that *Mycoplasma mycoides* subsp. *mycoides* can produce capsular galactans-like (Bertin et al. 2013). Besides, the literature is little explicit concerning the potential of fungi as galactan producers. *Pleurotus* is the only clearly reported genus able to produce galactan structures such as a 6-α-D-gal*p* by *Pleurotus eryngii* (Carbonero 2008) or 4-α-D-gal*p* by *Pleurotus ostreatoroseus* (Rosado et al. 2002, 2003). Other fungi such as *Inonotus levis* have been reported for producing similar structures (Vinogradov and Wasser 2005). *Cordyceps dipterigena* BCC2073 can also produce

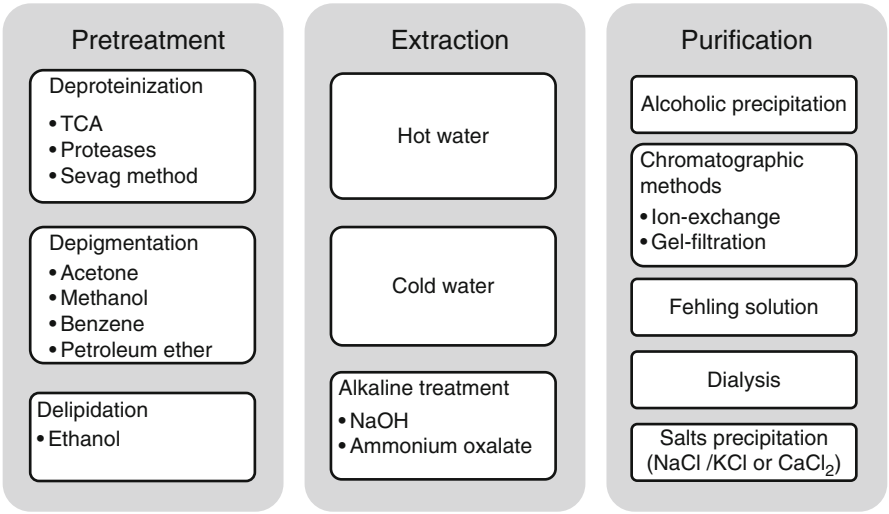

Fig. 7 Main methods used for galactan extraction and purification from seaweeds or superior plants

a (1,3)-galactan as described by Methacanon et al. (2005). Note that a rhamnogalactan was recently produced from *Fusarium solani* SD5 (Mahapatra and Banerjee 2013).

2.5 Extraction of Galactans

Depending on the galactan source (seaweeds, superior plants, or marine invertebrates), methods applied to recover polymers are different. However, all include three main steps: pretreatment, extraction, and purification. Main methods applied for galactan isolation and purification from seaweeds and plants are summarized on Fig. 7.

2.5.1 Pretreatments

Prior to extraction and depending on the polysaccharide source, some pretreatments should be needed. Before extraction of polysaccharides from seaweeds, a depigmentation step is often useful to prevent colorless extracts. The main method used is based on the use of acetone or methanol (Siddhanta et al. 1999). Nevertheless, some authors have used Sohxlet apparatus and benzene (Chattopadhyay et al. 2007) or petroleum ether (Karmakar et al. 2009) prior to acetone extraction. Moreover, it is often needed to remove lipids before extraction of galactans. The defatted fraction is then obtained by boiling sample in ethanol (Yang et al. 2011; Li et al. 2013). As main examples, deproteinization of samples can be achieved using Sevag method (n-butanol:chloroform, 1:4, v/v) (Staub 1965), conjunction of barium hydroxide and zinc sulfate (Pierre et al. 2011), or by acid precipitation with TCA

(Palacios et al. 2012). Moreover, proteases can also be used (Stevens and Selvandran 1984; Palacios et al. 2012). Finally, for plant extracts, processes can include a step consisting of a methanolic extraction in order to remove phenolic compounds (Palacios et al. 2012). In some cases, an increase in the effectiveness of extraction was observed (Park et al. 2009; Palacios et al. 2012).

2.5.2 Extraction of Galactans from Seaweeds

Galactans from seaweeds are generally extracted by two main methods: hot water extraction and sodium hydroxide extraction. In the two cases, dried and milled seaweeds are incubated in the corresponding solution at hot temperature (generally between 80 °C and 100 °C) for 1–4 h (Fenoradosoa et al. 2009). The mixture is then filtrated or centrifugated to remove non-soluble residue. The polysaccharides are then precipitated (generally three times) using alcohol prior to drying. As recently detailed by Prajapati et al. (2014), there are some differences in the processing of carrageenans. Kappa-carrageenans are generally extracted by using NaOH (5–10 %) treatment at 80–90 °C for 1–4 h depending on the texture of the macroalgae. As in the case of *Eucheuma* or *Furcellaria*, kappa-carrageenans are precipitated using cold 1–1.5 % of KCl solution followed by: washing with water, dehydrating (using pressing method), drying, and milling in order to get kappa-carrageenan in powder form. Lambda- and iota-carrageenans are generally extracted in alkali solution (NaOH or KOH) at 80–100 °C during 1–4 h. After filtration, the carrageenan solution is concentrated using evaporator and finally precipitated using alcohol (propyl or ethyl alcohol).

Alkaline extraction often leads to higher extraction yields; nevertheless, these polymers have shown a modified structure, with alteration of monosaccharide composition and substitution (Greer and Yaphe 1984; Fenoradosoa et al. 2009) and then a modification of final properties, as for example, a modification of the gel strength of agar (Lahaye and Yaphe 1988).

2.5.3 Extraction of Galactans from Terrestrial Plants and Mushrooms

Concerning terrestrial plants, different extraction methods exist, but as plants can contain several different polysaccharides (cellulose, hemicelluloses, pectin substances. . .), isolation of galactans generally require several steps to obtain a purified fraction. The first extraction step generally implies cold (Siddiqui and Wood 1972; Srivastava and Pande 1974; Gupta et al. 1979; Antonova and Usov 1984) or hot water (Mandal and Das 1980; Tomoda et al. 1990; Chen et al. 2008; Braz de Oliveira et al. 2013) as the easiest methods. Alternatives are the use of phosphate buffers (Tsumuraya et al. 1987), ammonium oxalate (Das et al. 1977; Stevens and Selvandran 1984; Mikshina et al. 2012), and sodium hydroxide. In some cases, comparative extraction with hot water and ammonium oxalate has shown modified composition of polymer mixture, with a proportion of uronic acids greater in the case of ammonium oxalate (Das et al. 1977). Palacios et al. (2012) have tested cold water, hot water, and hot aqueous NaOH extractions on fruiting bodies of the edible mushroom *Pleurotus ostreatus*. Polysaccharides recovered after alcoholic precipitation were analyzed, showing

that only the cold water fraction was constituted of α-(1,3)(1,6)-linked galactopyranosyl residues on a linear backbone, whereas the others consisted of glucose-linked units.

Because the extracted compounds are a heterogeneous mixture, homogeneous polysaccharides are further purified by various methods: treatment with Fehling solution (Unrau 1964; Braz de Oliveira et al. 2013), dialysis (Lahaye and Yaphe 1988), fractional precipitation with alcohol (Sarkar and Chatterjee 1983; Shimizu et al. 1990), and NaCl (Das et al. 1977) or $CaCl_2$ solutions (Mandal and Das 1980). But most of time, chromatographic separations are achieved, using various resins: ion-exchange resins (Blake et al. 1983; Tsumuraya et al. 1987) such as Sephadex (Sarkar and Chatterjee 1983; Shimizu and Tomoda 1987; Tomoda et al. 1990; Zhang et al. 2014) or DEAE cellulose (Siddiqui and Wood 1972; Das et al. 1977; Ghosh and Das 1984; Shimizu et al. 1990). In some cases, gel filtration separation has been applied, using Sepharose (Das et al. 1977; Blake et al. 1983) or Sephacryl (Blake et al. 1983; Tomoda et al. 1990).

2.5.4 Extraction of Galactans from Marine Invertebrates

Some galactans have been identified in animals, especially from marine organisms such as mollusks. Methods applied generally include a protease digestion step. For extraction of polysaccharides from ascidians (tunicates), Albano and Mourao (1986) incubated tissues with papain at 60 °C during 48 h. After centrifugation, polysaccharide was collected from supernatant by ethanol precipitation and dialyzed extensively against water. Similar protocol has been successfully applied to eggs and ovaries of sea urchins (Cinelli et al. 2010), leading to obtain sulfated L-galactans. To extract galactans from sponges, Esteves et al. (2011) have tested four methods. The most efficient one comprised a papain digestion step, followed by incubation with DNase during 24 h. After centrifugation, 10 % (w/v) cetylpyridinium chloride (CPC) was added to the supernatant to recover a precipitate. The resulting cetylpyridinium-polysaccharide complexes were dissolved in 2 M NaCl-ethanol (100:15, v:v) to obtain the corresponding sodium salt-polysaccharides, before precipitation with ethanol.

3 Biological Properties of Galactans

As described by Delattre et al. (2011), galactans from algae, plant, animal, and microorganisms could be considered as pharmaceutical key in human biological systems. As mentioned by Misurcova et al. (2012), there was increasing interest of functional food ingredients such as galactan for its putative health benefits during the last years. Generally speaking, lots of examples have related the biological activities of polysaccharides and particularly, SGs as antithrombotic (Mourao and Pereira 1999; Berteau and Mulloy 2003), antimetastatic (Coombe et al. 1987), antiviral (Harrop et al. 1992); anti-inflammatory (Berteau and Mulloy 2003), anticoagulant (Pereira et al. 2002), and antiadhesive (Berteau and Mulloy 2003) agents. The biological activities of SGs can be correlated to their chemical

composition and mainly to their sulfation patterns (Delattre et al. 2011; Misurcova et al. 2012). Due to their diverse health advantageous effects, a great agreement of significance has been observed by the people consumers toward natural bioactive molecules as practical food ingredients. Therefore, it can be recommended that bio-processed products from marine origin could be very interesting alternative sources as surrogates of synthetic food ingredients in order to provide health benefits and well-being to consumers. This paragraph gives an overview of our present knowledge about the potential application of galactans as bioactive molecules.

3.1 Antiviral Activities

All over the world, one of the most important causes of human death is the pathogenic virus infections (Kim et al. 2006). Even if lots of antivirus agents have been proposed and developed for treatment of viral diseases, there are needs of new natural ones because of synthetic drug resistance and bad side effect. As Kim et al. (2006) explained, the current searches for antivirus compounds focus more and more on polysaccharides from natural products with an important attention for marine algae. In this context, antiherpes virus galactans extracted from marine algae have been used and considered as potential therapeutical food antiviral ingredients (Kim et al. 2006).

The last years, several structural kinds of marine galactans such as carrageenan have revealed effective antiviral activities against viruses counting more especially: *i*) the human pathogen herpes simplex virus type 1 (HSV-1), (*ii*) the human pathogen herpes simplex virus type 2 (HSV-2), (*iii*) the dengue virus (DENV), (*iv*) human cytomegalovirus (RSV), (*v*) respiratory syncytial virus (RSV), and (*vi*) human immunodeficiency virus type 1 (HIV-1) (Carlucci et al. 1999; Gunay and Linhardt 1999; Talarico et al. 2004, 2005; De SF-Tischer et al. 2006; Carlucci et al. 1997b). It was clearly established by the studies of Chen et al. (1997) that these viruses could interact with glycosaminoglycan-type polysaccharides as heparan sulfate during the initial binding with the host cell. For that reason, some studies propose carrageenans as antiviral agents at the early event viral infection. De SF-Tischer et al. (2006) have shown that carrageenans derived from *Meristiella gelidium* (*Solieriaceae*) were more effective inhibitors of DENV than carrageenans from *Gymnogongrus griffithsiae* (Talarico et al. 2004) and other reference antiviral polysaccharides such as heparin and dextran sulfate 8000 (DS 8000). In the same way, studies have revealed the antiviral properties of lots of SGs as those of the red seaweeds: *Grateloupia indica* (Chattopadhyay et al. 2007), *Schizymenia binderi* (Matsuhiro et al. 2005), *Callophyllis variegata (*Rodríguez et al. 2005), *Stenogramme interrupta* (Cáceres et al. 2000), *Gymnogongrus torulosus (*Pujol et al. 2002), *Bostrychia montagnei* (Duarte et al. 2001), or even *Cryptonemia seminervis* (Mendes et al. 2014).

Nevertheless, according to literature (Damonte et al. 2004), galactans extracted from the red seaweed *Meristiella gelidium* could be considered as among the most effective antiviral sulfated polysaccharides against herpes virus.

Note to mention that another study revealed that galactan derivatives from terrestrial plants have been as well proposed as antivirus agents. In fact, for example, pectic arabinogalactan containing β-(1,6)-linked D-Gal*p* and extracted from leaves of *Stevia rebaudiana* has been described for its antiviral activities against herpes simplex virus type 1 (De oliveira et al. 2013). In addition, Dong et al. (2012) have proposed galactans from the edible plant *Basella rubra L.* as antiviral agents against herpes simplex virus type 2.

Therefore, the prospective of galactans as antiviral agents continues to be of considerable interest for therapeutical applications. Finally, it is also clear that as stated by Lee et al. (2004), in antiviral field using natural polysaccharides, there is a large diversity of structure correlated with antiviral potencies. Comparative researches on biological effect of polysaccharides have largely revealed that the antiviral activities appeared to be strongly associated with glycosidic linkage and monosaccharide composition as well as molecular weight and sulfate contents.

3.2 Antioxidant Activities

When we speak of human oxidative stress, we are talking about an extreme generation of reactive oxygen species called ROS. As a result, oxidative damage of macromolecules among nucleic acids, lipids, and proteins led to cellular tissue injuries (Halliwell and Auroma 1991). Usually, mainly organisms are able to protect themselves against adverse effects of oxidation. Nonetheless, for more serious oxidative stresses of human body, the innate defense mechanisms cannot be sufficient. In fact, as mentioned by Cuzzocrea et al. (2001), oxygen-derived free radicals such as hydroxyl radical and superoxide anion radical are well known to play an important role as mediators in the development of carcinogenesis and aging mechanism. With the aim to protect both food and human body against oxidative damages, antioxidant agents are used. These exogenous antioxidant molecules are necessary to regulate the amount of ROS (Cuzzocrea et al. 2001). Currently, synthetic antioxidants like tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), propyl gallate (PG), or butylated hydroxytoluene (BHT) are commonly employed in food industry (Qi et al. 2005). However, as related by Elboutachfaiti et al. (2011), BHA, BHT, and TBHQ are more and more restricted by legislation in food because of their possible impact in carcinogenesis. For this reason, the last years, there is a growing attention in using natural antioxidants. Among natural sources, marine algae represent one of the richest sources of bioactive compounds. One particularly interesting feature of marine algae is their richness in polysaccharides such as galactans. These polysaccharides have been largely described to be effective nontoxic compounds and have been confirmed to play a significant role as antioxidants and radical scavengers against oxidative damage in human living organisms (Zhang et al. 2003, 2004; Souza et al. 2007; Jimenez-Escrig et al. 2012). Generally speaking, antioxidant activities of polysaccharides have been evaluated by using diverse methods such as lipid peroxide inhibition, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical

scavenging, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP), or superoxide and hydroxyl radical scavenging assays (Ngo and Kim 2013).

In this context, lots of SGs from red seaweeds have been described for their antioxidant properties. For example, Costa et al. (2010) have highlighted the antioxidant effect of carrageenans from tropical seaweeds and more especially from the Rhodophyta *G. caudata*. In the same way, Souza et al. (2012) revealed the antioxidant activities of SGs from red seaweed *Gracilaria birdiae* and Zhang et al. (2003) studied the in vivo applicability of SGs from marine red alga *Porphyra haitanensis* in aging mice. In addition, other SGs from red seaweeds were investigated for their antioxidant capacity related to their sulfate amount (Jiao et al. 2011; Jiménez-Escrig et al. 2012). In another study, Souza et al. (2007) have investigated the in vitro antioxidant activities of iota (from *Eucheuma spinosum*), kappa (from *Eucheuma cottonii*), and lambda (from *Gigartina acicularis* and *G. pistillata*) carrageenans. It was shown that lambda-carrageenan was the highest antioxidant agent and free radical scavenger. It should be noted as well that the antioxidant properties of polysaccharide depend not only on degree of sulfation but also on the molecular weight. Indeed, previous works have shown that carrageenans and their oligomers have antioxidant properties (Abad et al. 2013). Moreover, it has been clearly demonstrated that the lower molecular weight (LMW) carrageenans exhibited better antioxidant abilities. Overall, it has to be noted that all these studies clearly show once again that the antioxidant activities of galactans strongly depend on the combination of sulfation as well as molecular weight.

Even now, many scientists still continue to isolate antioxidant polysaccharides since more recently, a sulfated galactan from the red macroalga *Mastocarpus stellatus* has been reported for its antioxidant ability (Gómez-Ordonez et al. 2014).

3.3 Anticoagulant and Antithrombotic Activities

As mentioned by Ngo and Kim (2013), the blood coagulation mechanism is well known as processed by factor of coagulation so as to end the flow of blood though the ill vessel wall during occurrence of an abnormal vascular condition. Therefore, since exogenous or endogenous anticoagulant agents interacted with the coagulation factor, the coagulation of blood can be blocked. Commonly, heparin, which is a glycosaminoglycan currently extracted from porcine tissues, was the most anticoagulant/antithrombotic drug used (Fareed et al. 2000). Nevertheless, heparin has been largely criticized for its limitations caused by collateral effects linked to the contamination of heparin preparation with chondroitin sulfate (Guerrini et al. 2008; Pomin and Mourao 2008). As related by Kishimoto et al. (2008), when heparin was administered by intravenous injection, the contamination by chondroitin sulfate induced a phenomenon of hypotension. Moreover, other side effects have been observed and described after administration of heparin as for examples: hemorrhagic effect, antithrombin deficiencies, or thrombocytopenia (Costa et al. 2010). Consequently, research has been investigated to discover new heparinoid-like

molecules as coagulators, and various galactans from marine algae have been described for their anticoagulant/antithrombotic properties (Farias et al. 2000; Fonseca et al. 2008; Pereira et al. 2002; Pomin and Mourao 2008). In that sense, different types of anticoagulant galactans have been extracted from macroalgae such as *Codium cylindricum* (Matsubara et al. 2001), *Botryocladia occidentalis* (Farias et al. 2000; Pereira et al. 2005), *Gelidium crinale* (Fonseca et al. 2008), *Grateloupia indica* (Sen et al. 1994), *Gigartina skottsbergii* (Carlucci et al. 1997a), *Schizymenia binderi* (Zuniga et al. 2006), *Lomentaria catenata* (Pushpamali et al. 2008), and *Porphyra haitanensis* (Zhang et al. 2010). Note to mention that in addition to SGs from macroalgae, other SGs with anticoagulant properties have been isolated from the egg jelly coat of sea urchin of *Echinometra lucunter* (Alves et al. 1997) or from the tunic of ascidians such as *Herdmania momus* (Santos et al. 1992) and *Styela plicata* (Mourão and Perlin 1987).

In a general way, as mainly revealed by Pomin and Mourao (2008), the activities of galactans were strongly mediated by heparin cofactor II as well as antithrombin. In some cases, a serpin-independent anticoagulant effect was exhibited, probably correlated to the fibrin polymerization inhabitation as supposed by Matsubara et al. (2001). Besides, in their study, Fonseca et al. (2008) have clearly shown that minor differences in sulfation level and sulfation distribution of algal galactans could lead to differences in their anticoagulant and venous antithrombotic activities.

Moreover, as mentioned by Pomin and Mourao (2008), the slight difference in sulfation pattern of galactans was described as critical for interactions between some activators, proteases, and inhibitors during coagulation, which could cause diverse patterns in antithrombotic activity as well as pro- and anticoagulant properties. For this reason, sulfated galactans possess procoagulant as well as anticoagulant effect depending on the sulfation distribution. Finally, from all these studies, new perspectives are opened for the development of galactans as pharmaceutical drugs in thrombosis field.

3.4 Anticancer Activities

Nowadays, cancer is considered as one of the first reason of human death, and globally, in the last decades, its incidence has doubled according to Amaro et al. (2013). Overall, cancer was classified as a disparity in the levels of cellular proliferation and cell apoptosis. The last one is well known as a biological mechanism which implicated several protein families such as caspases taking part in tumor growth at several stages of the carcinogenesis (Guedes et al. 2011). Inhibition of tumor cell mediated by specific protease activities led to the cell tumor suppression mechanism. More, the attenuation of tumor involved angiogenesis, immunostimulation, and cell apoptosis (Amaro et al. 2013). As related by Ehrke (2003), therapeutical methodologies using the chemotherapy technology was habitually used for the cancer treatment. Nevertheless, for the most part, anticancer agents currently employed are particularly cytotoxic. Consequently, lots of natural

compounds have been proposed for their beneficial effects as cancer-preventive agents (Ooi and Liu 2000; Schepetkin and Quinn 2006; Tzianabos 2000; Fedorov et al. 2013).

Then, the anticancer activities of sulfated galactans from red seaweeds have been recognized many times by in vivo and in vitro experimentations. For example, a sulfated galactan from the toxic marine dinoflagellate *Gymnodinium* sp. A3 (called GA3P) has been revealed as a potent inhibitor of specific enzyme such as DNA topoisomerase I and topoisomerase II (Umemura et al. 2003). Moreover, in their study, Umemura et al. (2003) have shown the growth inhibition of human cancer cell line by using this SG from these marine microalgae. For that reason, GA3P could be a good candidate for the development of anticancer chemotherapeutic agent. In recent times, several studies have reported that SGs from red macroalgae have inhibitory activity against tumor growing in mice as well as antiproliferative activity in cancer cell lines (Khotimchenko 2010). Furthermore, Lins et al. (2009) have proved the antitumor activities of SGs extracted from the red seaweed *Champia feldmannii*. A galactan from red algae *Porphyra yezoensis* could induce cancer cell death via apoptosis in a dose-dependent manner in vitro (Kwon and Nam 2007). In this work, it was particularly reported that when HT-29 colon cancer cells and AGS gastric cancer cells were cultured in presence of low concentration of purified galactans, the growth of cancer cells was inhibited by around 50 %. Note that low molecular weight (LMW) sulfated galactans from *Porphyra yezoensis* have been already reported to have immunoregulatory and antitumor activities (Osumi et al. 1998). In this study, LMW SGs were produced by enzymatic digestion of sulfated galactans using enzymes from *Arthrobacter* sp. and tested for their in vitro antitumor activities against WI-38 and VA-13 cells. It was then observed a very important antitumor activity against Meth-A fibrosarcoma and Ehrlich ascites. In addition, numerous works have reported that sulfated galactans such as carrageenans could have inhibitory activity of tumor growth in mice as well as interesting antiproliferative activity in cancer cell lines in vitro (Yuan et al. 2006; Zhou et al. 2006, 2004). In their studies, Zhou et al. (2004, 2006) investigated the positive effect of lambda-carrageenan from *Chondrus ocellatus* on the antitumor H-22 activity. Besides, Yamamoto et al. (1986) have already mentioned a considerable decrease in the impact of carcinogenesis after the oral administration of several red seaweeds. Indeed, they have shown the inhibition of growth of sarcoma-180 cells subcutaneously implanted into mice after dietary but also intraperitoneally injected red seaweed polysaccharides. On the other hand, Hagiwara et al. (2001) investigated the modifying effects of carrageenans on colonic carcinogenesis in male rats. Hu et al. (2006) have shown that low molecular weight carrageenans from the red alga *Kappaphycus striatum* may exert antitumor activities by immune system promoting on mice inoculated with a suspension of S180 tumors cells.

Finally, as already detailed above, galactans were largely described for their important antioxidant and free radical scavengers for the prevention of oxidative injuries which is one of the most important providers in carcinogenesis. Hence, these polysaccharides could be added as functional and active agents in food as cancer-preventive substances.

3.5 Antibacterial Activities

As related by Pierre et al. (2011), lots of microorganisms have adapted resistance against antimicrobial drugs. In fact, these microbial pathogens have developed specific multiple drug resistance during repeating treatment of infectious diseases. For that reason, there are strongly needs to find alternative antimicrobial compounds. Then, researches have been focused on the seaweed field and notably on the utilization of polysaccharides such as galactan from marine algae (Al-Haj et al. 2009; Kim et al. 2007).

According to literature, several seaweeds have antibacterial properties. For example, a galactan from the red algae *Hypnea musciformis* has been described for its antimicrobial activities against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Selvin and Lipton 2004). In addition, Yamashita et al. (2001) have revealed that commercial iota-carragenans showed significant growth inhibitory effect on *Salmonella enteritidis, Escherichia coli, and Staphylococcus aureus.*

In their study, Pierre et al. (2011) highlighted the antibacterial activities of a sulfated galactan from *Chaetomorpha aerea* which is a green filamentous alga from the French oyster ponds (Marennes-Oléron, France). Authors have investigated antimicrobial properties of galactans against some microorganisms, such as *Candida glabrata* (DSMZ 6425), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (CIP 103214), *Staphylococcus aureus* (ATCC 25923), *Salmonella enteritidis* (ATCC 13076), *Micrococcus luteus* (ATCC 4698), and *Bacillus subtilis* (CIP 5262). It was shown that this sulfated galactan from *Chaetomorpha aerea* was efficient only against *S. aureus*, *M. luteus*, and *B. subtilis*. So, these results confirmed the potential use of the green algae *Chaetomorpha aerea* as a source of new antibacterial agent. In the same way, iota-carrageenan extracted from the red algae *Eucheuma denticulatum* has shown interesting antibacterial activity against *Streptococcus pyogenes* and *Staphylococcus aureus* (Al-Haj et al. 2009). Additionally, in recent works, it was revealed that carrageenan was efficient in producing antibacterial defense when it was used as raw material in the edible food (Plotto et al. 2006, 2010; Bico et al. 2009).

Aqueous extract of red seaweed such as *Gracilaria chilensis*, *Porphyra columbina*, and *Gigartina skottsbergii* have been described by Jimenez et al. (2011) for anti-phytopathogenic activities against the fungal plant pathogenic strain *Phytophthora cinnamomi*. In their study, authors described that extracts from *G. chilensis* led to an inhibition of the *P. cinnamomi* growing in a dose- and season-dependent manner. Note to mention that this extract was not efficient against *Botrytis cinerea*. As suggested by the authors, the main effective components in these extracts were polysaccharides such as sulfated galactans. Besides, Amorim et al. (2012) have tested a sulfated galactan from the red seaweed *Gracilaria ornata* against the growth of bacteria including *Staphylococcus aureus, Enterobacter aerogenes*, *Bacillus subtilis*, *Salmonella choleraesuis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. In this study, data have clearly showed that the sulfated galactan from *G. ornata* inhibited only the *E. coli* growing. It should be noted that research continues in this area since very recently, Raposo et al. (2014)

observed that sulfated galactans from the red microalgae *Porphyridium cruentum* had significant ability to inhibit the growth of *Salmonella enteritidis* bacterium.

According to Pierre et al. (2011), several hypotheses have been proposed to explain why sulfated galactans are active against microbial strains: (i) the interaction of polysaccharides with specific target from the cell wall of the bacteria or (ii) the ability of the bacteria to modify the structure of the galactans. However, there are publications which suppose antimicrobial mechanisms may be linked to the binding properties of the sulfated galactans to positive regions of microbial membrane during the interaction step (Pomin and Mourao 2008; Pomin 2010; Amorim et al. 2012). Nevertheless, even if there is evidence that galactans could act as antibacterial agents, no real mechanism proofs have been confirmed up to now (Pomin 2010).

4 Physicochemical Properties

Carrageenans and agars are the generic names for a large family of gel-forming and viscosifying polysaccharides that are obtained commercially from red seaweeds (*Rhodophyceae*). These hydrocolloids are the most important polysaccharides exploited as texturing agents in food and in other industrial areas (Hoffman 2002; Campo et al. 2009). They can bind water, promote gel formation, and thicken, stabilize, and improve tastiness and appearance through interaction with other substances (Piculell 2006). The wide employments of these sulfated polysaccharides are essentially based on their properties to form strong aqueous gels (Rees et al. 1969; Lahaye 2001). It was largely defined that higher levels of 3,6-anhydro-α-L-galactopyranosyl units and lower sulfate contents were the major structural requirements for gelling (Lahaye 2001). Moreover, it is well known that modifications in the backbone of SGs allowed to greatly changes in their physicochemical and biological properties (Lahaye 2001).

4.1 Conformation, Properties in Solution, and Mechanism of Gelation of Agars

Agar, also previously named agar-agar, is extracted from red seaweeds. It is known from the seventeenth century in Japan for its gelling properties, and now farming is developed in Indonesia. Agar gels melt between 60 °C and 97 °C and solidify in the range from 30 °C to 45 °C. As described above, agar is a generic name and several kinds of compounds are recognized depending on sulfate, pyruvate, and 3,6-anhydro-α-L-galactopyranose contents. Gelation of agars is an exothermic phenomenon based on a coil-helix transition followed by aggregation of double helices each other. The gels, thermoreversible, are stabilized by cooperative hydrogen bonds. Agar is known to form a very porous gel. Gelation temperatures are related to sulfate but also methoxyl contents of polymers that adversely influence gelation. The alkali-treated agar showed higher melting and gelling temperatures

but also higher gel strength compared to non-alkali-treated ones (Rinaudo 2007). This phenomenon was explained by the hydrolysis of sulfate groups after conversion of (1,4)-linked galactose-6-sulfate to anhydrogalactose units. However, gel strength of agars decreased with an increase in the duration of alkali treatments as the 3,6-anhydrogalactose content decreased (Higuera 2008). Agars differ from other hydrocolloids by their high gel strength and their wide range of hysteresis (defined as the difference between the transition temperatures measured upon heating and cooling). Among agars, agarose is the better gel-forming polysaccharide and is the component responsible for gelation of agars. Agaropectin with higher sulfate content is a poorer gelling agent compared to it. The stronger gels are obtained with pure agarose, but they exhibit high levels of syneresis (separation of water). Even if agaroses are globally uncharged, they are sensitive to salts such as NaCl for lyotropic effects (increasing of H bonds) (Rinaudo 2007; Delattre et al. 2011).

As other polysaccharides and notably carrageenans, the texturing properties of agars can be modulated by their interaction with other components in complex matrix such as food media. High sugar concentration (glucose or sucrose) can increase gelling and melting temperatures of agars but also gel strength (Meena et al. 2006). Recent experiments clearly indicated a competition of the different carbohydrates for water (Russ et al. 2014). This phenomenon is called "sugar reactivity" by authors (Meena et al. 2006). The gelation of agars is also modified by interactions with other hydrocolloids. With locust bean gum (a galactomannan), a synergistic effect is observed with some agars increasing the gel strength (Lundin and Hermansson 1997) and leading to a more effective and economical use of these two polysaccharides. In contrary, the mechanical performances of films and gels obtained with mix of agar and starch and with agar and arabinoxylan blends are degraded compared to those obtained with agars alone (Phan The et al. 2009). Blends of agar with alginate give films that are more flexible and easy to manage than pure agar, but the strength of gels obtained with these blends decreased as a consequence (Laurienzo 2010). In the same way, the mixed gels composed of κ-carrageenans and agars were much more deformable, had a higher failure strain, and lower strength (Norzia et al. 2006). They can be stabilized by cross-linking with genipin, showing remarkable swelling capacity and solution stability in a wide range of pH (Meena et al. 2009). Agar does not show any protein reactivity and has a good acidic stability compared to other polysaccharides and notably to carrageenans. This property is a significative advantage for its use in dairy products where carrageenan would cause excessive flocculation due to its protein reactivity.

4.2 Conformation, Properties in Solution, and Mechanism of Gelation of Carrageenans

For several hundred years, carrageenans have been used as gellifying, thickening, and stabilizing agents in food (note that carrageenans has no food value) and food

industries in Europe and the Far East (Van de Velde and De Ruiter 2002). The first use of carrageenans started more than 600 years ago in Ireland in the village of Carrageenan where flans where made by cooking red seaweed in milk.

Carrageenans are water soluble, but their solubility depends on the type of carrageenans, temperature, pH, and type and concentrations of counter ions (McHugh 2003). The three most prevalent, and of highest commercial interest, carrageenans are iota, kappa, and lambda species, having different properties due to their high capacity of water retention and mechanical strength of their gels (Van de Velde and De Ruiter 2002). These three biopolymers are biodegradable and biocompatible.

The most soluble carrageenan is λ-carrageenan lacking hydrophobic 3,6 anhydrogalactose in its structure and having a high sulfate ester content. This carrageenan species is present in a random coil conformation and cannot form gel but can be used as a thickener.

In gelling applications, only κ- and ι-carrageenans form thermoreversible gels in aqueous solutions as the other carrageenans lack the essential $^1C_4$-conformation that results from the 3,6-anhydro bridge. κ-carrageenan sodium salt is soluble with difficulty in cold water, whereas κ-carrageenan potassium salt is only soluble in hot water solutions. It can occur in two forms: an unstructured random coil conformation at elevated temperature (above temperature around 60 °C called coil-helix transition temperature) and a structured double helix formed upon cooling. ι-carrageenan has an intermediate solubility, but as κ-carrageenan, it can occur in two forms. The main difference between ι-carrageenan and κ-carrageenan is their behavior upon cooling. At temperature above 60 °C, ι- and κ -carrageenans are always in the random coil conformation. Depending on the chemical characteristics of κ-carrageenan and of nature of its physicochemical environment, its coil-helix transition temperature is around 38 °C. In the presence of potassium ions, the helices of κ-carrageenan cluster together into bigger units where potassium forms electrostatic interactions with the sulfate groups and anhydro oxygen atom (Tako and Nakamura 1986). The properties of the obtained gels depend on the strength of these electrostatic interactions and then on the level of aggregation of double helices. This association explains also the hysteresis observed during rheological measurements of κ-carrageenan. The gelation of κ-carrageenan is also strongly promoted by other monovalent cations such as rubidium and cesium (Takemasa and Chiba 2001). κ-carrageenan gels are strong and brittle.

For ι-carrageenan, the coil-helix transition temperature is around 45 °C. Even if helices are formed below this temperature, they are not dependent of cations, and they do not form aggregates as suggested by the thermal conformational transition of ι-carrageenan showing no hysteresis. However, helices of ι-carrageenan can be bridged by calcium. In this case, the strength of the gel is related to the amount of calcium. Gels obtained from ι-carrageenan are weak and thixotropic (Van de Velde and De Ruiter 2002).

As described for agars, the texturing properties of carrageenans can be modified after interaction with other components in food systems. κ-carrageenan has a stabilizing effect on proteins resulting in mixed-gel large deformation behavior

which depends on physicochemical properties of the system. Protein addition increases gelation temperatures, melting temperatures, and storage moduli of carrageenan gels. Moreover, some proteins are protected by carrageenans which enhance their temperature of denaturation. The mechanism involved in this phenomenon is poorly known, but carrageenans could prevent interactions between hydrophobic regions of proteins and stabilize them after formation of hydrogen bonds. In milk, very low concentrations of carrageenans are required to obtain a gel. The synergistic effect of proteins and polysaccharides is related to the negatively charged polysaccharides and positively charged amino acids of caseins. Synergisms are also observed between κ-carrageenan and other polysaccharides such as galactomannans (locust bean gums). The mechanism at the origin of this synergism is based on the alignment of the non-branched mannan backbone regions of the galactomannan with the aggregates of the double helices of κ-carrageenan (Arda et al. 2009). These interactions lead to elastic gels with low syneresis compared to those of κ-carrageenan alone. Interactions with starch have been also described by authors. Carrageenan, as other polysaccharides, can interact with starches leading to a modulation of their gelatinization and retrogradation and then to their viscosity. The impact of carrageenans on starch is highly dependent to the type of carrageenan. Globally, carrageenans increased the apparent pasting temperature of starch and increase its apparent viscosity. Carrageenan was found to act as a protector for starch granules mostly against shear degradation (Appelqvist and Debet 1997). Funami et al. (2007) investigated the gelatinization and retrogradation of the aqueous starch/ι-carrageenan composite systems and their dependence on salinity. They explained the different behaviors as a combination of two phenomena: a similarity in structure between iota-carrageenan and amylose, leading to both a thermodynamic incompatibility and phase arrangements for the heating behavior, and physical interactions (both molecular complexes and steric hindrance) for the cooling behavior.

5 Conclusion

Often described as well-known macromolecules, galactans and sulfated galactans from various origins appeared as very complex polymers exhibiting a large diversity of biological activities and techno-functional activities. At this time, the rheological properties of sulfated galactans are well known and abundantly employed in industry. The future advances for this family of polysaccharides are probably a better understanding of relationships between their structures and the biological activities observed in various models. For this, progresses are necessary in the field of structural analysis but also in preparation of well-defined galactan sequences with controlled addition of noncarbohydrate groups and other substitution on the galactan backbone. These well-defined oligo- and polysaccharidic structures are not available in nature or only in small quantities after fastidious strategies of extraction, fractionation, and purification leading often to the collection of nonnative compounds. Indeed, their obtaining implies the development of

chemical and/or enzymatic tools to modify and/or synthesize galactans patterns. These galactan libraries could be then used in glycoarrays with a goal of building a systemic and biological approach of glycan structure-function relationships.

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Functional Components in *Zizyphus* with Emphasis on Polysaccharides

27

Tsai Hua Kao and Bing Huei Chen

Contents

Abstract

Zizyphus jujuba (Chinese jujube), mainly distributed in the subtropical regions of Asia, has been cultivated for over 4,000 years and used as food, food additive, flavor, as well as traditional Chinese herb, as it possesses analeptic, palliative, and antibechic functions. The fruit of jujube is quite popular due to high nutritional value and presence of many functional components, in which polysaccharide is the most important one. Polysaccharides, being essential constituents of all living organisms, are naturally occurring polymers of aldoses and ketoses linked together through glycosidic linkages. In recent years, polysaccharides of plant origin have emerged as a vital class of bioactive nature products; however, there is a lack of information regarding analysis and biological activity of jujube polysaccharides. Moreover, the composition of polysaccharides in jujube may vary with different species, growth environment, cultivar variety, and extraction and separation methods, which in turn may affect the overall biological activity. The objective of this manuscript is to review the current status associated with jujube polysaccharide, including nutritional and functional components; methods of extraction, purification, separation, identification, and quantitation; as well as biological activity.

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1 Introduction to *Zizyphus*

Zizyphus, a kind of spiny shrubs (Fig. 1), belongs to the Rhamnaceae family, with the leaves being alternate and entire. Several species of *Zizyphus* are deciduous, while the others are overgreen. The flowers are small and possess yellow-green appearance (Fig. 2). In general, the florescence is about from April to May, while the fruit maturation period is from July to September. Also, the fruits contain drupe and possess yellow-brown, red, or black appearance, with shape being globose or ovoid, size up to 6 × 4 cm, skin smooth or rough, glossy, thin but tough, and taste crisp and juicy, subacid to sweet (Pareek 2013). *Zizyphus* is mainly grown

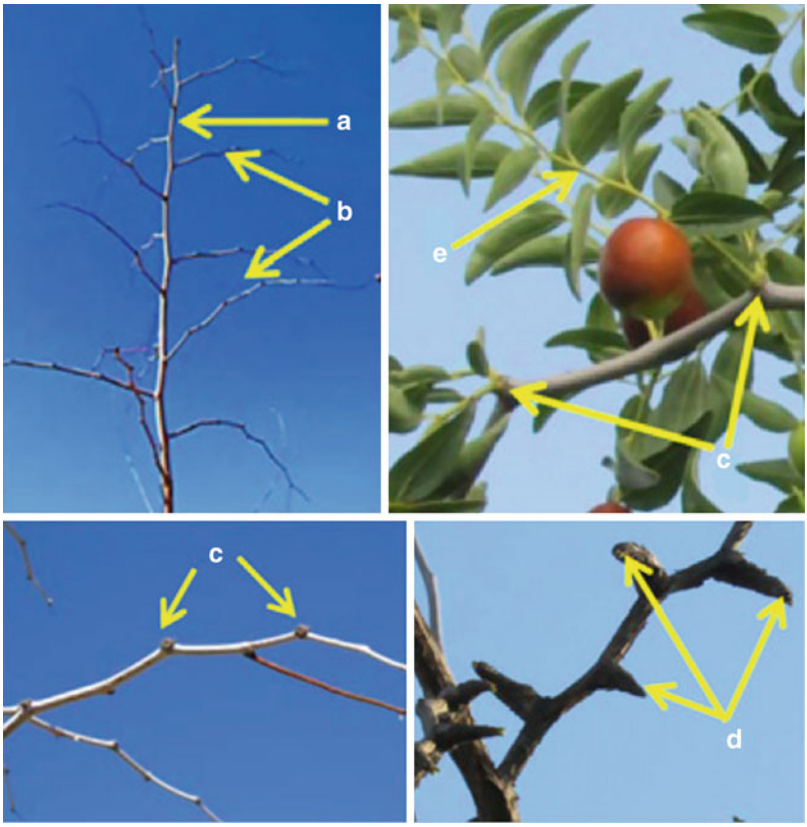

Fig. 1 Jujube shoot structure: **a** primary shoot, **b** secondary shoot, **c** mother-bearing shoot (young fruiting spur), **d** old fruiting spur, and **e** fruit-bearing shoot (branchlet) (Yao 2013)

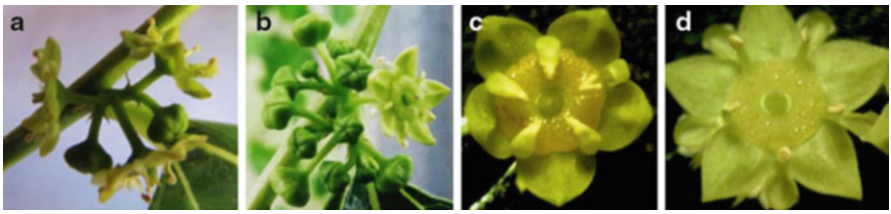

Fig. 2 Jujube flowers: **a** a simple cyme, **b** a large cyme, **c** a half-opened flower, and **d** a fully opened flower (Yao 2013)

Fig. 3 Changes in color with maturity and ripening of Indian jujube fruits (Pareek 2013)

in warm subtropic area, originally distributed in Asia, Africa, Europe, and America, possessing more than 700 species, including the most common *Z. jujuba* (jujube), *Z. spina-christi* from southwestern Asia, *Z. lotus* from the Mediterranean region, and *Z. mauritiana* from western Africa to India. In addition, *Z. joazeiro* is grown in Caatinga of Brazil, while *Z. celata* is listed as an endangered species in the United States (Wikipedia 2014). Botanically, *Zizyphus* is derived from its wild relative sour jujube or wild jujube. In ancient times, people selected and cultivated sour jujubes with big fruit and good flavor, which then gradually became the modern jujube species (*Z. jujuba*). Nevertheless, there are still semicultivated sour jujubes like "Tiger Eye" and "Yanjishan," both of which are popular in Beijing and Shandong Province of China, respectively (Guo and Shan 2010). Fresh jujube fruits are often consumed as fruits and can be processed into dried jujube, candied jujube (honey jujube), spirited jujube (drunk jujube), smoked jujube, roasted jujube, or jujube paste/filling (Yao 2013). Nonetheless, in Asia dried jujube fruits are often used as traditional herbs (Fig. 4).

China is the main production country of *Zizyphus*, with *Z. jujuba* being the major species, also named as Chinese date, red date, or zao, which has been grown over 4,000 years, and the production yield accounts for 90 % of total yield in the world. The cultivation area is widely distributed in mainland China, in which Gansu, Hebei, Henan, Ningxia, Shandong, Shaanxi, Shanxi, and Xinjiang produce the

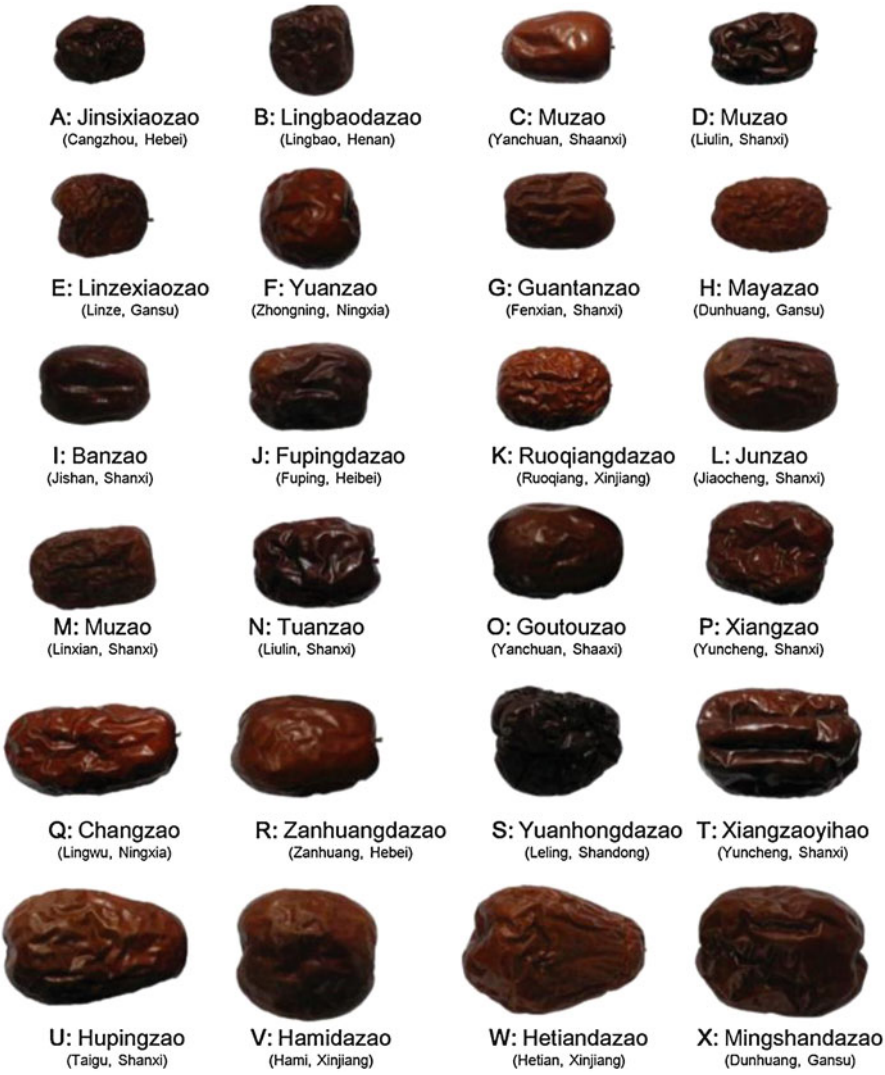

Fig. 4 The geographical locations and photographs of Chinese dates (Chen et al. 2013)

largest yield (Guo and Shan 2010; Yao 2013). Some other countries such as Korea and Japan also cultivate a small amount of jujube, and the main production area of jujube in Taiwan is located in Miaoli County. In addition, there is one more common species of *Zizyphus* named as *Ziziphi spinosi* semen (sour jujube seed), which is made from the dried seed of *Z. jujuba* var. *spinosa*. Although both jujube and sour jujube seeds are from the same species, the consumption mode and therapeutic efficiency can be varied greatly (Lee et al. 2012).

Jujube is often subject to direct sun-drying after maturation, or oven-drying until peel becomes soft followed by sun-drying, or boiling in hot water until pulp becomes soft followed by sun-drying (Wang et al. 2003). Jujube not only tastes sweet but also rich in nutrients, and thus it can be used as a common food and a vital drug source of the Chinese Materia Medica. According to the Divine Husbandman's Herbal Foundation Canon and the Compendium of Materia Medica, jujube possesses many important biological functions such as strengthening of the spleen and stomach, improvement of digestion and blood circulation, treatment of insomnia, and prolongation of life (Tsai 2004; Lee at al. 2012), all of which may be associated with the presence of functional compounds such as anthocyanin and polysaccharides in jujube (Lee et al. 2012). Additionally, in recent years, several studies have shown that jujube is protective against cancer, inflammation, allergy, and diabetes (Goyal et al. 2011; Lee et al. 2012). The traditional therapeutic effect of sour jujube is for treatment of insomnia and anxiety, as well as for pain-relieving and anticonvulsant (Guo et al. 2011; Han et al. 2009; Ma et al. 2008). As for functional components, they include alkaloids (sanjoinine A), flavonoids, saponins (jujubosides), and fatty acids (Cao et al. 2010; Ma et al. 2008; Zhao et al. 2006a).

2 Nutrients in Jujube

2.1 Basic Composition

According to the Chinese Academy of Agricultural Sciences, the basic composition of dried jujube is moisture (25–30 %), carbohydrate (55–80 %), crude protein (2.92 %), crude fiber (2.41 %), crude fat (0.96 %), phosphorus (0.09–1.27 %), potassium (0.61–1.05 %), calcium (0.03–0.06 %), magnesium (0.03–0.05 %), and trace minerals such as iron, manganese, copper, zinc, and selenium (Lei et al. 2006). Li et al. (2007a) determined the main composition of the five species of Chinese jujubes including Jinsixiaozao, Yazao, Jianzao, Junzao, and Sanbianhong, and moisture was found to range from 17.38 % to 22.52 %, carbohydrate from 80.86 % to 85.63 %, protein from 4.75 % to 6.86 %, fat from 0.37 % to 1.02 %, soluble fiber from 0.57 % to 2.79 %, insoluble fiber from 5.24 % to 7.18 %, reducing sugar from 57.61 % to 77.93 %, and ash from 2.26 % to 3.01 % (Table 1). Fructose and glucose are the major soluble sugars in all the five species, while rhamnose, sorbitol, and sucrose are present in a lesser amount. For minerals, potassium is present at 79.2–458 mg/100 g, calcium 45.6–118 mg/100 g, manganese 24.6–51.2 mg/100 g, and trace amounts of iron, sodium, copper, and zinc as mentioned above. Total phenolic contents are from 5.18 % to 8.53 mg/g (Li et al. 2007a).

According to a report by Pareek (2013), the jujube fruits contain 81–83 % moisture, 17.0 % carbohydrate, 0.8 % protein, 0.07 % fat, 0.76–1.8 % iron, 0.03 % each of calcium and phosphorus, 0.02 mg/100 g of carotene and thiamine, 0.020–0.038 mg/100 g of riboflavin, 0.7–0.9 mg/100 g of niacin, 0.2–1.1 mg/100 g of citric acid, 65–76 mg/100 g of ascorbic acid, 22 g/100 g of sugar, 1.3 g/100 g of

Table 1 Chemical component of five cultivars of Chinese jujube

Component (basis on 100 g of fresh weight)	*Zizyphus jujuba* cv.				
	Jinsixiaozao	Yazao	Jianzao	Junzao	Sanbianhong
Carbohydrate (g)	81.62 ± 3.12a	80.86 ± 3.55a	84.85 ± 1.83a	82.17 ± 1.94a	85.63 ± 0.96a
Reducing sugar (g)	57.61 ± 2.71c	60.24 ± 2.56c	77.93 ± 0.61a	58.73 ± 1.13c	67.32 ± 0.64b
Soluble fiber (g)	2.79 ± 0.24a	1.46 ± 0.19b	1.51 ± 0.11b	1.07 ± 0.22c	0.57 ± 0.03d
Insoluble fiber (g)	6.11 ± 0.30a	7.18 ± 0.16a	5.24 ± 0.13b	5.83 ± 0.49b	5.56 ± 0.28b
Lipid (g)	0.37 ± 0.01c	1.02 ± 0.05a	0.39 ± 0.02c	0.71 ± 0.07b	0.65 ± 0.03b
Protein (g)	5.01 ± 0.05d	6.86 ± 0.02a	4.75 ± 0.03e	6.43 ± 0.02c	6.60 ± 0.04b
Moisture (g)	18.99 ± 1.23ab	20.98 ± 1.12ab	17.38 ± 1.21b	21.09 ± 1.39ab	22.52 ± 1.43a
Ash (g)	2.26 ± 0.03e	2.78 ± 0.05b	2.41 ± 0.09d	3.01 ± 0.06a	2.56 ± 0.02c
Potassium (mg)	79.2 ± 5.2a	458 ± 5.0e	375 ± 6.3d	201 ± 2.7b	224 ± 3.2c
Phosphorus (mg)	110 ± 2.3e	59.3 ± 1.5a	72.3 ± 2.7b	105 ± 1.8d	79.7 ± 1.4c
Calcium (mg)	65.2 ± 3.2b	91.0 ± 2.2d	45.6 ± 2.1a	118 ± 3.1e	76.9 ± 1.9c
Manganese (mg)	39.7 ± 4.1bc	36.5 ± 1.1b	51.2 ± 2.8d	24.6 ± 2.5a	42.1 ± 1.6c
Iron (mg)	4.68 ± 0.11a	6.93 ± 0.22d	6.42 ± 0.12c	7.90 ± 0.13e	6.01 ± 0.21b
Sodium (mg)	6.34 ± 0.31c	7.61 ± 0.28d	6.21 ± 0.24c	5.96 ± 0.23b	3.22 ± 0.11a
Zinc (mg)	0.55 ± 0.04d	0.63 ± 0.03c	0.47 ± 0.06b	0.42 ± 0.01b	0.35 ± 0.02a
Copper (mg)	0.26 ± 0.04b	0.27 ± 0.01b	0.42 ± 0.02c	0.31 ± 0.05b	0.19 ± 0.02a
Selenium (mg)	nd	nd	nd	nd	nd

Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$) by Bonferroni *t-test* (Li et al. 2007a)

fiber, and 0.2 g/100 g of fat, with a caloric value of 104 kcal/100 g. Galactose, fructose, and glucose are the major sugars found in jujube fruit (Muchuweti et al. 2005).

Guil-Guerrero et al. (2004) analyzed several jujube varieties from Spain for fatty acid composition and found that triglycerides having medium-chain fatty acids were the most abundant in all samples. The main fatty acids included lauric acid (12:0), capric acid (10:0), linoleic acid (18:2 n6), palmitoleic acid (16:1 n7), palmitic acid (16:0), and oleic acid (18:1 n9) in total saponifiable oil. On an average, 1.3 g/100 g of saponifiable oil was found in fruit on a dry weight basis. In another study, San and Yildirim (2010) reported that jujube fruit also contained

methyl caproate (6:0), benzoic acid (6:3), methyl caprylate (8:0), ethyl hexanol (8:0), 2-octenoic acid (8:1), nonoic acid (9:0), benzenepropanoic acid (9:3), methyl caprinate (10:0), undecanoic acid (11:0), methyl laurate (12:0), methyl myristate (14:0), palmitic acid (16:0), *cis*-palmitoleic acid (16:1), *trans*-palmitoleic acid (16:1), stearic acid (18:0), 11-octadecenoic acid (18:1 n7), oleic acid (18:1 n9c), linoleic acid (18:2 n6c), linolenic acid (18:3 n3), and methyl heptacosanoate (28:0).

2.2 Functional Components

In addition to the nutrients mentioned above, jujube also contains some other functional components such as organic acid, carotenoid, nucleoside, vitamin, flavonoid, triterpenic acid, and polysaccharide, all of which will be discussed below. The general composition of jujube fruit is given in Table 2.

2.2.1 Organic Acids and Carotenoids

Citric, malonic, and malic acids were identified as the major organic acids in jujube fruit (Muchuweti et al. 2005). Carotenes were also found in jujube fruit; however, the contents were lower when compared to some other fruits. San and Yildirim (2010) analyzed four varieties of four jujube fruits, and β-carotene was present at a level from 7 to 35 μg/100 g based on fresh weight, whereas the amount of carotenes was from 4.12 to 5.98 mg/100 g of dry weight as reported by Guil-Guerrero et al. (2004). It was also shown that a much higher amount of β-carotene (15.6 mg/100 g) could be retained by freeze-drying (Gao et al. 2012).

2.2.2 Nucleosides and Nucleobases

It is well known that nucleosides and their bases (nucleobases) are involved in the regulation and modulation of various physiological processes in the body (Jacobson et al. 2002) and thus exhibit multiple bioactivities such as antiplatelet aggregation (Anfossi et al. 2002), antiarrhythmic (Conti et al. 1995), antioxidant (Virag and Szabo 2001), antiseizure (Schmidt et al. 2000), and antitumor (Kinahan et al. 1981). Jujube fruit is also a rich source of cAMP and cGMP, both of which possess the same physicochemical properties as pure cAMP and cGMP. The cAMP contents of jujube fruits ranged from 100 to 150 nmol/g of fresh fruit and 100 to 600 nmol/g of dried jujube fruit, with the cGMP content being 30–60 nmol/g of dried fruit and rose by 90-fold as the fruit ripened (Cyong and Takahashi 1982; Qu et al. 1987). Liu and Wang (1991) reported that cAMP in jujube cultivar Muzao was present in largest amount (303 nmol/g) based on fresh weight, which is the highest ever found in higher plants. Cyong and Hanabusa (1980) found the jujube cAMP content to be ten times higher than all the other fruits tested. According to a report by Guo et al. (2010a), both nucleosides and nucleobases are abundant in 49 varieties of jujube fruits, which ranged from 287.79 to 1239.23 μg/g. The contents of both cAMP and cGMP can be affected by species, climate, or geography. In a recent study, Chen et al. (2013) determined the chemical composition of 24 jujube fruits in mainland China, with uracil and cytidine being found in Hamidazao; uridine,

Table 2 Nutritional composition of jujube fruit (on fresh weight basis)

Constituents	Amount (per 100 g)	Ref
Moisture (g)	81.6–83.0	Pareek 2013
Protein (g)	0.8	Pareek 2013
Fat (g)	0.07	Pareek 2013
Fiber (g)	0.6	Pareek 2013
Carbohydrates (g)	17.0	Pareek 2013
Total sugar (g)	5.4–10.5	Pareek 2013
Reducing sugar (g)	1.4–6.2	Pareek 2013
Nonreducing sugar (g)	3.2–8.0	Pareek 2013
Ash (g)	0.3–0.6	Pareek 2013
Calcium (mg)	25.6	Pareek 2013
Phosphorus (mg)	26.8	Pareek 2013
Iron (mg)	0.76–1.80	Pareek 2013
Carotene (mg)	0.021	Pareek 2013
β-Carotene (mg)	0.007–0.035 (0.004–15.6 on dry weight basis)	Guil-Guerrero et al. 2004; San and Yildirim 2010; Gao et al. 2012
Thiamine (mg)	0.02–0.08	Pareek 2013; Li et al. 2007a
Riboflavin (mg)	0.02–0.09	Pareek 2013; Li et al. 2007a
α-Tocopherol (mg)	0.04–0.07	San and Yildirim 2010
Niacin (mg)	0.7–0.873	Pareek 2013
Citric acid (mg)	0.2–1.1	Pa reek 2013
Ascorbic acid (mg)	65.8–534.9	Pareek 2013; Yao and Yin 2006; Bi et al. 1990
Flavonoids (mg)	3,300	Lei et al. 2006
Fluoride (ppm)	0.1–0.2	Pareek 2013
Nucleosides and nucleobases (mg)	28.78–123.92	Guo et al. 2010a
Pectin (% dry basis)	2.2–3.4	Pareek 2013

xanthine, and guanine in Jinsixiaozao; cAMP and guanosine in Goutouzao; cGMP in Yuanzao; and adenine in Hupingzao.

2.2.3 Vitamins

Jujube fruits also contain many vitamins such as A, B complex, C, E, and P, in which both vitamins C and P are present in the largest amount (Yao and Yin 2006). Bi et al. (1990) analyzed the vitamin C content in 121 jujube cultivars and reported an average of 412 mg/100 g of fresh weight with fresh-eating cultivars containing a lower amount of vitamin C than drying cultivars. However, most vitamin C underwent degradation during sun-drying, and only about 10 % could be retained. Thus, the consumption of fresh jujube fruits should be much better than dried ones. In addition to vitamin C, vitamin P is also present at a high amount

(3,300 mg/100 g) in the pulp of jujube fruit. Conversely, thiamine, riboflavin and vitamin E are present at a lower amount, which equal 0.04–0.08, 0.05–0.09, and 0.04–0.07 mg/100 g, respectively (San and Yildirim 2010). Nevertheless, the consumption of one fresh jujube fruit per day can meet the daily requirement of both vitamins C and B complex for an adult as recommended by WHO (Pareek 2013).

2.2.4 Polyphenols and Flavonoids

According to literature reports, total phenolic contents in jujube fruits ranged from 5.18 to 8.53 mg/g, with *p*-hydroxybenzoic, caffeic, ferulic, and *p*-coumaric acids being the most abundant at a concentration of 366, 31, 20, and 19 mg/kg DW, respectively, whereas vanillic acid was present at a lesser amount of 2.5 mg/kg DW (Pareek 2013). Wang et al. (2010a) determined polyphenol compounds in five varieties of jujube fruits – Hamidazao, Muzao, Goutouzao, Jinsixiaozao, and Tanzao in Xi'An, China – and a total of six compounds including gallic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid, rutin, and quercetin were found, with caffeic acid and rutin being the dominant ones. Likewise, the polyphenol compounds in jujube fruits produced in Turkey were shown to contain catechin, caffeic acid, epicatechin, ferulic acid, rutin, *p*-hydroxybenzoic acid, and chlorogenic acid, with catechin and rutin being the major ones (San and Yildirim 2010). Similar outcome was also reported by Hudina et al. (2008) and Liu (2006). However, in a recent report, some more phenolic compounds such as gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, cinnamic acid, epicatechin, catechin, quercetin, and rutin were found in jujube fruits (Du et al. 2013). Also, a total of 24 varieties of jujube fruits were found to contain (-)-catechin, procyanidin B2, (-)-epicatechin, quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-β-D-glucoside, and kaempferol 3-*O*-rutinoside by Chen et al. (2013). In a later study, Liao et al. (2012) further reported the presence of several flavonoids including isospinosin, spinosin, 6‴-sinapoylspinosin, 6‴-feruloylspinosin, and 6‴-coumaroylspinosin in jujube fruits produced in China. In jujube seeds, the flavonoids such as spinosin, 6″-feruloylspinosin, 6‴-*p*-hydroxybenzoylspinosin, saponarin, swertish, and vitexin were present (Choi et al. 2011).

2.2.5 Triterpenic Acids

Triterpenic acids are widespread in plants in the form of free acids or aglycones for triterpenoid saponins, which have been reported to possess multiple biological effects such as anti-inflammatory, antimicrobial, hepatoprotective, antioxidative, and anticarcinogenic activities (Liu et al. 1998; Fan et al. 2004; Gbaguidi et al. 2005; Martin et al. 2009; Kalogeropoulos et al. 2010). According to a report by Lee et al. (2004), jujube fruits were found to contain 11 terpenoides, including colubrinic acid, zizyberenalic acid, alphitolic acid, 3-*O*-*cis*-*p*-coumaroyl alphitolic acid, 3-*O*-*trans*-*p*-coumaroyl alphitolic acid, betulinic acid, betulonic acid, 3-*O*-*cis*-*p*-coumaroyl maslinic acid, 3-*O*-*trans*-*p*-coumaroyl maslinic acid, oleanolic acid, and oleanonic acid. However, some more triterpenic acids such as ceanothic,

alphitolic, zizyberanal, zizyberanalic, epiceanothic, ceanothenic, betulinic, oleanolic, ursonic, and zizyberenalic acids were shown to be present in dried jujube fruits by Guo et al. (2009a, b). Apparently, the composition and amount of triterpenic acids in jujube fruits can be affected by species, growing season, as well as geographical and environmental conditions (Guo et al. 2010b). In addition, both jujubosides A and B are also present in jujube fruits, which have been demonstrated to possess biological functions such as anti-proliferation of cancer cells (Xu et al. 2014).

3 Polysaccharides in Jujube

3.1 Introduction to Polysaccharides

Polysaccharides contain neutral and acidic polysaccharides, both of which are composed of one specific monosaccharide or two to six different monosaccharides connected with each other through glycosidic bond. The degree of polymerization is about between 100 and 1,000, and the physical and chemical properties of polysaccharides can be varied with monosaccharide variety and bonding mode. In general, polysaccharides possessing biological activity often carry negative charge and contain galacturonic acid (Paulsen 2001). Polysaccharides are widely distributed in plant, animal, and microorganism tissues, with more than 300 polysaccharides being characterized in nature. There are two main functions of polysaccharides: one is the basic structural substance of tissues such as cellulose in the cell wall of plants and chitosan in crustaceans for maintenance of tissue strength; the other one is to provide energy such as starch in plant tissue and glycogen in animal. In addition, several polysaccharides derived from plants or fungi participate in the regulation of cell signal transduction for life maintenance. Among the various polysaccharides, polysaccharides from Chinese herb are of particular importance as they have been shown to be nontoxic in vivo and effective in inhibiting tumor growth, enhancing immunity and antioxidant activity, as well as lowering blood sugar.

3.1.1 Factors Affecting Pharmaceutical Activity

Bonding Mode of Main Chain

Most polysaccharides possessing antitumor activity are found to have 1,3-β-D-glucan linkage. Bohn and BeMiller (1995) pointed out that the inhibition percentage of 1,3-β-D-glucan-containing polysaccharides derived from fungi toward tumor growth could reach 99–100 %, while the inhibition percentage of the other polysaccharides was only 10–40 %. In an early study, Matsuzaki et al. (1986) combined curdlan (1,3-β-D-glucan) and cellulose (1,4-β-D-glucan) for the synthesis of β-D-glucan containing a D-mannosyl and D-arabinofuranosyl branch chain, which was shown to possess high antitumor activity. This outcome implied that the bonding mode of main chain could affect biological activity.

Molecular Weight

The size of polysaccharides is another important factor which can affect biological activity. For instance, polysaccharides with MW 9,000 Da of dextrose were reported to exhibit biological activity, which could decrease sharply when the MW was higher or lower than 9,000 Da (Wang et al. 2004). Likewise, the antitumor activity could lose completely when the MW of fungi polysaccharides decreased to 10,000 Da by sonication mode (Kojima et al. 1986). In a study dealing with the inhibition of sarcoma 180 cell, Maruyama et al. (1989) reported that polysaccharides from *fungal mycelia* with MW $>$ 10,000 Da did not show inhibition effect. Gao et al. (1997) also found that the anticoagulation activity of sulfated polysaccharides increased following a rise in MW. For enhancement of immunity, polysaccharides with MW in the range of 200–1,000 kDa were the most effective (Borchers et al. 1999). As controversial outcomes over the antitumor activity as affected by MW of polysaccharides have been reported in the literature, more researches need to be done in clarifying this issue.

Functional Group

The physiological activity of polysaccharides can also be affected by displacement position and amount of functional groups such as sulfated, acetyl, and alkyl groups. Most sulfuric acid-esterified polysaccharides are effective in antivirus, antitumor, and anticoagulation. When the O-3 position was bonded with acetyl group in a polysaccharide molecule, the highest antitumor activity was shown. However, when the O-5 position was bonded with acetyl group, the antitumor activity could diminish. Under the condition of complete acetylation, the antitumor activity could lose completely as more hydroxyl groups are exposed, resulting in a change of polysaccharide activity (Wang et al. 2004). The antioxidant activity of polysaccharides extracted form longan peel was studied by Yang et al. (2009), reporting that the scavenging effect toward DPPH and superoxide radicals could decrease following a rise in degree of methylation.

Monosaccharide Composition

A study on mushroom indicated that polysaccharides with monosaccharide composition such as glucose, galactose, mannose, xylose, arabinose, fructose, ribose, and glucuronic acid could have antitumor activity (Zhang et al. 2007). In a similar study, Manning and Gibson (2004) found that polysaccharides containing glucose, galactose, xylose, and fructose could promote growth of probiotics. Also, the scavenging effect of carbohydrates did not correlate with the type of intrachain linkages, molecular weight, or degree of polymer branching but did appear to correlate with monosaccharide composition of polymer. A portion of the activity seems to be associated with monosaccharides, as both dextrose and mannose exhibited weak free radical scavenging activity. However, polymers were significantly better free radical scavengers than the polymeric structure confers additional free radical scavenging ability (Tsiapali et al. 2001).

Configuration

In addition to the factors mentioned above, the biological activity of polysaccharides can also be associated with configuration. For instance, a triple helical structure formed for schizophyllan with high MW 90,000 Da could be responsible for biological activity (Kojima et al. 1986). This finding was supported by a study by Maeda et al. (1988), who reported a decline in antitumor activity following breakdown of triple helical structure of mushroom polysaccharide (lentinan). Ohno et al. (2001) further reported that polysaccharides possessing a triple helical structure of 1,3-β-D-glucan could regulate immune function. Conversely, compared to a triple helical structure, a single helical structure was shown to exhibit a higher biological activity (Saito et al. 1991). Thus, more studies need to be conducted in clarifying this issue.

3.2 Extraction of Jujube Polysaccharides

The extraction methods of polysaccharides from plant tissues have been well documented and can be different due to variation in structure, location, and MW. In general, prior to polysaccharide extraction, solvents such as ethanol, acetonitrile, or methanol can be applied to remove interfering substances such as lipids, pigments, or proteins.

In an early study, Hormadova et al. (1999) used hot water or alkaline to extract polysaccharides from *Salvia officinalis* by sonication. Luo et al. (2004) extracted polysaccharides from dried *Lycium barbarum* L., followed by grinding into powder (<710 μm), using chloroform–methanol (2:1, v/v) for defatting, refluxing with 80 % ethanol, extraction with hot water (90 °C), precipitation with 95 % ethanol, 100 % ethanol and acetone separately, centrifugation, and drying to obtain crude polysaccharide. Zhang et al. (2004) compared the effect of two extraction methods (100 °C hot water for 3 h and sonication for 2 h) on MW distribution and antitumor activity of polysaccharides from *Pleurotus tuber-regium* and observed a higher antitumor activity for the former method and a larger MW for the latter method. Obviously, the variety and biological activity of polysaccharides can be affected by extraction method.

By summarizing all the results in the literature reports, it can be concluded that the extraction of jujube polysaccharides is usually conducted by 80–95 % ethanol with refluxing for removal of lipids, proteins, monosaccharides, or pigments (Zhao et al. 2006a; Wang et al. 2012a; Li et al. 2013), followed by hot water (80–100 °C) extraction, precipitation with 75–95 % ethanol, centrifugation or filtration, and freeze-drying (Zhao et al. 2006a; Chang et al. 2010; Wang et al. 2012a; Li et al. 2013). In addition, some more new methods, such as application of enzyme, sonication, and flocculation for polysaccharide extraction, have been developed, which are described below.

3.2.1 Hot Water Extraction and Ethanol Precipitation

As mentioned above, the most common method used for the extraction of polysaccharides from plant tissue is to employ hot water for extraction, followed by

precipitation with ethanol, and centrifugation for collection. Thus, the extraction efficiency of polysaccharides can be affected by variety and volume of solvent as well as extraction time and temperature (Li et al. 2007b). In a study dealing with the extraction of jujube polysaccharides in Taiwan, Chang et al. (2010) used 75 mL of hot water (90 °C) for extraction for 6 h, followed by precipitate removal by centrifugation, collection of supernatant for concentration, addition of 95 % ethanol for polysaccharide precipitation for 5 h at 4 °C, protein hydrolysis by proteinase, dialysis, and column chromatography for fractionation into neutral and acidic polysaccharides. Several similar methods were also used by some other authors (Li et al. 2013). The main advantage of this method is that not only many impurities such as fat, protein, pigment, and monosaccharide can be discarded but also only simple equipment is required. Nonetheless, this method may suffer a partial loss of immunity-enhanced polysaccharides. Also, the yields of both acidic and high MW polysaccharides can be reduced, while solvent consumption can be too high and extraction lengthy (Chang 2008).

3.2.2 Alkaline Extraction

The extraction efficiency of high MW and acidic polysaccharides can be enhanced by alkaline treatment as its solubility in hot water is low. Several alkalis such as sodium hydroxide, potassium hydroxide, and sodium carbonate in a concentration ranged from 0.1 to 1 mol/L are frequently used for polysaccharide extraction followed by ethanol precipitation (Li et al. 2007b). Based on a report by Yang et al. (2004), who compared the extraction efficiency of polysaccharides from jujube waste by several alkalis, the following order was observed: sodium carbonate > calcium oxide > sodium hydroxide. Similarly, a high yield (12.7 %) of polysaccharide could be attained by employing sodium carbonate solution (0.5 mol/L) with extraction temperature at 80 °C, raw material and solvent ratio at 1:20 (g/mL), and extraction time 3 h (Hu and Chen 2011). Most importantly, the solvent concentration has to be carefully controlled for alkaline extraction as several polysaccharides can be degraded through hydrolysis under strong base condition. Additionally, long extraction time and high solvent consumption are the major drawbacks for alkaline extraction. To overcome this, polysaccharides should subject to neutralization or dialysis immediately after alkaline extraction (Li et al. 2007b).

3.2.3 Enzyme Extraction

Enzyme extraction is a new technique widely used for polysaccharide extraction from plant tissues in recent years. Compared to some other extraction methods, the condition of this method is much milder, and the release of polysaccharides can be faster (Li et al. 2007b). Also, the polysaccharide purity can be greatly raised as the major interfering components such as starch, pectin, lipid, and protein can be degraded (Chang 2008). Moreover, the extraction can proceed at lower temperature and energy due to decrease of activation energy (Chang 2008). Thus, the major drawbacks associated with polysaccharide extraction such as high solvent consumption, low purity, and low in polysaccharide extraction recovery can be

remedied (Hou et al. 2013). Nevertheless, enzyme is very sensitive to temperature and pH changes, as well as the presence of inhibitors, such as Cu^{+2}, Hg^{+2}, and Al^{+3}. Also, the extraction cost can be relatively high, making enzyme extraction more difficult for commercialization (Chang 2008).

Due to the presence of thick cell wall and abundant amount of pectic substance and protein in jujube fruits, the polysaccharide release can be more difficult. Thus, during extraction process, several enzymes such as cellulase, pectinase, and protease can be added to facilitate polysaccharide release (Shi 2006). There are several proteases including neutral protease, acidic protease, trypsin, and pepsin that can be selected (Shi 2006). Cellulase can be used to degrade cellulose in the cell wall, pectinase can hydrolyze high MW pectin to reduce viscosity caused by dissolution of mucilaginous materials in hot water, and protease can hydrolyze protein to facilitate release of polysaccharides (Shi 2006; Li et al. 2013).

Accordingly, enzyme extraction can be divided into single enzyme extraction and complex enzyme extraction. The former aims to select only one enzyme for a particular characteristic of plant tissue, while the latter can select more than two enzymes for enlargement of limitation in enzyme extraction (Shi 2006). As described above, a combination of enzymes including cellulase, pectinase, and protease is often applied for jujube polysaccharide extraction. In addition, there are several commercially available proteases containing a small amount of pectinase and amylase, which can decrease capital cost and enhance convenience in application.

There are only a few literature reports dealing with jujube polysaccharide extraction by enzyme. For single enzyme extraction, Yang and Sun (2005) used papain to extract jujube polysaccharide and reported that the highest yield could be reached with papain concentration at 0.3 % (w/v), extraction temperature at 70 °C, and extraction time at 3 h. A similar outcome was shown by Meng et al. (2006), who used papain to extract jujube polysaccharides with papain concentration at 0.5 mg/mL, extraction temperature at 55 °C, pH at 7, and extraction time at 2.5 h. Interestingly, Yang and Yan (2012) employed a different enzyme (pectinase) for jujube polysaccharide extraction and observed a high yield of 7.29 % with pectinase concentration at 0.05 g/L, extraction temperature at 52 °C, and extraction time at 45 min.

For complex enzyme extraction, Yang et al. (2003) compared the effect of five enzyme systems, i.e., pepsin, papain, trypsin, papain plus trypsin, and trypsin plus papain (trypsin first followed by papain), on the yield of jujube polysaccharides and reported that the most efficient extraction could be attained at pH 7, trypsin concentration at 3 % (w/v), and extraction temperature at 65 °C for 1.5 h, followed by addition of 2.5 % (w/v) papain and extraction at 55 °C for 1 h. In a later study, a total of 3 complex enzyme systems, i.e., acidic protease containing a small amount of pectinase and amylase, cellulase containing a small amount of pectinase and semi-cellulase, as well as pectinase containing a small amount of semi-cellulase and amylase, were compared with traditional aqueous extraction for jujube polysaccharide yield (Shi 2006). Results showed that the application of acidic protease

system is the most efficient as the extraction time could be shortened to be within 2 h with extraction temperature at 50 °C, protease concentration at 1 % (w/v), and pH at 3.2, while a polysaccharide yield of 4.9 % with a purity of 47.9 % was obtained. Comparatively, the extraction time can exceed 3 h for traditional aqueous extraction method. Likewise, a combination of acidic protease, cellulase, and pectinase was adopted by Shi et al. (2010) for jujube polysaccharide extraction, which was shown to be superior to hot water extraction under the condition of enzyme concentration 1 % (w/v), temperature 48 °C, pH 5.2, and extraction time 2.2 h, with a polysaccharide yield of 4.61% being attained.

3.2.4 Ultrasonic Extraction

The ultrasonic extraction efficiency is based on its high frequency, short wavelength, and strong penetration. Theoretically, ultrasonic belongs to longitudinal wave, which can produce numerous minute vacuum bubbles through pressure change in liquid during extraction. A phenomenon of cavitation can be produced by the explosion of these vacuum bubbles, which can generate great impact to enhance the extraction efficiency largely. Additionally, ultrasonic extraction can be regarded as a physical disruption process through high-speed vibration during ultrasonic extraction, which in turn results in instant breakdown of the cell wall to allow components to dissolve into solvents (Li et al. 2007b). Also, ultrasonic can produce heat to accelerate extraction efficiency (Sun et al. 2011). Many recent studies have shown that with application of high strength and energy of ultrasonic, many bioactive compounds such as alkaloids, flavonoids, polysaccharides, proteins, chlorophylls, and essential oils can be extracted from *Panax ginseng*, *Salvia officinalis*, *Coptis chinensis* Franch, *Gynostemma pentaphyllum* (Thunb.) Makino, *Lycium chinense* Miller, *Humulus lupulus*, *Cinnamomum camphora*, and peanut (Li et al. 2007b).

The advantages of ultrasonic extraction are to shorten extraction time, lower solvent consumption, increase yield of polysaccharides, prevent heat loss due to operation at low temperature, and avoid vaporization of bioactive compounds with low boiling point (Li et al. 2007b).

Yao et al. (2007) reported that with ultrasonic frequency 28 KHz, extraction time 2.5 h, and temperature 70 °C, the jujube polysaccharide yield was higher than that without ultrasonic treatment by 48.7 %. In another study, Sun et al. (2011) employed ultrasonic extraction initially, followed by enzyme treatment of jujube polysaccharides by papain, trypsin, and neutral protease, and reported a high yield (21.95 %) and purity (13.05 %) to be reached with frequency at 70 W for 25 min at 70 °C, 0.15 % (w/v) papain, pH 6, and extraction temperature 70 °C for 2 h. In a recent report, Qu et al. (2013a) studied the effect of ultrasonic extraction on the yield and antioxidant activity of jujube polysaccharides and found that the experimental data fitted the quadratic response surface model by multiple regression analysis. The largest yield of polysaccharides was obtained by ultrasonic frequency at 120 W, extraction time 15 min, and temperature 55 °C, whereas the highest hydroxyl radical scavenging activity was observed at 80 W, 15 min and 40 °C.

3.2.5 Microwave Extraction

Microwave is an electromagnetic wave, consisting of electric field and magnetic field which oscillate perpendicularly to each other in a frequency ranged from 0.3 to 300 GHz. Microwave energy can not only penetrate into certain materials and interact with polar components to generate heat but also act directly on molecules through ionic conduction and dipole rotation. Thus, only selective and targeted materials can be heated based on their dielectric constants (Chen et al. 2011). Ionic interaction denotes that foods contain water or some other salts which can be dissolved in water to form ions for attraction caused by difference in electric poles in electric fields. Then positive ions can move toward negative pole and negative ions toward positive pole. However, the formation of microwave energy can be dependent upon the production of alternating field. Thus, when there is a fluctuation in electric field, positive and negative poles can exchange each other which lead to migration of positive and negative ions in foods toward to a 180° direction difference, after which heat caused by friction can be generated. On the other hand, the polar water molecules belong to a V-shaped structure with a clip angle at 105°. Both the positive charge (H^+) and negative charge (O^-) in water molecules can be affected by fluctuation in electric field to produce rotation, the so-called dipolar interaction, which lead to heat generation and subsequent temperature elevation during rotation (Buffler 1999).

The major advantages of microwave extraction are reduction of extraction time through temperature elevation as well as moderation of capital cost and improvement of performance under atmospheric condition (Chen et al. 2011). The efficiency of microwave treatment depends upon dielectric loss (ε'') and dielectric constant (ε') of materials. The larger the dielectric loss, the higher the efficiency of microwave conversion into heat. Likewise, a higher dielectric constant of a material implies a better absorption capacity of microwave. However, due to the presence of more than one component in foods, the difference in ε'' and ε' between food components can result in non-homogenicity of microwave energy and thus more difficult to control. From the cellular point of view, the pressure generated through water evaporation during microwave cooking can disrupt cell wall for tiny pore formation. After successive heating, cells can undergo shrinkage and form wrinkle on surface, making the penetration of solvents into cells more readily to leach active substances out (Lu 2003). In a review report, Chen et al. (2011) pointed out that the factors affecting microwave extraction included solvent nature, solvent to feed ratio, microwave power and extraction temperature, extraction time and cycle, plant matrix characteristics, and condition of stirring. Many commercial extraction equipments are now available, and the manufacturers include Milestone, CEM, Sineo, etc.

Microwave extraction is basically a novel extraction technology and now applied to the extraction of bioactive components like polysaccharides. Shi et al. (2008) reported that the best extraction efficiency of jujube polysaccharides could be attained by mixing 20 g of jujube powder with 200 mL of water, followed by microwave extraction at 480 W for 4 min. The optimum pH range was from 6.0 to 7.2, and a polysaccharide purity (83.62 %) and yield (4.07 %) were obtained.

A higher polysaccharide yield (7.99 %) in jujubes could be reached by controlling material to solvent ratio at 1:50 (g/mL) and microwave energy at 420 W for 8 min (Han and Ma 2013).

3.2.6 Flocculation

Flocculation is a process wherein colloids come out of suspension in the form of floc or flake, either spontaneously or due to the addition of a clarifying agent (An 2006). The action differs from precipitation in that, prior to flocculation, colloids are merely suspended in liquid and not actually dissolved in solution. In the flocculated system, there is no formation of cake, since all the flocs are in the suspension. According to the IUPAC definition, flocculation is "a process of contact and adhesion whereby the particles of a dispersion form larger-size clusters" and is synonymous with agglomeration, aggregation, and coagulation/coalescence (IUPAC 2006). During flocculation, gentle mixing accelerates the rate of particle collision, and the destabilized particles are further aggregated and enmeshed into larger precipitates. Flocculation is affected by several parameters, including mixing speed, mixing intensity, and mixing time.

The advantages of flocculation process are low capital cost, high efficiency, simple operation, and management. It has been widely applied to wastewater treatment, mineral substance separation, molasses and protein recovery, as well as drug manufacture (Liu 2005). The most commonly used flocculating agents include chitosan, gelatin, sodium alginate, and some other commercial products such as ZTC1 + 1 and 101. Factors affecting flocculation efficiency include MW, dose, contact time, temperature, and pH. Chang (2008) compared the efficiency of various flocculating agents and found both chitosan and ZTC1 + 1 possessed the best clarification efficiency, while a high polysaccharide recovery was maintained. Nevertheless, more studies need to be done to strengthen its application.

3.2.7 Comparison of Various Extraction Methods

Lin et al. (2005) compared the extraction efficiency of jujube polysaccharides by hot water, sonication, and microwave, with the latter two shown to be fast, safe, convenient, low in capital cost, stable in components, and high in yield, which equaled 0.899 %, 0.982 %, and 1.137 %, respectively. Similarly, in a later study, Qi et al. (2012) compared the extraction efficiency of jujube polysaccharides by the same extraction methods, but a higher yield of 11.33 %, 12.40 %, and 10.98 %, respectively, was obtained. Comparatively, there is a large difference in polysaccharide yield even with the same extraction method, which can be due to the difference in hot water temperature, extraction time, sonication and microwave power, ratio of solvent to raw material, and species of raw material. More specifically, the factors affecting degree in extraction efficiency of jujube polysaccharides by hot water extraction were in the following order: pH > soaking temperature > raw material to solvent ratio > soaking time; by sonication, pH > time > temperature > power; and by microwave, soaking time > microwave time > pH. In addition, the polysaccharides extracted by hot water and microwave showed a similar hydroxyl radical scavenging capacity, while sonication showed the least capacity.

3.3 Purification of Jujube Polysaccharides

As mentioned above, the crude polysaccharides obtained by various extraction methods still contain some other impurities like proteins, mucilaginous materials, and small-molecule substances.

3.3.1 Protein Removal

Organic Solvent

Sevag method is the most common and mildest method to remove protein based on protein denaturation in the presence of organic solvents such as chloroform. A suitable amount of chloroform-*n*-butanol (4:1, v/v) can be added to polysaccharide extract, followed by shaking vigorously, settling, or centrifuging. At this time, denatured protein will form colloidal substance between organic solvent and water layer, after which protein can be removed to obtain polysaccharide extract. Alternatively, protein denaturation can be accelerated by lowering pH of crude extract to 4–5. However, this method suffers a major drawback of low efficiency and repetitive operation. Thus, some other methods can be adopted to remove most protein, followed by residual protein removal with Sevag method to minimize polysaccharide loss (Li and Zhou 2007).

In addition to chloroform, trichlorotrifluoroethane can be used to denature protein. However, this solvent cannot be used in large quantity due to its low boiling point and high volatility. Some other solvents such as trichloroacetic acid can also be used to denature protein; however, this solvent may cause polysaccharide degradation. It is worth pointing out that for the production of polysaccharide as functional food, the toxicity of organic solvents has to be taken into account.

Enzyme Hydrolysis and Solvent Addition

As mentioned above, protease can be used to hydrolyze protein in crude polysaccharide extract and peptide linkage (Peng and Zhang 2003; Liu et al 2006). The protein removal step can be omitted if enzyme hydrolysis is carried out in the beginning. The most commonly used proteases are pepsin, trypsin, and papain. The advantages of enzymes are high specificity, low toxicity, and prevention of polysaccharide degradation. However, the capital cost is high for enzyme treatment but may be compromised by employing immobilized enzyme for repetitive use.

3.3.2 Defatting

The fat content in crude polysaccharide extract is very low; however, some authors used petroleum ether or diethyl ether with Soxhlet extraction apparatus to remove fat and enhance polysaccharide purity (Chen et al. 2006). Usually, the fat removal step has to be conducted before extraction.

3.3.3 Bleaching

Jujubes also contain a large quantity of red pigments. According to a report by Lu (2003), the pigments in jujubes can be applied to food, cosmetics, or medicine as

coloring agent, but the pigment characteristics need further investigation. As aqueous solutions are often adopted for polysaccharide extraction, lipid-soluble pigments should only present in small amount. Most pigments belong to phenolic-type compounds and anthraquinone derivatives. Crude polysaccharide extracts often possess brown or brown red appearance, which make pigment extraction more difficult because of high viscosity of crude extract. The current bleaching methods include ethanol precipitation, active carbon adsorption, resin adsorption, and hydrogen peroxide bleaching. The principle of ethanol precipitation method is based on the repetitive use of ethanol until the precipitate color becomes pale (Li 2004). Active carbon adsorption has been applied to bleaching of many materials like fat or oil through physical adsorption amid its porous and high surface area characteristics; however, the residual powdered active carbon is difficult to remove. Also, active carbon can not only adsorb a portion of polysaccharides, but also a low adsorption efficiency is shown for pigments carrying negative charge (Lu 2003). Thus, in recent years, some other adsorbents such as weak base resin DEAE-cellulose, Duolite A-7 (Wang et al. 2010b), and macroporous resins (LSA-800B or AB-8) are used for pigment adsorption (Qiu et al. 2007; Liu et al. 2007). The bleaching efficiency of resin is good but only suitable for crude extract containing a low amount of pigments and with low viscosity; otherwise, the regeneration of resins will be more difficult (Liu et al. 2007). Some other drawbacks of resins are as follows: the capital cost is high, the use of a chromatographic column is a must, and a large solvent consumption can create a solvent residual problem. Hydrogen peroxide is highly soluble in water, and the bleaching effect is based on the release of ion HO_2^- ($ka = 1.78 \times 10^{-12}$) in water, with the bleaching efficiency even being better under alkaline condition. Nonetheless, the concentration of hydrogen peroxide has to be carefully controlled; otherwise, it can create a residual problem and affect the integrity of polysaccharide structure. Meanwhile, the hydrogen peroxide degradation into water has to be prevented (Lu 2003).

3.3.4 Desalting

After bleaching, dialysis technique is often used to remove salt and other small-molecule substances in crude extract by a semipermeable membrane with designated MW. Based on osmotic pressure principle, large molecules like polysaccharides can be retained inside the membrane, while small molecules like salt can migrate toward outside of the membrane (Feng et al. 2004).

3.4 Separation of Jujube Polysaccharides

The purity of polysaccharides can be enhanced by removal of impurities; however, difference still exists in chemical composition, degree of polymerization, and molecular shape of various polysaccharides in crude extracts. Thus, polysaccharides in jujubes have to be further separated by employing chromatographic techniques such as ion exchange, gel filtration, affinity, etc.

Most water-soluble polysaccharides are weak acidic, present as charge-carrying complex in aqueous solution. The separation principle of ion-exchange chromatography is based on the difference in polarity of solutes, showing different affinity toward ion-exchange resins. Thus, through change in ionic strength and pH of mobile phase, solutes can be separated in column. In practice, initially a suitable buffer solution pours into a column for elution to attain equilibrium, followed by pouring sample solution for adsorption with stationary phase, and the solutes can be separated successively through increase in ionic strength or change in pH of mobile phase. The separation of different solutes can be dependent upon characteristics, density, and surface distribution of charges, resulting in different degree of interaction with ion-exchange resins. Usually the solute with weak interaction can be eluted first. The most commonly used negative ion-exchange resins are DEAE-cellulose, DEAE-Sepharose CL-4B or CL-6B, and Toyopearl DEAE-650 M (Li et al. 2011a). For positive ion-exchange resin, the SP Sepharose can be adopted (Wang et al. 2010b). Mobile phase can be composed of water, sodium chloride, sodium acetate, sodium bicarbonate, or salt of boric acid. After separation, the eluates containing polysaccharides can be dialyzed, concentrated, and freeze-dried (Wang and Fang 2004).

Gel filtration chromatography (GFC) or gel permeation chromatography (GPC), a kind of size-exclusion chromatography, is applicable to high MW compounds. Packing material for GLC/GPC can be small (~10 μm) silica or polymer particles containing a network of uniform pores into which solute and solvent molecules can diffuse (Skoog et al. 2007). The various polysaccharides can be separated based on MW or size by GFC/GPC with quickness, high selectivity, and good repeatability. The polysaccharide with large MW can be eluted first, which is then measured via a calibration curve prepared from a series of standards possessing different MW (Wang and Fang 2004). In general, the stationary phases include Sephadex (G-100, G-75, G-50, or G-25), Sephacryl (S-200 or S-300), TSK (G5000-PW or G3000-PW), Toyopearl HW-65 F, Sepharose CL-6B, and Fractogel HW-65, whereas the mobile phase can be distilled water, 0.1–0.2 M NaCl, phosphate, etc. (Wang and Fang 2004; Li et al. 2011a; Gao et al. 2013).

In addition, affinity chromatography is regarded as an efficient method for purification of protein–polysaccharide complex, with ConA or Sepharose 4B column and water or sodium chloride solution as eluent being frequently used (Huie and Di 2004).

3.5 Qualitative and Quantitative Analyses of Jujube Polysaccharides

Compared to monosaccharides, disaccharides, and oligosaccharides, the analysis of polysaccharides is more difficult due to large molecular weight, complex structure, and inert chemical activation. Colorimetry with different chromogenic systems, such as carbazole–sulfuric acid, anthracenone–sulfuric acid, phenol–sulfuric acid, etc. can be applied to analyze total polysaccharides. However, the quantitative data

can be less accurate. For identification, several chromatographic methods are extensively applied to the compositional and structural analysis of polysaccharides when combined with some other detection methods such as laser induced fluorescence (LIF), electrochemical detection (ED), UV, IR, MS, NMR, etc. Thus, the structure of polysaccharides can only be elucidated by a combination of various techniques, which are described below.

3.5.1 Molecular Distribution of Polysaccharides by GFC/GPC

The MW distribution of polysaccharides can be determined by GPC or GFC based on the standard curve of polydextrin or some other polysaccharides with known MW (Yang et al. 2006). Chang et al. (2010) reported the average MW of polysaccharides in *Zizyphus jujuba* to be 129,518 and 46,740 Da for crude polysaccharide, 131,825 and 40,566 Da for deproteinated polysaccharide, 47,100 Da for neutral polysaccharide, and 55,522, 60,053 and 52,407 Da for three acidic polysaccharides obtained by column chromatography. This outcome is different from that reported by Zhao et al. (2006a), showing the average MW of the acidic polysaccharide fraction in Jinsixiaozao to be 2×10^6 Da, but a lower MW (1.4×10^5 Da) was reported by Li et al. (2007b). Apparently, the difference in MW distribution may be accounted for by variation in *Z. jujuba* species, extraction, purification, as well as separation conditions. In Chang's study (2010), a total of one neutral polysaccharide fraction and three acidic polysaccharide fractions were obtained, which should explain why a wide range of MW was observed.

3.5.2 IR

If the difference of rotational or vibrational energy level in a molecule conforms to infrared frequency, this molecule can absorb infrared light. This phenomenon can be used to analyze polysaccharide structure through absorption, reflection, and emission spectra. Generally purified polysaccharides can be dried, ground into powder, and mixed with KBr for compression into pastille for IR spectrum scanning.

Several researches have shown that the IR absorption spectra of jujube polysaccharides are as follows: 3,600 and 2,500 cm^{-1} refers to O-H stretching absorption due to inter- and intramolecular hydrogen bonds. The O-H stretching vibrations that occur within a broad range of frequencies indicate several features of a compound: free hydroxyl group stretching bands occur in samples in vapor phase and bonded O-H bands of carboxylic acid, the absorbance band at 3,414 cm^{-1} represented the stretching vibration of O-H in the constituent sugar residues, and the small band around 2,935 cm^{-1} refers to C-H absorption, including CH, CH_2, and CH_3 stretching and bending vibrations (Li et al. 2013).

The FT-IR spectra in the wave number between 850 and 1,200 cm^{-1} are regarded as the "fingerprint" region for carbohydrate, which is unique to a compound. These bands are usually difficult to interpret, but this region allows the identification of major chemical groups in polysaccharides as the position and intensity of these bands are specific to each polysaccharide (Cerna et al. 2003). The attributions of the main absorptions are characteristics of glycosidic structures

and related to CO stretching (1,097 cm^{-1} and 1,020 cm^{-1}) as well as anomeric C_1H group vibration (831 cm^{-1}). The small absorption bands at about 831 cm^{-1} in the spectrum can be associated with α-glycosidic linkage between the sugars units. The broader band of 1,149 cm^{-1} represents C-O-C and -OH in pyran structure. The bands representing ester carbonyl (1,760–1,745 cm^{-1}) and free carboxylate groups (1,640–1,620 cm^{-1}) are important in the identification and quantitation of polysaccharides. The bands occurring between 1,751 and 1,629 $cm^{-1}$are derived from the ester carbonyl (-COOR) groups and carboxylate ion stretching band (-COO-), respectively (Li et al. 2013).

According to Wang's study (2011), the absorption band at around 3290.20 cm^{-1} can be assigned to the stretching vibrations of hydrogen-bonded OH groups. The weak absorption bands at about 3,000–2,900 cm^{-1} are related to the C-H stretching vibration of free sugar. The absorption at 1616.20 cm^{-1} is assigned to the stretching vibrations of the CHO and C=O bonds. The broad absorption bands with strong intensities at 1,407.35, 1,342.96, and 1,254.35 cm^{-1} can be assigned to deforming vibrations of C-H bond. Similarly, the absorption bands at about 1,188.65, 1,143.50, and 1,025.43 cm^{-1} can be assignable mainly to the C-O-C stretching vibrations and C-O-H bending vibrations. The weak absorption bands at 917.28 and 865.20 cm^{-1} can be related to the C-H deforming vibrations in β-pyran ring, whereas the absorption at 816.57 cm^{-1} is related to the C-H deforming vibrations in α-pyran ring. Taken together, these outcomes suggest that Chinese jujube polysaccharides possess typical IR absorption peaks of polysaccharides. In a similar study, the IR spectra data for the identification of jujube polysaccharides were found as follows (Chang et al. 2010): all the polysaccharide fractions showed the same absorption bands at 3,600–3,200, 3,000–2,800, and 1,400–1,000 cm^{-1}, which are characteristics of stretching vibration of O-H, C-H, and bending vibration of C-H, respectively. Also, there are three absorption peaks present at 1,100–1,010 cm^{-1}, implying the presence of pyranose ring. A weak absorption peak at 1,250 cm^{-1} may be due to the stretching vibration of nonsymmetrical C-O-C. Likewise, the absorption peaks at 1,018 cm^{-1} and 1,146 cm^{-1} may be caused by stretching vibration of C=O and C-O-C, respectively. The α-glycosidic linkage in glycosyl residue may account for the absorption peak at 860 cm^{-1}. Another absorption peak at 1,250–950 cm^{-1} should be due to stretching vibration of O-H and C-O in pyranose ring.

3.5.3 Methylation

Methylation analysis has been used to determine carbohydrate structure for over a century, and today it is still the most powerful method in determining the sugar linkage of polysaccharides (Cui 2005). The methylated derivatization of a polysaccharide includes conversion of all free hydroxyl groups into methoxyls followed by acid hydrolysis, resulting in cleavage of the inter-glycosidic linkage in poly-methyl-ether while leaving the methyl-ether bonds intact. The hydrolyzed monomers are thus reduced and acetylated to give volatile products, such as partially methylated alditol acetate (PMAA), which can be identified and quantified by GC–MS. The substitution pattern of the *o*-methyl and acetyl groups in PMAA

reflects the linkage pattern and ring size of the corresponding sugar in the original polymer (Li et al. 2013).

In this technique, saccharides are reduced with sodium borodeuteride prior to per-*O*-methylation. Prior to per-*O*-methylation, the polysaccharides containing uranic acid residues are allowed to pass through a cation-exchange resin (H^+ form) in order to convert all the carboxyl groups into protonated form, thus permitting a more complete permethylation of polysaccharides containing uranic acid residues. High recovery of the per-*O*-methylated products can be attained by employing reversed-phase liquid chromatography after cleanup by a Sep-Pak C18 cartridge. Identification of microscale partially *O*-methylated alditol acetates by GC–MS with multiple, selected ion-mode electron-impact MS was found to be about sevenfold as sensitive as standard (Srivastava and Kulshreshtha 1989).

3.5.4 Mass Spectrometry

Following the development of new ion source like desorption ionization, mass spectrometry (MS) can be applied to analysis of nonvolatile, highly polar, and large MW components.

Methane and isobutane chemical ionization (CI) mass spectra of saccharides generally carry a large number of fragment ions; in the ammonia CI mass spectra, ammonia clusters and sample adduct ions are abundantly observed. Some other important features of the ammonia CI mass spectra are the higher stability of molecular ion adduct. Improvements in sensitivity and specificity have been reported by using methane chemical ionization MS and selected ion monitoring (Srivastava and Kulshreshtha 1989).

With field desorption mass spectrometry (FDMS), di- and trisaccharides give rise to $[M+H]^+$ and $[M+H\text{-}H_2O]^+$ ions with little fragmentation at a lower emitter current. A higher emitter current can be used to produce cleavage on either side of the glycosidic oxygens leading to sequence information. While the lower MW oligosaccharides can be analyzed without much difficulty, saccharides in the MW from range of 2,500 can be handled after partial methylation (Srivastava and Kulshreshtha 1989). Fast atom bombardment (FAB) MS and laser desorption (LD) MS are now in extensive use in the analysis of biopolymers such as polysaccharide. This approach utilizes a beam of projectiles or photons to ionize and desorb sample molecules deposited on a solid support (Srivastava and Kulshreshtha 1989).

3.5.5 NMR

The determination of anomeric configuration of the number of different sugars present in a repeating unit, the presence of amino and deoxy sugars, and the identification of *O*-acetyl groups and other nonsugar substituents are now routinely performed by ^1H and ^{13}C NMR spectroscopy, but the former can only provide limited information due to increase in size and complexity of molecules. Moreover, single broadening resulting from the decreased spin–spin relaxation time (T2) further adds to the intractability of the spectra. In the helical form, the C-3 signal of β-glucosyl moiety appeared at δ89, whereas in the random coil structure,

C-3 signal resonated at a higher field (δ86) in the ^{13}C NMR spectrum (Srivastava and Kulshreshtha 1989).

3.6 Sugar Composition in Jujube Polysaccharides

The average MW of neutral polysaccharides in jujube dates is about 23,000, which is mainly composed of arabinose, galactose, and glucose (Lei et al 2006), whereas the average MW of acidic polysaccharide is about 263,000, with the monosaccharide composition being rhamnose, arabinose, galactose, mannose, and galacturonic acid (Lei et al 2006). The monosaccharide composition can be analyzed by HPLC with refractive index (RI) detection, evaporative light scattering detection (ELSD), capillary zone electrophoresis combined with amperometric detection (CZE-AD), and thin-layer chromatography (TLC) (Zhao et al. 2006b).

In a study conducted by Yao et al. (2007), the monosaccharide composition of jujube polysaccharides after extraction by ultrasonic was shown to be L-rhamnose, D-fructose, glucose, D-galactose, and L-arabinose. In a later study, Chang et al. (2010) reported that after identification and quantitation by GC-FID, six monosaccharides including rhamnose, arabinose, xylose, mannose, glucose, and galactose were present in deproteinated polysaccharide at a molar ratio of 1:3.7:0.4:0.2:1:1.8, respectively. The same monosaccharides were also found in both ZJPa1 and ZJPa2 (2 acidic polysaccharides), with a molar ratio of 0.3:9.6:0.1:0.4:1:12.1 for the former and 3:16.8:1.2:0.2:1:12.2 for the latter. However, only 5 monosaccharides of arabinose, xylose, mannose, glucose, and galactose were present in neutral polysaccharide at a molar ratio of 0.3:0.2:0.2:1:0.7, respectively, as were rhamnose, arabinose, xylose, glucose, and galactose at 21:24:2:1:20 in ZJPa3 (one acidic polysaccharide). By comparison, galactose was the most abundant sugar in all the polysaccharide fractions. In several similar studies, Zhao et al. (2006a) reported a molar ratio of 2:1:1 for rhamnose, arabinose, and galactose, respectively, in the acidic polysaccharide fraction isolated from *Z. jujuba* cv. Jinsixiaozao fruits. But for the same fruit variety, Li et al. (2007a) reported the presence of rhamnose, arabinose, mannose, and galactose at a molar ratio of 13.8:4:3:8, respectively. This phenomenon implied that the monosaccharide composition might be different even for the same variety of *Z. jujuba*, which should be caused by the difference in extraction, purification, and separation conditions.

3.7 Physiological Activity of Jujube Polysaccharides

The pharmacological activity of jujubes has been well documented in many traditional Chinese herb books, with most functions focusing on improvement in insomnia, digestion, and blood circulation (Lee et al. 2012). In recent years, the researches on physiological activity of jujube polysaccharides have risen gradually. However, the scientific evidence associated with biological activity of jujube polysaccharides remains inadequate. Most jujube polysaccharide-related studies are

carried out in mainland China. The major challenge associated with biological activity research for jujube is the purity of polysaccharides. As noted before, even after extraction and purification, there are still many impurities present in polysaccharide fractions. Thus, it is possible that in addition to polysaccharides, some other functional components in jujube matrix may also play a vital role in maintaining biological functions. In view of this, more studies need to be done in terms of the relationship between function and structures such as position of activation and functional group, as well as metabolic route and products, so that the underlying mechanism on biological functions of jujube polysaccharides can be well elucidated.

3.7.1 Antioxidation

Compared to other research topics, the antioxidative activity of jujube polysaccharides has been studied more often. For instance, the antioxidative activity of hot water extract of jujubes was studied by Fan (1995), reporting the efficiency in inhibiting precursor formation of reactive oxygen species. Likewise, the activities of superoxide dismutase (SOD) and catalase (CAT) in mice blood could be raised by jujube polysaccharides, while the lipid oxidation product malondialdehyde in the blood, liver, and brain of mice could be lowered (Lee et al. 2005). In a later study, Chang et al. (2010) developed an appropriate analytical method for the isolation of polysaccharides from *Z. jujuba* fruits and evaluated their antioxidant activities *in vitro*. Results showed that all the four polysaccharide fractions including three acidic and one neutral polysaccharides were more effective in scavenging superoxide anions than hydroxyl radicals, while the acidic polysaccharides showed a more pronounced effect in chelating ferrous ion. Similarly, Li et al. (2011b) isolated four water-soluble polysaccharide fractions from *Z. jujuba* cv. Jinsixiaozao by DEAE-Sepharose CL-6B and Sepharose CL-6B columns and determined the free radical scavenging activity of each fraction. Data obtained in this study suggested uronic acid to be a reliable indicator of antioxidant activity. Also, the more the uronic acid in the structure, the higher the free radical scavenging activity. Wang et al. (2012b) measured the antioxidant activity of crude polysaccharide prepared from Z. jujuba cv. Shaanbeitanzao and reported a strong free radical scavenging effect that could prevent or ameliorate oxidative stress. More recently, Qu et al. (2013b) compared the antioxidant activity of jujube polysaccharides extracted by different methods and found that the polysaccharides extracted by ultrasonic showed a higher hydroxyl free radical scavenging activity than that extracted by hot water or microwave.

3.7.2 Immunomodulation

Many studies have pointed out that polysaccharides are effective in enhancing killing action of macrophage cells; inducing formation of TNF-α, IL-1, and NO; promoting stimulation of TNF-α by macrophage cells (Chang et al. 1999); and increasing anticomplementary activity (Yamada et al. 1985; Li et al. 2013).

Zhao et al. (2006b) isolated two pectic polysaccharides (Ju-B-3 and Ju-B-2) from fruits of *Z. jujuba* Mill. cv. Jinsixiaozao, with the latter showing a larger

proliferation effect of spleen cells at a higher dose (>30 μg/mL), whereas the former did not show any proliferation activity compared with control. From the structural point of view, rhamnogalacturonan and side chains (arabinan and galactan at O-4 of rhamnose residues) were proposed as the major contributors in stimulating immune responses. Li et al. (2011c) studied immunological activity of polysaccharide fractions from *Z. jujuba* cv. Jinsixiaozao (ZSP) and reported that the crude ZSP not only dramatically increased thymus and spleen indices in mice but also enhanced proliferation of splenocytes and peritoneal macrophages. Two fractions of ZSP (ZSP3c and ZSP4b) were believed to be responsible for immunological activity.

Zhang et al. (2013) purified four polysaccharides through protein removal and column chromatography, followed by addition of chlorosulfonic acid and pyridine under an optimal condition to obtain four sulfated polysaccharides $sOPS_{80}$, $sOPS_t$, $sJPS_{50}$, and $sJPS_t$, for comparison of immune-enhancing activity, with four unmodified polysaccharides as control. The *in vivo* finding showed that the sulfated polysaccharides could stimulate lymphocyte proliferation more effectively than their corresponding unmodified polysaccharides, with $sOPS_{80}$ possessing the highest efficiency. For in vivo experiment, the sulfated polysaccharides could also increase serum antibody titer. In a recent study, Hsu et al. (2014) studied the immunomodulatory effect of deproteinated polysaccharide isolated from *Z. jujuba* and observed a decline in IL-2 production in phytohemagglutinin-activated Jurkat T cells in a dose-dependent manner after 48 h incubation.

3.7.3 Hepatoprotection

Chang and Wu (2006) studied the effect of jujube polysaccharides on carbon tetrachloride-induced liver damage and exercise-induced fatigue in mice and found that with medium (200 mg/kg) and high (400 mg/kg) doses, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) decreased and the pathological change in liver tissue improved. Also, both muscle and liver glycogens rose in mice. With administration of high dose, the activity of lactic acid dehydrogenase (LDH) after exercise was elevated. Obviously, the intake of jujube polysaccharides can be protective against acute liver damage and fatigue. In a similar study, Wang et al. (2012b) showed that administration of the polysaccharides from *Zizyphus jujube* cv. Shaanbeitanzao significantly reduced the activities of CCl_4-elevated ALT, AST, and LDH in serum, as well as the level of hepatic malondialdehyde (MDA) level. Furthermore, mice treated with ZSP showed a better profile of hepatosomatic index (HI) and antioxidant system with normal glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities in the liver. All these findings suggested that jujube polysaccharides could be protective against CCl_4-induced hepatic injury through mediation of antioxidative and free radical scavenging activities.

3.7.4 Others

In addition to the findings shown above, some other studies have shown the neutral polysaccharides isolated from jujubes to be efficient in improving chest thickness,

increasing spleen lymphocytes in mice, and alleviating shrinkage and aging of immune organs in mice, as well as in antiaging (Miao and Shen 2001). Moreover, jujube polysaccharides could promote hemopoietic modulation system, stimulate proliferation and differentiation of hemopoietic stem cell and hematopoietic progenitor cells, and finally enrich blood (Xu et al. 2004). Also, the swimming time of mice could be extended through administration of jujube polysaccharides, and thus the antifatigue activity was enhanced (Wang 2009). Additionally, during exercise, jujube polysaccharides could provide energy source, avoid excessive utilization of protein, raise antioxidant activity, and elevate the amount of sugar stored (Wang 2009).

4 Conclusion and Future Perspective

Owing to modernization of extraction and analytical technique, the development of functional food with therapeutic efficiency has become a vital research area all over the world. Jujube polysaccharides represent an important class of health foods with potential therapy of chronic diseases. However, the studies dealing with biological activity of jujube polysaccharides remain inadequate. More researches need to be done in elucidating the relationship between physiological activity and polysaccharide structure. Also, new methods have to be developed to remove impurities and enhance polysaccharide purity. To overcome this, it is feasible to develop a botanic drug with jujube polysaccharide as raw material in the future.

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Chitosan-Based Edible Films

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Maher Z. Elsabee

Contents

Abstract

What is an edible packaging? An edible film or coating is simply defined as a thin film of edible material formed and sprayed on foods or food components. This package can be eaten as a part of the whole food product; it is also biodegradable, so if dumped it will disintegrate in reasonable short time.

Edible films and coatings offer extra advantages such as edibility, biocompatibility, esthetic appearance, barrier to gas properties, nontoxicity, nonpolluting, and having low cost (No et al. J Food Sci 72(5):87–100, 2007). In addition, biofilms and coatings by themselves are acting as carriers of food additives (i.e., antioxidants, antimicrobials) and have been particularly considered in food preservation due to their ability to extend the shelf life.

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This chapter will focus mainly on edible films based on chitosan – a wonderful amazing material which is derived from the naturally occurring polymer chitin.

Keywords
Chitosan • Chitosan blends • Starch • Essential oils • Antibacterial • Shelf life

1 Introduction

According to the US Environmental Protection Agency, 32 million tons of plastic wastes were generated in 2012, representing 12.7 % of the total municipal solid waste (MSW). In 2012, the United States generated almost 14 million tons of plastics as containers and packaging, about 11 million tons as durable goods such as appliances, and almost 7 million tons as nondurable goods, such as plates and cups. Almost every year 1.6 million metric tons of packaging waste is dumped into landfills in the United States. Packaging waste is app. 30 % of municipal waste by weight, and 13 % is due to plastic material which is not biodegradable. This dumped packaging includes mainly plastics which reduce moisture and O_2 transfer rate of soil and deteriorates the quality of land. Plastics are nonbiodegradable and they are the biggest threats to environment in present world. Edible package can be a partial solution to these environmental problems. Recently, considerable research has been conducted to develop and apply bio-based polymers made from a variety of agricultural commodities and/or of food waste products (Vásconez et al. 2009). Numerous review articles have described the nature of these films and their antimicrobial constituents, construction, and general effectiveness (Elsabee and Abdou 2013; No et al. 2007; Joerger 2007; Alvarez 2000; Cutter 2002, 2006; Appendini and Hotchkiss 2002; Quintavalla and Vicini 2002; Suppakul et al. 2003; Ozdemir and Floros 2004; Cha and Chinnan 2004).

This increased interest was intensified due to concerns about limited natural resources of the fossil fuel reserve and the environmental impact caused by the use of nonbiodegradable plastic-based packaging materials (Catarina et al. 2009). Such biopolymers include starches, cellulose derivatives, chitosan/chitin, gums, proteins (animal or plant based), and lipids (Cutter 2006). These materials offer the possibility of obtaining thin films and coatings to cover fresh or processed foods to extend their shelf life.

Chitin, a naturally abundant polymer, consists of 2-acetamido 2-deoxy-b-D-glucose through a β (1–4) linkage. In spite of the presence of nitrogen, it may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group (Abdou et al. 2008). Like cellulose, it functions as structural polysaccharides. Its natural production is inexhaustible; arthropods, by themselves, account for more than 106 species from the 1.2×10^6 of total species compiled for animal kingdom, constituting a permanent and large biomass source. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. Chitin is usually isolated from the exoskeletons of crustaceans and more particularly from shrimps and crabs where a-chitin is produced (Minke and Blackwell 1978; Austin et al. 1989). Squid is another important source of chitin in which

it exists in the β form which was found to be more amenable for deacetylation. It also shows higher solubility, higher reactivity, and higher affinity toward solvents and swelling than α-chitin due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains (Pawadee et al. 2003; Gardner and Blakwell 1975; Hunt and Elsherief 1990; Chandumpaia et al. 2004). The production of chitosan from crustacean shells obtained as a food waste is economically feasible, especially if it includes the recovery of carotenoids. The shells contain considerable amount of astaxanthin, a carotenoid that has so far not been synthesized and that is marked as a fish food additive in aquaculture. Chitosan itself was directly extracted from fungi by alkaline and acid treatment (Rane and Hoover 1993; Cai et al. 2006; Suntornsuk et al. 2002; Chatterjee et al. 2005). Some authors (Wang et al. 2006; Gagne and Simpson 1993; Oh et al. 2000; Yang et al. 2000) have developed methods to use microorganisms or proteolytic enzymes for the deproteinization of the crustacean chitin wastes; in this way a more economic production of chitin and chitosan can be achieved. The major procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong alkaline solution. Isolation of chitin itself from different sources is affected by the source. Generally the raw material is crushed, washed with water or detergent, and cut into small pieces. The mineral content of the exoskeleton of the different crustaceans is not the same, and consequently different treatments may be used.

The potential of chitosan to act as a food preservative of natural origin has been widely reported on the basis of in vitro trials as well as through direct application on real complex matrix foods (Coma et al. 2002, 2003; Durango et al. 2006; Han et al. 2004; Park et al. 2004; Ribeiro et al. 2007). Chitosan is also an excellent film-forming material (Domard and Domard 2001). Chitosan films have a selective permeability to gases (CO_2 and O_2) and good mechanical properties. However, the fact that chitosan films are highly permeable to water vapor limits their use as being an important drawback since an effective control of moisture transfer is a desirable property for most foods, especially in moist environments. Therefore, several strategies have been used to improve the physical properties of biopolymer-based films.

2 Chitosan

The incorporation of natural antimicrobial substances in the packaging film is an alternative way to reduce the chemical substances used in food preservation (Cissé Mohamed et al. 2013). These antimicrobial-enhanced packaging films can ensure the safety of food surfaces through controlled release of antimicrobial substances from the carrier film structure to food surface. The antimicrobial compounds and their incorporation into packaging materials have been well reviewed (Appendini and Hotchkiss 2002; Han 2000; Park et al. 2004). These films have advantages over the direct application of antibacterial agents onto food, because edible films can be designed to slow antimicrobial diffusion to the surface of food (Dawson et al. 2002; Sebti and Coma 2002). Therefore, smaller amounts of antimicrobial agents would

be needed in edible films to achieve a target shelf life, compared with direct application on the food surface. Cisse et al. have prepared chitosan films using dilute lactic acid as a solvent at pH 5.5 and added a lactoperoxidase system (LPOS) which consists of lactoperoxidase (LPO; 140 U/mg), glucose oxidase (GO; 158.9 U/mg), D-glucose (Glu), and potassium thiocyanate (KSCN), not potassium iodide. This system possesses a broad antimicrobial spectrum and showed a bactericidal effect on gram-negative bacteria and a bacteriostatic effect on gram-positive bacteria also, which has antifungal and antiviral activity. The mechanical properties of the films showed slight enhancement due to the incorporation of the enzyme system; however, the water vapor permeability, which is a key parameter to ensure the organoleptic qualities of food and the film capability to fight against dehydration or rehydration, was not improved with this modification. The chitosan films obtained containing different amounts of the peroxidase system were used for mango packaging and coating to protect them against pathogens. Its use can help preventing the loss of mango due to fungi and bacteria during storage and transport.

A comprehensive review dealing with the application of chitosan in food protection applications has been published by No et al. (2007). The application of chitosan in food industry is mainly due to its film-forming ability and its antimicrobial activity against a wide range of food-borne filamentous fungi, yeast, and bacteria. Three mechanisms were given to explain the antimicrobial mechanism of chitosan albeit being not very exclusive. The first hypothesis attributes the antimicrobial property of chitosan to a change in cell permeability due to interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes. This interaction leads to the leakage of proteinaceous and other intracellular constituents. Other mechanisms are the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis. Finally a chelation of metals and spore elements with chitosan results in depriving the cells from their essential nutrients, leading to their starvation and death. The review discussed the effect of chitosan on several food items 1- bread; applications of chitosan for extension of shelf life of baguette bread by retarding starch retrogradation and/or by inhibiting microbial growth. Park and others (2002b) coated the surface of dough with 0.5 %, 1.0 %, or 1.5 % chitosan in 1.0 % acetic acid using a brush after molding. The baguette, with 1 % chitosan, showed less weight loss, hardness, and retrogradation than the control during storage for 36 h at 25 °C, and the shelf life of 1 % chitosan coated was extended by 24 h compared to the control. In another experiment by Ahn and others (2003), it has been shown that bread coated with 1 % and 2 % chitosan (120 kDa, DD = 85 %) dissolved in 0.3 % lactic acid showed lower total bacterial counts and thiobarbituric acid-reactive substances (TBARS) and higher water content than those of the control after 8 days of storage at room temperature. Mold growth was detected in the control after 4 days of storage. Also, Lee and Lee (1997) reported that the shelf life of fermented pan bread containing carboxymethyl chitosan was extended by retarding retrogradation and by inhibiting growth of microorganisms. Therefore, the improved shelf life and quality of bread by chitosan coating or addition is attributed to moisture barrier property and ability to retard retrogradation and

microbial growth of chitosan. The review contains a compiled data of scientific publications reporting the antimicrobial activity of chitosan and its applications in food science. The second item in the review was the application of chitosan for the protection and extending the shelf life of eggs. It has been shown by several workers (Lee et al. 1996; Bhale et al. 2003; Caner 2005) that chitosan coating is effective in preserving the internal quality of eggs without affecting consumer acceptance. Using chitosan with different molecular weights in 1 and 2 % concentration in 1 and 2 % acetic acid with or without glycerol and coating eggs with brush or sponge or by immersion lead to reducing weight loss and obtaining more desirable albumen (higher Haugh unit) and yolk (higher yolk index) quality during a 5-week storage period. The Haugh unit and yolk index values indicated that the albumen and yolk quality of chitosan-coated eggs could be preserved up to 5 weeks at 25 °C, which was at least 3 weeks longer than observed for the control noncoated eggs. Caner (2005) observed that the albumen pH of chitosan-coated eggs (3 % in 1 % acetic acid) increased from 7.49 to 8.83, while that of uncoated eggs increased from 7.48 to 9.3 after 4-week storage at 25 °C. These results indicate that chitosan coating decreased carbon dioxide release through the shell by acting as a gas barrier. These results indicate that chitosan coating decreased carbon dioxide release through the shell by acting as a gas barrier. This provides evidence that the improved quality and extended shelf life of eggs by chitosan coating is attributed to the protective barrier properties of chitosan film for moisture and gas transfer through the egg shell.

The review discussed also the role of chitosan as a protective film to extend the shelf life of fruit and vegetable and provided several references dealing with this subject.

Among the interesting points in the review is the use of chitosan for extending the shelf life of milk, and a negative effect of chitosan was found; however, banana-flavored milk showed complete inhibition of microbial growth in contrast to that observed in control milk (without chitosan) during storage for 15 days at 4 °C and 10 °C. The banana-flavored milk containing chitosan also maintained relatively higher pH than that of control milk during storage for 15 days at both temperatures. The review deals also with many other items which should be inspected in more details for each individual subject for the interested researcher. A quick summary is given below: Kimchi is a traditional Korean fermented vegetable food, made mainly from Chinese cabbage with various ingredients such as garlic, red pepper powder, ginger, green onion, and fermented fish sauce. Several workers have reported that chitosan was effective in extending the edible periods of kimchi (No et al. 1995; Hur et al. 1997; Lee and Jo 1998; Yoo et al. 1998; Lee and Lee 2000). The shelf life of kimchi supplemented with chitosan oligosaccharide (0.005 %, 0.02 %, and 0.2 % in water) could be extended by 2–6 times compared to 2 days of the control kimchi at 20°. For mayonnaise, chitosan was found to increase the emulsifying capacity of egg yolk, and the addition of chitosan enhanced the emulsion stability of mayonnaise by 9.4 % compared to the control (Lee 1996). For meat, chitosan possesses antioxidant in addition to its antimicrobial properties (Kamil et al. 2002; No et al. 2002). The addition of chitosan to meat may retard and

inhibit the growth of bacteria and fungi during storage. Noodle and rice cake were found to half longer shelf life after treatment with chitosan solution with different concentrations and molecular weight (Lee and No 2002). Soybean curd (tofu) soybean sprouts were treated with chitosan solution, and it was found that this treatment extends the shelf life of the curd and increased the growth rate and weight of the sprout. The use of chitosan addition to sausage was found to be useful in reducing the amount of sodium nitrite which is usually used as a preservative and curing agent. However, the nitrite has a strong toxicant effect to human health (Park et al. 1999; Youn et al. 1999, 2000, 2001b).

The use of chitosan as an antimicrobial agent to extend the shelf life of starch jelly has been demonstrated by Moon et al. (1997). The shelf life of acorn starch jelly containing 0.5 % chitosan (44 kDa, DD = 75.2 %) dissolved in 1.0 % acetic acid was extended to 6 days at room temperature, twice longer than that of the control. The addition of chitosan to the acorn starch jelly formulation also increased its hardness compared with that of the control.

Since seafoods and seafood products are highly susceptible to quality deterioration due to lipid oxidation of unsaturated fatty acids, catalyzed by the presence of high concentrations of hematin compounds and metal ions in the fish muscle (Decker and Hultin 1992), the review devoted thorough and detailed studies regarding the use of chitosan as a protective coating and antioxidant to salmon and other seafood product. The main conclusion here is that the antioxidant efficacy of chitosan is highly dependent on the molecular weight of chitosan and its concentration (Kim and Thomas 2007; Peng et al. 1998; Kamil et al. 2002). The protective action of chitosan is also effective when it is applied as a protective film, where it retards lipid oxidation and microbial spoilage by acting as a barrier against oxygen (Jeon et al. 2002).

Another review dealing with antibacterial films used in packaging and food protection was also published in 2007 by Rolf D. Joerger. The review catalogues and analyzes the outcome of researches conducted in the last decade to develop and test films with antimicrobial properties to improve food safety and shelf life. It discussed the methodologies for measuring antimicrobial activity of both edible and inedible films. The article contains interesting data and comparison between edible and inedible films used as packaging materials carrying antimicrobial compounds. The data are represented as a pie diagram with figures representing the number of articles dealing with each specific type of polymer, in which chitosan occupied the largest number of articles describing its application as edible films with antimicrobial property. Many diagrams and figures were included in the review describing the use of particular antimicrobial compounds used in packaging films with antimicrobial applications and the organisms that were subjected to antimicrobial film tests which were inoculated into nonfood media or onto food surfaces and those that are indigenous to foods. Also the media described in the literature are reviewed in another pie diagram. Numerous figures were given compiling data regarding the antimicrobial efficacy for the different antibiotics and natural materials used in corporation with packaging films both edible and inedible.

The effect of chitosan coating as a barrier to gases, water vapor, solutes, and microbiological safety has been demonstrated by covering minimally processed

garlic with agar–agar-based (1 %) coatings incorporated with 0.2 % chitosan and 0.2 % acetic acid (Geraldine et al. 2008). Filamentous fungi and aerobic mesophilic were inhibited on the coated garlic cloves. Coated garlic cloves had a respiration rate halved compared to the noncoated garlic cloves. Water vapor transmission was lower for the films added with chitosan. The uncoated garlic samples turned brown rapidly due to the effect of polyphenol oxidase enzyme that oxidizes the phenolic compounds in the garlic in the presence of O_2, causing browning in tissues.

Coating has probably reduced the O_2 concentration around the clove tissues, making it unavailable for the browning reactions (Geraldine et al. 2008). The data obtained by the authors indicated that coating of garlic leads to a reduction of log (UFC g^{-1}) from 10^{-8} to 10^{-5} after 9 days of storage at 25 °C as compared to the uncoated samples. No noticeable effect of coating has been found regarding mesophilic count.

The authors concluded that the active coatings with chitosan and agar–agar can guarantee the quality of minimally processed garlic for an extended period, especially with respect to microbiological aspects; ensured lower color variation, moisture loss, and respiration rate; and prolonged shelf life (Geraldine et al. 2008).

Chitosan (CH) and chitosan lactate (CL) have been used in a powder coating technique to preserve and extend the shelf life of shredded radish (Pushkala et al. 2013). The effects of the two forms of chitosan as a powder coating on the physicochemical parameters, bioactive compounds, microbial quality, consumer acceptance, and marketability of radish shreds stored in macro-perforated LDPE packages have been investigated. Radish shreds were spread as a thin layer in polypropylene trays. Chitosan powder 0.2 % was applied uniformly over the surface of the shreds, followed by thorough mixing to ensure adequate coating.

The physiological loss in weight (%) of radish shreds stored for 10 days at 10 °C for the untreated control was found to be 8–10 %, while that for the coated samples with 0.2 % purified CH and CL was 4–6 % which was attributed to the ability of chitosan to act as water vapor barrier preventing the migration of moisture to the environment. Vitamin C content of 147.5 mg/kg was recorded in all the radish shreds samples immediately after processing. A gradual decrease in ascorbic acid content during storage was observed, which was attributed to the stress induced during shredding operation. The CH- and CL-treated samples exhibited 53 % and 72 % higher vitamin C content, respectively, compared to the control after 10 days of storage. Better retention of vitamin C has been also demonstrated in chitosan-coated fresh-cut mango (Chien et al. 2007), chestnut, and pear (Pen and Jiang 2003; Xiao et al. 2010). Mango pulp is very perishable and so has a short shelf life, which both marketers and consumers would like to be longer. Manually sliced mango was treated with aqueous solutions of 0 %, 0.5 %, 1 %, or 2 % chitosan; placed into plastic trays; and over-wrapped with PVDC film and then stored at 6 °C. Chitosan coating retarded water loss and the drop in sensory quality, increasing the soluble solid content, titratable acidity, and ascorbic acid content. It also inhibited the growth of microorganisms.

This has been related to the reduced respiration rate as a result of coating treatment (Jiang and Li 2001). An association was observed between the bioactive

compounds and antioxidant activity, with the chitosan-coated samples recording higher bioactive compounds and correspondingly higher antioxidant activity especially on the fifth and tenth day of storage.

Chitosan-coated samples, CH and CL, exhibited a lower microbial mesophilic aerobic bacterial count and yeast and mold count in the samples during storage. On the seventh day of storage, the microbial load was found to increase to 6.74 log CFU g − 1 in control samples, whereas in both CH and CL samples, 2 log reductions in microbial counts (4.3 and 4.6 log CFU g − 1) were witnessed, respectively. Application of chitosan coating also led to a significant reduction in the yeast and mold count. An increase in the yeast and mold population was observed in control samples on the 7 days (4.4 log CFU g − 1), whereas treatment with chitosan brought about a 2 log decrease in the yeast and mold numbers.

The authors concluded finally that both CH- and CL-coated samples showed comparable results with regard to physicochemical parameters, bioactive compounds, microbial quality, and sensory acceptability throughout the 10 days storage period, significantly better than the control. Chitosan coatings were also beneficial in maintaining a higher product quality during the storage period. Hence, a powder coating technique using chitosan biopolymer and macroperforation is proposed as a practical method to preserve the quality and to extend the shelf life of fresh-cut radish (Pushkala et al. 2013).

Chitosan was successful in inhibiting the growth of *Pe. chrysogenum*, *Fusarium oxysporum*, and *Aspergillus parasiticus* in in vitro study; chitosan coating also gave good results for table grapes and honey-cut melons against *F. oxysporum* growth. There were no significant differences between 1.5 % and 3.0 % concentrations of the coating, and better hunter color values were obtained from fruits with chitosan coating. It was concluded that 1.5 % of chitosan coating may be considered as an optimum edible coating concentration in the preservation of cut fruits or fruit salads (Irkin and Guldas 2013).

The effects of chitosan edible coating on microbiological and sensory quality of refrigerated broccoli were studied by Moreira et al. 2011. The antimicrobial effects of chitosan on the native microflora (mesophilic, psychrotrophic, yeast and molds, lactic acid bacteria, and coliforms) and on the survival of *E. coli* O157:H7 inoculated in broccoli were evaluated. Chitosan treatments resulted in a significant reduction in total mesophilic and psychrotrophic bacteria counts with respect to the control samples during the entire storage period. Chitosan coating inhibited the growth of total coliform throughout the storage time. Also, chitosan treatments resulted in a bactericidal effect on endogenous *E. coli* and a significant decrease in total *E. coli* counts (endogenous and O157:H7). The application of chitosan coating on fresh-cut broccoli inhibited the yellowing and opening of florets.

Chitosan CH was also used to protect white leg shrimp from microbial spoilage and endogenous enzymatic browning during 0 ± 1 °C storage and could prolong shelf life up to 10 days (Huang et al. 2012). Carboxymethyl chitosan (CMC), a water-soluble derivative of chitosan, can keep working against spoilage bacteria throughout 10 days storage and thus extend shelf life of shrimp. Both CMC and CH can be applied onto the shrimp surface then slowly diffuse into the shrimp

after the treatment. However, CMC can be less efficient on melanosis that maybe because it gradually dissolved under high-humidity storage in fridge. Either CH or CMC and refrigerated storage have a synergistic effect on extending shelf life of white shrimp. The authors of this work recommend more research on soluble, compatible, antimicrobial, and antioxidative CH derivatives and foresee a growing interest in natural potential bio-preservatives in the future.

In another study chitosan-based solutions were applied on frozen salmon as edible coatings and water glazing preservation by Soares et al. (2013). For this purpose, three chitosan solutions (0.25 %, 0.50 %, and 0.75 % w/v) and water were applied in different amounts (6 %, 8 %, and 11 % of coated fillet weight) directly on the surface of frozen salmon. The results obtained showed that chitosan coatings can be a good barrier to protect frozen fish from deterioration. Microbial growth, assessed by total viable counts (TVC), and total volatile basic nitrogen (TVB-N) were maintained below the maximum limits recommended which are 5×10^5 CFU/g and 35 mg nitrogen/100 g fish, respectively. The use of 0.50 % and 0.75 % chitosan solutions generally demonstrated to be more efficient in preventing salmon weight loss (Soares et al. 2013).

Listeria monocytogenes, a gram-positive rod, is a bacterium that can cause illness brought about by a variety of contaminated food products. Eating foods contaminated with *L. monocytogenes* normally causes the disease listeriosis which is more serious for elderly adults and adults with compromised immune systems and can cause meningitis (Roberts and Greenwood 2003). In pregnant women, the disease may cause spontaneous abortions or stillborn babies (Anonymous 2003).

Beverly et al. (2008) evaluated the antimicrobial effect of chitosan (high or low molecular weight), as an edible film, that was dissolved in lactic acid or acetic acid at 0.5 % (w/v) or 1 % (w/v) against *L. monocytogenes* on RTE roast beef. The roast beef samples were dipped into the chitosan solution, and the bacterial counts were determined on days 0, 7, 14, 21, and 28. It was found that on day 14, *L. monocytogenes* counts were significantly different for all the chitosan-coated samples from the control counts by 2–3 log CFU/g and remained significantly different on day 28. Their data have shown that the acetic acid chitosan coating was more effective in reducing *L. monocytogenes* counts than the lactic acid chitosan coating. Hence chitosan coatings could be used to control *L. monocytogenes* on the surface of RTE roast beef (Beverly et al. 2008). The use of the edible coating containing chitosan preserved the overall visual quality and reduced surface whiteness of carrot sticks during storage. Microbial populations were very low and not influenced by coating or modified atmosphere packaging (MAP) (Adriano et al. 2009).

The conclusion of this work was that the combined application of edible coating containing chitosan and moderate O_2 and CO_2 levels could maintain the quality and enhance the phenolic content in carrot sticks (Adriano et al. 2009).

A quite high efficiency of chitosan in food preservation was demonstrated by Mohan et al. (2012) by inhibiting bacterial growth and reducing the formation of volatile bases and oxidation products of Indian oil sardine (*Sardinella longiceps*) in iced condition significantly. Chitosan with high degree of deacetylation (83 %) in

1–2 % concentration was used in this study. The chitosan coating improved the water holding capacity, drip loss, and textural properties significantly compared to untreated sample. The eating quality was maintained up to ~8 and 10 days for 1 and 2 % chitosan-treated sardine, respectively, compared to only 5 days for untreated samples (Mohan et al. 2012).

An interesting study to improve the homogeneity and transport properties of chitosan films by using electric field was performed by Souza et al. 2009. Ohmic heating is based on the passage of electrical current through a sample that has electrical resistance. The electrical energy is directly converted to heat and instant heating occurs, at a rate which depends on the intensity of the current passing through the material. Four different field strengths were tested (50, 100, 150, 200 V cm^{-1}), and for each electric field treatment, the water vapor, oxygen, and carbon dioxide permeabilities of the films formed were determined, together with their color, opacity, and solubility in water. The results showed that ohmic heating had statistically significant effects on film's physical properties and structure. The most pronounced effect of the field strength was observed for treatments made at 100 V cm^{-1} or higher, a positive correlation being found between the water vapor, oxygen, and carbon dioxide permeability coefficients and field strength. The surface of chitosan films is much more uniform when an electric field is applied, which may be related with a more uniform gel structure leading to the differences observed in terms of transport properties (Souza et al. 2009).

In a recent study the effect of chitosan on blue mold caused by *Penicillium expansum* on jujube fruit has been investigated (Wang et al. 2014). The study showed that application of chitosan reduced disease development of blue mold caused by *P. expansum* in wounded and inoculated jujube fruit at 25 °C. Chitosan also provided an inhibitory effect on natural decay of jujube fruit during storage at 0 °C. The results indicated that spore germination, germ tube length, and mycelial growth of *P. expansum* were significantly inhibited by chitosan in a concentration-dependent mode. Electron microscopy observation and fluorescent measurements indicated that plasma membrane of *P. expansum* was gradually disrupted after chitosan application (Wang et al. 2014).

3 Blending of Chitosan

The functional properties of chitosan-based films can be improved by combining them with other hydrocolloids. Chitosan-/pectin-laminated films have been developed by the interaction of the cationic groups of chitosan with the anionic groups of pectin. A decrease in water vapor transmission rates (WVTRs) by combining chitosan with two thermally gelatinized cornstarches (waxy starch and regular starch with 25 % amylose) has been observed. However, composite film made with regular starch showed higher TS and *E* than those with waxy starch. The addition of starch decreased WVTRs of the composite films (Xu et al. 2005). An alternative way to improve the mechanical and physical properties of these biofilms is by combining proteins (e.g., milk proteins, soy protein, collagen, and gelatin)

with polysaccharides (e.g., starches, alginates, cellulose, and chitosan). Chitosan–gelatin blend films have been shown to be homogeneous due to the good miscibility between both biopolymers (Haider et al. 2008; Silva et al. 2007; Pereda et al. 2008) leading to improved material properties of the blend films as compared to those obtained from the pure polymers. This is explained by the formation of electrostatic interactions between the ammonium groups of the chitosan and the carboxylate groups of the gelatin. On the other hand, chitosan-/soy protein-blended membranes (Silva et al. 2007) are not completely miscible. The blended membranes became more brittle with increasing soy protein content and showed a rougher surface morphology; this is probably related to phase separation among blend components. Chitosan/sodium caseinate films have also been studied; in this case no phase separation was observed due to the complexation of the two polymers within the blend film matrix (Pereda et al. 2008). Chitosan/whey protein films have been prepared at pH 6 with different protein concentrations, in the absence or presence of transglutaminase as a cross-linking agent. The chitosan was the main film component, and its amount was kept constant, the protein was from a spray-dried whey product still rich in lactose, and the amount of whey protein did not exceed the proportion of 1:9 (protein: chitosan) in the final films. The films prepared in the presence of the enzyme showed low solubility at a wide range of pH, a lower degree of swelling, and good biodegradability following protease treatments. The presence of transglutaminase induced also an enhancement in film mechanical resistance and a reduction in their deformability. Finally, the barrier efficiency toward oxygen and carbon dioxide was found to be markedly improved in the cross-linked films which showed also a lower permeability to water vapor. Catarina et al. (2009) prepared films of chitosan/whey protein blend with a high amount of protein in order to obtain a blend with new functionality out of the interaction of the cationic polyelectrolyte chitosan with protein. The study was aimed also at preparing an edible film-forming material with antimicrobial properties.

4 Chitosan–Starch Blends

Considering the advantages and unique characteristics of starch and chitosan, it is expected that a blend of chitosan–starch would be able to form a biodegradable film that would show improved mechanical properties (better strength and flexibility), lower water permeability, and antibacterial properties compared to starch-based films without chitosan.

Antimicrobial activity of edible coating solutions based on chitosan and blends of chitosan–tapioca starch with or without potassium sorbate (KS) addition was studied (Vásconez et al. 2009). This study showed an antagonist effect on the efficiency of chitosan against *Lactobacillus* spp. when KS and/or tapioca starch was present. A salmon slice coating assay showed that the chitosan solution was the best coating since aerobic mesophilic and psychrophilic cell counts were reduced and pH and weight loss remained acceptable throughout refrigerated storage,

extending global quality to 6 days. Chitosan–tapioca starch-based films reduced *Zygosaccharomyces bailii* external spoilage in a semisolid product but were not effective against *Lactobacillus* spp.

The results suggest that antibacterial action depended on the application technique, due to the fact that chitosan is more available in a coating solution than in a film matrix. The addition of chitosan reduced water vapor permeability and solubility of starch films. Starch-based films have been particularly considered for the reason that they exhibit physical characteristics similar to synthetic polymers: transparent, odorless, tasteless, semipermeable to CO_2, and resistant to O_2 passage (Nísperos-Carriedo 1994).

The main differences between starch and chitosan are the glucoside linkage – α (1, 4) for starch and β (1, 4) for chitosan – and the hydroxyl group of the second carbon that is replaced by the amine group which appears acetylated in the case of the natural polymer chitin.

Studies about the production and application of edible starch-based films incorporating preservatives have confirmed the availability of the latter to extend the shelf life of fresh and minimally processed vegetables (Durango et al. 2006; Garcia et al. 1998). According to the Food and Agriculture Organization (FAO 2004), one important source of starch in South America is tapioca.

Fernandez et al. (2004) studied the physical stability and moisture sorption of aqueous chitosan–amylase starch films plasticized with polyols. They used high, medium, and low molecular weight chitosan with amylose-rich cornstarch as a co-film former in the presence of glycerol and i-erythritol. In comparison to regular cornstarch which contains approximately 28 % amylose, Hylon VII is a corn hybrid containing approximately 70 % amylose. Since amylose is a linear polymer, it can closely align or associate through hydrogen bonding. This characteristic of amylose is primarily responsible for the gelling and film-forming ability of starches. Since Hylon VII contains more than twice as much amylose as regular cornstarch, it can form more rigid gels and contribute to the formation of stronger and tougher films. Chitosan–Hylon VII solutions plasticized with glycerol or erythritol were prepared in a high-pressure reactor equipped with a blade mixer.

Edible chitosan–starch film has been used for extending the shelf life of Mongolian cheese (Mei et al. 2013). In this work the physicochemical, mechanical, optical, and structural properties of edible films from mung bean/water chestnut starch and chitosan containing glycerol/perilla oil have been investigated. The shelf life of Mongolian cheese coated by the films was monitored by the microbiological and physicochemical changes of Mongolian cheese after 30 days of storage period. The obtained results showed that the incorporation of perilla oil resulted in a decrease in moisture content, solubility, and mechanical properties and an increase in total color difference (ΔE*). High water vapor permeability (WVP), good transparency, and low solubility were observed with the addition of glycerol. Meanwhile, the film based on mung bean chitosan–starch (MSC) exhibited higher moisture content, WVP values, ΔE* and less transparency than that based on water chestnut chitosan–starch (WSC). The morphology of films was also different. The results showed that the cheese coated by WSC film containing perilla oil presented

better treatment performance in terms of microbial growth delay, weight loss, and shelf life length. Mung bean is the seed of *Vigna radiata* and is traditionally used in soup, pancake, and cold noodle in China. Water chestnut (*Eleocharis dulcis*) is a fruit crop grown in China, India, and Southeast Asia, and the cake made from its flour is a traditional dessert in the south of China (Mei et al. 2013).

In order to enhance the antibacterial capacity of chitosan–starch films, irradiation of compression-molded starch-based mixture in physical gel state with electron beam (EB) at room temperature was performed (Zhai et al. 2004). The tensile strength and the flexibility of starch film were improved largely after incorporation of 20 % chitosan into starch film. X-ray diffraction and scanning electron microscope analyses indicated that there was interaction and microphase separation between starch and chitosan molecules. The antibacterial activity of the chitosan–starch blend films against *Escherichia coli* (*E. coli*) was measured via optical density method. After irradiation, there is no obvious change in the structure of chitosan–starch blend films, but antibacterial activity was induced even when the content of chitosan was only 5 % due to the degradation of chitosan in blend films under the action of irradiation.

Biodegradable blend films from rice chitosan–starch were developed by casting film solution (Bourtoom and Chinnan 2008). The film from rice chitosan–starch showed an increase in tensile strength (TS), water vapor permeability (WVP), lighter color and yellowness and a decreasing elongation at the break (*E*), and film solubility (FS) after incorporation of chitosan. The introduction of chitosan increased the crystalline peak structure of starch film; however, too high chitosan concentration yielded phase separation between starch and chitosan. However, the water vapor permeability of rice chitosan–starch biodegradable blend film was characterized by relatively lower water vapor permeability than chitosan films but higher than polyolefin; these films could be a potential candidate for edible packaging.

Edible film with antimicrobial property was developed based on poly(lactic acid)/starch in which chitosan was the active antimicrobial ingredient which is released slowly from the poly(lactic acid)/starch matrix (Bie et al. 2013). An increase in the starch content drastically improved the hydrophilicity of the blends, which was favorable for the diffusion of the embedded chitosan. Moreover, the release of chitosan was observed to occur in two stages, with a very fast release stage initially and a slow but durable release stage as the latter. These two stages exhibited the effectiveness and long residual action of antimicrobial property of the blends, respectively, demonstrating the suitability to be used for foods with high water activity, such as fresh meat. The authors recommend the use of the blend material in packaging (Bie et al. 2013).

Chitosan–starch (high amylose) blend film (1:1), via microfluidization, was prepared by casting with different glycerol concentrations (0 %, 2.5 %, 5 %, and 10 %) (Liu et al. 2013). Microfluidization is a well-documented promising high-energy homogenization method that had been widely applied in the emulsion preparing process in various areas including personal care, cosmetics, health care, pharmaceuticals, and agrochemicals to improve the stability of the emulsion,

attributing to its ability to produce exceptionally fine and stable emulsion (Jafari et al. 2007). During microfluidization, two steams of flow that collide with each other at a pressure of up to 150 MPa and force them through microchannels toward an impingement area create a tremendous shearing action (Salvia-Trujillo et al. 2012).

The films were characterized for their mechanical, thermal, and morphological properties. The addition of glycerol at 5 % (w/w) and higher concentrations resulted in decrease in tensile strength and increase in elongation at break due to plasticization.

An antiplasticization effect was observed in chitosan–starch–glycerol films when the glycerol concentration was 2.5 % (w/w). The antiplasticization was reflected in visible surface cracking in the SEM image and the high Tg value of 2.5 % (w/w) glycerol film. At glycerol concentration of 5 % (w/w) and above the chitosan–starch–glycerol films were typically plasticized by glycerol as evidenced from the significant ($p < 0.05$) decrease in both tensile strength and Tg (Liu et al. 2013).

Chitosan has been previously added to films prepared with starches from different origins, such as potato (Mathew and Abraham 2008; Shen et al. 2010), tapioca (Vásconez et al. 2009), corn (Xu et al. 2005), and kudzu (Zhong et al. 2011). In this work, blends of wheat starch WS and chitosan CH have been used for the preparation of films with antimicrobial activity and tested on minced pork meat (Bonilla et al. 2013).

The incorporation of CH to WS films did not provoke a significant increase in their oxygen permeability OP values at 5 °C. At 25 °C, OP values tend to increase as the CH content is increased in the film, although this was only significant when more than 30 % CH is present in the blend and the differences were very small. This behavior represents an advantage of the blend films, since CH incorporation led to an enhancement of starch films, inhibiting retrogradation and increasing mechanical resistance, but did not improve their barrier properties notably. The lower requirement of glycerol in blend films plays an important role because glycerol contributes to promote mass transfer processes due to the promotion of molecular mobility. With lower ratio of glycerol, chitosan-containing films showed similar barrier properties to wheat starch–glycerol films. The replacement of wheat starch–glycerol with chitosan affected the properties of film-forming dispersions and films. Chitosan addition led to a significant increase of the particle surface charge and apparent viscosity of the film-forming dispersion and yielded glossier and thicker films. The mechanical properties were significantly improved as the chitosan ratio increased in the films. Water vapor permeability (WVP) and OP values tend to slightly increase when chitosan was incorporated to the film, although induced differences are not relevant. A notable antimicrobial effect was detected in the blend films when the proportion of chitosan was 50 %. The obtained results suggest that it would be possible to formulate edible antibacterial films with thermoplastic properties by combining wheat starch and chitosan (Bonilla et al. 2013). In a further improvement of the previous work, Bonilla et al. have added four antioxidant ingredients, namely, basil essential oil, thyme essential oil,

citric acid, and α-tocopherol, to the edible WS/CH blends. The starch–antioxidant mass ratio was 1:0.1. The incorporation of antioxidants led to a heterogeneous film microstructure, mainly in those containing α-tocopherol, which affected the surface roughness. Yellowness was induced in films when α-tocopherol was added, and no notable color changes were observed in the other cases, although all the antioxidants increased the transparency of the films. Despite of the fact that the mechanical properties were barely affected by the incorporation of antioxidants, citric acid promoted an increase in the elastic modulus but a decrease in film stretchability. The water vapor barrier properties of the films were only slightly improved when citric acid and α-tocopherol were added, whereas the oxygen barrier properties were significantly improved in all cases. The greatest antioxidant capacity of the films was reported for films containing α-tocopherol, which exhibited the highest antioxidant power (Bonilla et al. 2013).

Li et al. 2013 investigated the effect of cross-linking using glutaraldehyde on the physical and mechanical properties of CH–starch blend films. They found that the compatibility of the blends deteriorated after cross-linking but showed improved water barrier performance. This was further confirmed by the results of mechanical properties. The use of trace concentrations of glutaraldehyde in chitosan–starch films allows for possible application in the biomedical field (Li et al. 2013).

Zhong et al. 2011 investigated the effect of solvent on the mechanical and antibacterial behavior of chitosan/kudzu starch. The roots of kudzu plant contain starch, and its leaves show antioxidant activity that suggests food uses (Shurtleff and Aoyagi 1977). Acetic acid, lactic acid, and malic acid were used to dissolve chitosan. Fourier transform infrared spectra showed that kudzu starch and chitosan could form miscible films. X-ray diffraction data indicated that the crystalline of each single component was suppressed after film-forming process. The composite film using malic acid as a solvent showed the best antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, which may be due to the highest amount of dissolved amino group, the lowest water sorption ability, and the best water barrier property. The film that chose acetic acid as the solvent presented the strongest mechanical property, the smallest solubility, and the lightest yellowness and color. The film made from lactic acid solution displayed the greatest flexible property demonstrated by the maximum elongation (Zhong et al. 2011). These composite films could also find applications in bioactive packaging.

In an easy one step, a valuable thermoplastic material derived from secondary wheat B-starch, which is a low-quality by-product in the starch industry, had been prepared by Kelnar et al. (2013). The combination of a tiny amount of chitosan and montmorillonite represents a promising method to improve the mechanical and barrier properties of thermoplastic starch. The combination of 0.6 % CS and 3 % clay has a synergistic effect on the starch films. These effects were reflected in the reduced permeability for oxygen and enhanced thermal stability. However, the hydrophilicity of both added components resulted in increased water vapor absorption (Kelnar et al. 2013). The effect of CS primarily consists of modifying the matrix and improving the interfacial properties due to the strong potential of the amino groups in the CS for polar interaction/ionic exchange with the nanosilicate (Kelnar et al. 2013).

5 Chitosan with Alginate and Carrageenan

Alginate is linear copolymer extracted from brown seaweeds known as *Phaeophyceae* and composed of (1 → 4)-linked-β-D-mannuronate (M) and (1 → 4)-linked-α-L-guluronate (G) units. These units are arranged in G blocks, M blocks, and alternating sequences of GM blocks forming the polymeric structure, where the sequential arrangement depends on different factors such as the species, age, or parts of the seaweeds from which this material was obtained. It has been well characterized in both the liquid and gel state, making this biopolymer unique compared to other gelling polysaccharides. Alginates possess good film-forming property, producing uniform, transparent, and water-soluble films.

Alginate-based films are impervious to oils and fats but, as other hydrophilic polysaccharides, have high WVP. However, alginate gel coating can act as a sacrificing agent, where moisture is lost from the coating before the food significantly dehydrates. The coating can also improve the adhesion of batter to the surface of fruits and vegetables (Lin and Zhao 2007). Alginate coatings are good oxygen barriers that can retard lipid oxidation in various fruits and vegetables and have been found to reduce weight loss and natural microflora counts in minimally processed carrots (Amanatidou et al. 2000). Calcium alginate coatings were found to improve the quality of fruits and vegetables, such as reducing shrinkage, oxidative rancidity, moisture migration, oil absorption, and sealing in volatile flavors, improving appearance and color and reducing weight loss of fresh mushrooms in comparison with uncoated ones (Hershko and Nussinovitch 1998).

6 Chitosan- and Gelatin-Based Edible Films

Composite edible films and coatings can be formulated to combine the advantages of each component. Whereas biopolymers, such as proteins and polysaccharides, provide the supporting matrix, lipids provide a good barrier to water vapor (Anker et al. 2002). Since gelatin and chitosan are hydrophilic biopolymers with good affinity and compatibility, they are expected to form composite films with good properties (Sionkowska et al. 2004). Gelatin–chitosan blends have been used extensively for the production of scaffold and bilayers for biomedical applications (Pereda et al. 2011; Jiankang et al. 2009).

A bilayer and laminated biodegradable composite film based on gelatin and chitosan has been developed by Rivero et al. (2009). The aims of this work were to develop composite, bilayer, and laminated biodegradable films based on gelatin and chitosan. A distinction was made between them, calling bilayer the system prepared by the coating technique which is a two-step procedure and laminated the one formed by two independent films, placed one on the other. Gelatin and chitosan concentrations used were 7.5 % and 1 % (w/w), respectively. Glycerol (0.75 %) was added as plasticizer. In composite film, it was evident that the presence of chitosan led to stronger films, while glycerol improved film flexibility. Composite and bilayer systems showed a compact structure indicating a good compatibility

between components. Water vapor permeability (WVP) was independent of film thickness up to 120 μm for gelatin films and 60 μm for chitosan ones. Both bilayer and laminated systems resulted effective alternatives to reduce WVP of composite films (at least 42.5 %). Bilayer systems showed better mechanical properties than laminated ones. The resistance at break increased from 54.3 for composite to 77.2 MPa for bilayer films, whereas elongation at break values of both composite and bilayer films was similar (2.2–5.7 %).

A coating made in cold from a blend of a chitosan and a gelatin solution was applied to patties made of chilled cod, and its preservative effect was assessed by color measurements, rheological measurements (hardness, elasticity, cohesiveness, chewiness, gumminess, and adhesiveness), biochemical determinations (total volatile bases and thiobarbituric acid as measures of rancidity), and microbiological assays (total bacterial counts, luminescent bacteria, enterobacteria, *Pseudomonas*, lactic acid bacteria, and *Staphylococcus aureus*). The effect of dry powdered chitosan mixed into the patties was tested as well (López-Caballero et al. 2005). Chitosan was effective in preventing bacteria spoilage only when it was applied in the powder form in the cold. Gelatin made from fish skins offers an alternative to the more commonly used mammalian gelatin that is highly suitable for coating seafood products. Fish gelatins with a variety of attributes can be prepared for this purpose, depending on the raw material, i.e., source species and body parts used, and the manufacturing. Consequently, a mixture of fish gelatin and chitosan, both derived from marine sources, would seem to be especially suitable for use in the preparation of seafood products. The fish patty coating was quite natural looking and translucent, producing a more uniform and smooth surface. In the case of cooking the fish patties, the coating would turn liquid as the gelatin melted from the heat, leaving the mince patty completely uncoated with no remnants that could be seen or tasted (López-Caballero et al. 2005).

Fish gelatin, especially cold-water fish gelatins, is a good alternate to consider when dealing with composite edible films aimed at reducing water vapor transmission, and this is attributed to its higher hydrophobicity than bovine or porcine gelatin, since it has lower proline and hydroxyproline contents, as the hydroxyl group of hydroxyproline is normally available to form hydrogen bonds with water molecules (Avena-Bustillos et al. 2006). Hosseini et al. (2013) have prepared fish gelatin-based composite films with increasing concentrations of chitosan of 0, 20, 30, 40, and 100 % and investigated their physical and functional properties. It was found that fish gelatin and chitosan were totally compatible to form solutions and films. The addition of these positively charged polysaccharides improved the properties of films made from fish gelatin and improved mechanical properties and decreased water vapor permeability. The film prepared with the blend 60 % gelatin and 40% chitosan was found to be the best as it had lower water vapor permeability and film solubility. The addition of CH caused significant increase in the tensile strength (TS) and elastic modulus, leading to stronger films as compared with gelatin film, but significantly decreased the elongation at break. These results suggest a high potential of these films to be used as active packaging materials (Hosseini et al. 2013).

The effects of chitosan molecular weight (MW) and degree of deacetylation (DD) on the physicochemical properties of gelatin-based films were studied (Liu et al. 2012). The determination of the dynamic viscoelastic properties (elastic modulus G′ and viscous modulus G″) of the film-forming solutions revealed that the interactions between gelatin and chitosan were stronger in the blends made with chitosan of higher molecular weights or higher degrees of deacetylation than the blends made with lower molecular weights or degrees of deacetylation.

Films of chitosan and gelatin were prepared by casting their aqueous solutions (pHb4.0) at 60 °C and evaporating at 22 or 60 °C (low- and high-temperature methods). The physical, thermal, and mechanical properties and gas/water permeation of the water- or polyol-plasticized films have been investigated (Arvanitoyannisa et al. 1998). A considerable decrease in elasticity modus and tensile strength occurred with increasing the content of the plasticizer (30 % plasticizer was added), whereas the elongation percent increased up to 150 %. Higher percent crystallinity was obtained with the low-temperature method of preparation than high-temperature method. The higher crystallinity led to a decrease, by one or two orders of magnitude, of CO and O permeability in the chitosan/gelatin composite films, which is an advantage for edible packaging (Arvanitoyannisa et al. 1998).

Kołodziejska and Piotrowska (2007) design biodegradable material with good mechanical and barrier properties using fish gelatin–chitosan films plasticized with glycerol suitable for packages of many kinds of food products with different acidities and contents of moisture.

The physical characteristics of plain gelatin and chitosan films have been improved by using several gelatins of different origins (commercial bovine-hide gelatin and laboratory-made tuna-skin gelatin) (Gómez-Estaca et al. 2011). The dynamic viscoelastic properties of the film-forming solutions upon cooling and subsequent heating revealed that the interactions between gelatin and chitosan were stronger in the blends made with tuna-skin gelatin than in the blends made with bovine-hide gelatin. As a result, the fish gelatin–chitosan films were more water resistant (w18% water solubility for tuna vs. 30 % for bovine) and more deformable (68% breaking deformation for tuna vs. 11 % for bovine) than the bovine gelatin–chitosan films. The antimicrobial activity of the resulting films over *S. aureus* was also evaluated; the good inhibitory effects of the chitosan against *S. aureus* were maintained in spite of the gelatin–chitosan interactions observed (Gómez-Estaca et al. 2011). In a more recent work, Jridia et al. (2014) have demonstrated that mixing cuttlefish skin gelatin and chitosan rendered films of homogeneous structure, due to their high compatibility, as revealed by the thermal and microstructure analysis of composite films. Chitosan addition greatly reduced the extensibility, the thickness, and the solubility of composite films, while it increased their tensile strength. Furthermore, in composite films the presence of chitosan increases the thermal stability of the film and increased the antibacterial activity, while it decreased the antioxidant activities of composite edible films. The gelatin used in this work was obtained from cuttlefish (*Sepia officinalis*) skin gelatins by pretreatment with different pepsin concentrations at pH 2.0

(Jridi et al. 2013). The extraction of gelatin from alkali-pretreated skin of cuttlefish with pepsin affected the molecular weight and biophysical and functional properties of the resulting gelatins. Gelatin extracted without pepsin showed higher gel strength and transition, gelling, and melting temperatures. All of these values decreased with increasing enzyme concentration (Jridi et al. 2013).

7 Chitosan/Essential Oil Films

In spite of the extensive use of chitosan as edible films for coating, it still suffers from high water vapor permeation (WVP) which lowers its protective action. Therefore, the addition of oils may increase its hydrophobicity and improve its water vapor permeation. Vargas et al. (2006) reported data on the effect of unsaturated oils, such as olive oil, on the properties of chitosan-based films and the interactions between chitosan and olive oil or chitosan and olive oil components (Ham-Pichavant et al. 2005; Muzzarelli et al. 2000; Vargas et al. 2006). The study published by Vargas et al. (2006) focused on the improved physicochemical quality of the strawberries coated with the edible coatings. Muzzarelli et al. (2000) reported that the capacity of chitosan to alter the composition of olive oil is due to the percolation of the oil through a bed of chitosan powder. Ham-Pichavant et al. (2005) reported on the potential replacement of fluorocarbon treatment of paper-based materials by chitosan coating to produce oil barrier packaging.

Vargas et al. (2009) have further investigated the formation of edible films based on high molecular weight chitosan (CH) and different concentrations of oleic acid (OA). Film-forming dispersions (FFD) were characterized in terms of rheological properties, surface tension, particle size distribution, and ζ-potential. Their results showed that the increase in OA promoted changes in the size and surface charge of the FFD particles, which had an impact on the rheological properties of the FFD. As regards the film properties, the higher the OA content, the lower the WVP and the moisture sorption capacity.

In general, the addition of OA into the CH matrix leads to a significant increase in gloss and translucency and a decrease in the tensile strength, elongation at break, and elastic modulus of the composite films. The mechanical and optical properties of the films were related with their microstructure, which was observed by SEM.

Chitosan was mixed with olive oils in different concentrations to prepare homogeneous films which were found to have decreasing moisture sorption, lower water vapor permeation, and smaller effective diffusion coefficients of the films as the oil concentration increases (Pereda et al. 2012). All the tensile properties (Young modulus, strength, and maximum elongation) increased with olive oil concentration, which were explained considering the interactions developed between lipid and carbohydrate phases in addition to the lubricant action of the oil (Pereda et al. 2012). Physical characterization confirmed that the lipid globule distribution in the films was homogeneous, which was associated to the emulsion stability.

Contact angle measurements showed that increasing the olive oil concentration causes a slight increase of contact angle values, from approximately 60° for 0 %

olive oil to 65° for 15 % olive oil, which is due to the hydrophobic nature of the added lipid. In general when hydrocolloid and lipid ingredients are combined, they may interact favorably, resulting in edible films with improved structural and functional properties, as the mechanical and barrier properties depend not only on the compounds used in the polymer matrix but also on their compatibility.

Films prepared from quinoa protein extracts (Q) were mixed with chitosan and were obtained by solution casting (Valenzuela et al. 2013). From the optimal Q/CH mixture, the addition of three different concentrations of sunflower oil (SO) (2.9, 3.8, and 4.7 g/100 mL), and the optimal proportion of SO, g/100 mL was selected based on the mechanical and barrier properties of the films. The CH, Q/CH, and Q/CH/SO optimal blend films were characterized by FTIR, X-ray diffraction, and SEM. The 0.1 Q-8/CH blend was found to have high degree of interaction between the quinoa proteins and CH. The optimum concentration of SO used in the Q-8/CH/SO film was 2.9 g/100 mL. The addition of SO to the film improved the water vapor permeability (WVP) as a result of hydrophobic interactions and the presence of clusters of hydrophobic masses on the surfaces of these films but reduced the film's tensile strength and oxygen permeability due to the formation of micropores and microfractures detected by SEM. The elongation at break increased from Q-8/CH and Q-8/CH/2.9SO due to the plasticizing characteristics of the quinoa protein and lipid, in addition to the strong interactions developed between the lipid and hydrocolloid phases. The formation of the composite films in the absence of a plasticizer, producing good mechanical properties and lower WVP values, may lead to new applications in the food industry. The fields of application of Q/CH/SO blend may be on low-pH fresh fruits, to increase shelf life and consumer acceptability (Valenzuela et al. 2013).

Fresh fish is highly perishable due to its biological composition. Spoilage of fish muscle results from changes brought about by biological reactions such as oxidation of lipids, reactions due to activities of the fish's own enzymes, and metabolic activities of microorganisms. These activities lead to a short shelf life in fish and other seafood products (Arashisara et al. 2004). Ojagh et al. (2010) have used chitosan enriched with cinnamon oil (CH + C) to coat rainbow trout (*Oncorhynchus mykiss*) (Ojagh et al. 2010). *Cinnamomum zeylanicum* L., commonly known as cinnamon, is rich in cinnamaldehyde as well as b-caryophyllene, linalool, and other terpenes. Cinnamaldehyde is the major constituent of cinnamon leaf oil and provides the distinctive odor and flavor associated with cinnamon. It is used worldwide as a food additive. Studies have shown that cinnamon has a good antioxidant and antimicrobial potential (Ojagh et al. 2010). The quality of the coated rainbow trout was examined during refrigerated storage (4 ± 1 °C) over a period of 16 days. A solution of CH (2 %, w/v) and CH + C (2 %, w/v CH + 1.5 %, v/v C) was used for the coating. The control and the coated fish samples were analyzed periodically for microbiological (total viable count, psychrotrophic count), chemical (total volatile base nitrogen (TVB-N), peroxide value (PV), thiobarbituric acid (TBA)), and sensory (raw and cooked fish) characteristics. By the day 8 of storage, the total viable counts (TVC) in trout fillet for all of the different treatments were still below 6 log10 CFU/g, while that of controls attained a count of 7.88 at 12 days, which is

higher than the maximal recommended limit of 7 log10 CFU/g for TVC in raw fish (Ibrahim Sallam 2007), indicating a microbiological shelf life of about 9–10 days for the control samples. The results indicated that the effect of the CH + C coating on the fish samples was to enable the good quality characteristics to be retained longer and to extend the shelf life during the refrigerated storage. Adding cinnamon oil to chitosan coating, therefore, had a synergistic effect. In the case of most herbal extracts, their antioxidant activity has been attributed to their ability to break the free radical chain by donating a hydrogen atom (Ojagh et al. 2010).

The application of citrus essential oils to food preservation has received increased attention because of their use in food products and as safe at flavoring concentrations (GRAS). These factors made them into very promising compounds to be used as a natural alternative to chemical-based preservatives.

Bergamot oil, BO, is a citrus oil (from *Citrus bergamia*) whose major chemical compounds are volatile, such as limonene (32–45 %) and linalool (around 10.23 %) (Moufida and Marzouk 2003; Svoboda and Greenaway 2003). The antimicrobial efficiency of BO and its components, linalool and citral, has been found to be effective against *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Arcobacter butzleri*, and *Penicillium digitatum* (Fischer and Phillips 2006, 2008), among others, both when oil is applied directly and when in contact with the oil vapor. The mechanisms by which essential oils bring about their antimicrobial effect are not clear, but there are a number of proposed mechanisms (Holley and Patel 2005): terpenes have the ability to disrupt and penetrate not only the lipid structure of the cell membrane but also the mitochondrial membrane, leading to the denaturation of proteins and the destruction of cell membrane, cytoplasmatic leakage, cell lysis, and, eventually, cell death.

Sanchez-Gonzalez and coworkers (2011a) have published a review dealing with the use of essential oils in edible/biodegradable films, and they recommend that this strategy will lead to reduced doses of essential oils while maintaining their effectiveness. This review discusses the use of essential oils as natural antimicrobial and antioxidant compounds to obtain bioactive films or coatings. The advantages and limitations are also reviewed Sanchez-Gonzalez and coworkers (2011a).

Sanchez-Gonzalez et al. (2010a) prepared chitosan-based films containing bergamot essential oil (BO) at 0.5 %, 1 %, 2 %, and 3 % w/w to evaluate their physical and antifungal properties.

The incorporation of BO into the chitosan (CH) matrix provoked a decrease in the water vapor permeability, this reduction being around 50 % when using a CH–BO ratio of 3:1. The CH–BO composite films were less resistant to breakage, less deformable, and less glossy. The load parameters (TS and EM) decreased more than 50 %, and the percentage of elongation at break was also dramatically reduced from 22 % to 5 %, as compared with the pure chitosan films. CH–BO composite films showed a significant inhibitory effect on the growth of *P. italicum*, which depended on the BO concentration. Chitosan films with the maximum bergamot oil content (3:1 CH–BO ratio) led to a total inhibition of the fungus growth during the first 5 days at 20 °C. Although the antifungal effectiveness of the films decreased

throughout the storage time, a significant reduction of 2 logarithm units as compared with the control remained possible, after 12 days at 20 °C, using the highest BO content.

In a different report, Sanchez-Gonzalez et al. (2011b) prepared biodegradable coatings based on hydroxypropylmethylcellulose (HPMC) or chitosan (CH) with and without bergamot essential oil and applied them to table grapes, cv. muscatel, in order to find environmentally friendly, healthy treatments with which to better preserve fresh fruit quality and safety during postharvest cold storage The physicochemical properties (weight loss, Brix, total phenols, antioxidant activity, color, and texture), respiration rates, and microbial counts of the samples were determined throughout cold storage when using CS. CS coatings containing bergamot oil produced the most effective antimicrobial activity and showed the greatest inhibition of the respiration rates in terms of both O_2 consumption and CO_2 generation. Although the coatings did not seem to reduce the rate of grape browning during storage, they inhibited color development, thus improving the product appearance. Taking into account the overall results obtained, the most recommended coating for muscatel table grape is the CS-containing bergamot oil (Sanchez-Gonzalez et al. 2011b).

Among the great variety of EOs, lemon, thyme, and cinnamon EOs have gained greater acceptance among food technologists due to their better sensory evaluation and antimicrobial properties (Bagamboula et al. 2004; Fischer and Phillips 2006; Viuda-Martos and Fernández-López 2008).

The addition of lemon, thyme, and cinnamon EOs in chitosan film led to significant changes in the properties of film solutions and films, wherein the addition of a single EO caused the decrease in apparent viscosity of film-forming dispersions. The combined use of two kinds of EOs contributed to emulsion stability by a decrease in particle size and viscosity. Furthermore, the addition of EOs caused a significant decrease in WVP, color, transparency, and elongation at break of the chitosan films. The combined use of two kinds of essential oils balanced the antibacterial properties of the single essential oil. SEM analysis showed that the oil droplets were homogenously distributed across the film. The emulsification was obviously observed in chitosan/lemon/cinnamon essential oil composite films due to the electrostatic interaction of limonene and cinnamaldehyde. This study (Peng and Li 2014) revealed that an active chitosan film could be obtained by the combined use of two kinds of essential oils in the matrix, which might provide a new formulation option for developing antimicrobial film (Peng and Li 2014).

The essential oil of *Melaleuca alternifolia*, also named as tea tree oil (TTO), is a complex mixture of terpene hydrocarbons and tertiary alcohols (Sanchez-Gonzalez et al. 2010b). Tea polyphenols (TP) are extracted from tea and contain catechin, flavones, anthocyan, and phenolic acid, but catechin is the main component with more than 80 % content (Huang et al. 2007). Catechin mainly contains epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) (Yang et al. 2009). Other compounds responsible for the antimicrobial activity are terpinen-4-ol and 1, 8-cineole. TTO has been used

successfully in the management of oral candidosis in AIDS patients (Vazquez and Zawawi 2002) and other oral fungal infections in patients suffering from advanced cancer (Bagg et al. 2006). Research results demonstrated the antibacterial and antioxidant activities of TP, showing good prospects for their use as preservatives and antioxidants (He et al. 2006). It has been showed that using TP as antioxidant or preservative could extend the shelf life of fresh mutton and fresh-cut lettuce (Kumudavally et al. 2008; Martin-Diana et al. 2008). The antioxidant mechanism of polyphenols is principally attributed to their capacity in trapping reactive oxygen species and chelating metal ions, which could generate radicals through Fenton reaction.

Sanchez-Gonzalez et al. (2010b) incorporated TTO into chitosan matrix and investigated the physical and antibacterial behavior of the obtained composite. The CS films were rough and a reduction of its gloss occurred after the incorporation of TTO. Water vapor permeability was also reduced by 40 % when the CS/TTO ratio was 1:2. Likewise, the films' resistance to break was notably reduced by TTO incorporation due to the presence of discontinuities in the film matrix that affect its mechanical response. The poor mechanical properties obtained by the addition of TTO may be related with the structural arrangement of the lipid phase into the chitosan matrix. Thus, the structural discontinuities provoked by the incorporation of the oil could explain the lowest resistance to fracture of the composite films. Some of these results are in line with those reported by other authors when adding oils to a chitosan matrix (Srinivasa et al. 2007) but differ in some aspects due to the great influence of several, widely studied factors related to CS preparation. Only the composite films with CS/TTO ratios higher than 1 showed a limited antifungal effectiveness against *Penicillium*, which was notably reduced after 3 days of storage. Nevertheless, CS films presented a significant antimicrobial activity against *L. monocytogenes*, and the incorporation of TTO in the CS/TTO ratio of 1:2 improved the antibacterial properties of these films, showing a complete inhibition of the microbial growth during the fifth day at 10 °C.

Liyan et al. (2013) developed chitosan-incorporated tea polyphenol active films with different concentrations of TP. The incorporation of TP caused interactions between chitosan and TP and gave rise to the chitosan/TP films' darker appearance with reduced water vapor permeability and significant increase in the antioxidant activity of the chitosan film which makes the chitosan/TP films show great potential to be used for active food packaging (Liyan et al. 2013). Green tea extract was also used with chitosan to enhance the antioxidant efficacy of the composite edible films (Siripatrawan and Harte 2010).

Galangal extract was added to chitosan solution to prepare biodegradable films with improved antibacterial properties (Mayachiew et al. 2010). Galangal similar to ginger and turmeric is a member of the rhizome family. Rhizomes are knobby underground stems that are known for their pungent and flavorful flesh, and it is a traditional spice used extensively for flavoring and medicinal purposes. Galangal extract has also proved to be an effective natural antimicrobial agent against some food poisoning bacteria, e.g., *Staphylococcus aureus* (Mayachiew and Devahastin 2008). The main compounds of galangal extract are the terpenes, which have

potential antimicrobial activity. The aim of this work was to study the effects of drying methods and conditions (i.e., ambient drying, hot air drying at 40 °C, vacuum drying, and low-pressure superheated steam drying (LPSSD) within the temperature range of 70–90 °C at an absolute pressure of 10 kPa). Chitosan films containing galangal extract at 0.6 % and 0.9 % (w/w) were effective in inhibiting the growth of *Staphylococcus aureus* (*S. aureus*). No inhibition zone was observed when the extract concentration of 0.3 % (w/w) was used. This could be ascribed to a limited galangal extract release probably due to interaction between the extract and chitosan. The results showed that ambient dried film had the highest antimicrobial activity; this was followed by LPSSD films and vacuum-dried films. This may be due to the fact that the film temperature increased more rapidly and stayed at higher levels in the case of vacuum drying than in the case of LPSSD, thus inducing more thermal degradation of the antimicrobial compound (Mayachiew et al. 2010). In addition, different intermolecular interactions also contributed to the observed results.

Chitosan film incorporated with 0.9 % (w/w) galangal extract and prepared by ambient drying could reduce the number of *S. aureus* by about 3.6 log cycle within the contact time of 24 h. On the other hand, ambient dried film incorporated with 0.3 % (w/w) galangal extract exhibited lower cell reduction number of around 2.0 log cycle (Mayachiew et al. 2010).

Strawberry is a highly perishable fruit with a short postharvest life which is mainly due to fungal decay. The shelf life of cold-stored (0–4 °C) fresh strawberries is around 5 days (Perdones et al. 2012). Film-forming dispersions (FFD) were prepared with 1 % high molecular weight chitosan and 3 % lemon essential oil and were submitted to two different homogenization treatments. Lemon essential oil, which is extracted from *Citrus limon*, has limonene, valencene, and ocimene as major components. Limonene is used as a food additive or flavoring agent and is known to have fungicidal properties, including activity against *Botrytis* and *Aspergillus niger* (Perdones et al. 2012). The use of microfluidization to prepare chitosan-based FFD led to a significant reduction in the particle size and apparent viscosity of the dispersions, with no significant effect on the decrease in the WVP of the stand-alone coatings. Chitosan coatings did not show a significant effect in terms of the acidity, pH, and soluble solid content of strawberries throughout storage. In contrast, coatings slowed down the respiration rate of samples when lemon essential oil was added to the FFD. Adding lemon essential oil enhanced the chitosan antifungal activity both in in vitro tests and during cold storage in strawberries inoculated with a spore suspension of *Botrytis cinerea* (Perdones et al. 2012). In general the potential of chitosan coatings in maintaining strawberry flavor and aroma during cold storage has been demonstrated by Almenar et al. (2009) as a reduction in acetaldehyde and ethanol formation in chitosan-coated strawberry samples, which may lead to a prolonged shelf life. Another study (Kerch et al. 2011) indicated that coatings of chitosan and chitooligosaccharides applied to strawberries led to an improvement in fruit firmness and to a reduction in vitamin C and anthocyanin content. Campaniello et al. (2008) used chitosan coatings to control spoilage and browning of fresh-cut strawberries in combination with other preservation factors, such as low temperature and modified atmosphere packaging.

An essential oil that is extracted from Zataria multiflora Boiss is an aromatic medicinal plant that grows widely in warm and mountainous parts of Iran, Pakistan, and Afghanistan. This plant is called *Zataria multiflora* Boiss which belongs to the family Lamiaceae. The essential oil (EO) of this plant (ZEO) possesses significant quantities of phenolic oxygenated monoterpenes and exhibits antioxidant, antibacterial, and antifungal activity in vitro. Antioxidant chitosan-based edible films were developed incorporated with *Zataria multiflora* Boiss essential oil (ZEO) (5 and 10 g/L) and grape seed extract (GSE) (10 g/L) alone and in combination (Moradi et al. 2012). All films, with the exception of 10 g/L GSE + 10 g/L ZEO film, exhibited lower strength and elongation values, and only the addition of investigated 10 g/L ZEO to GSE film improved the water vapor transmission rate of chitosan films. Only GSE-containing films had higher swelling index. The incorporation of GSE and ZEO into chitosan film increased the wettability of the surface, total phenol, and antioxidant activity. Neat chitosan and ZEO-incorporated films had a light yellowish color, whereas GSE + ZEO films were gray. The results showed that GSE-formulated chitosan film may be used as active films at medium moisture products such as muscle food because of its excellent swelling index (%) and antioxidant properties.

A study has been conducted by Gómez-Estaca et al. (2010) in which chitosan–gelatin films containing sorbitol and glycerol as plasticizers were incorporated with several different essential oils. Essential oils of clove (*Syzygium aromaticum* L.), fennel (*Foeniculum vulgare* Miller), cypress (*Cupressus sempervirens* L.), lavender (*Lavandula angustifolia*), thyme (*Thymus vulgaris* L.), herb-of-the-cross (*Verbena officinalis* L.), pine (*Pinus sylvestris*), and rosemary (*Rosmarinus officinalis*) were tested for their antimicrobial activities on 18 genera of bacteria, which included some important food pathogen and spoilage bacteria. In an attempt to evaluate the usefulness of these essential oils as food preservatives, they were also tested on an extract made of fish, where clove and thyme essential oils were the most effective and tested for their antibacterial behavior against 18 different bacterial strains which included some important food pathogen and spoilage bacteria. Clove essential oil showed the highest inhibitory effect, followed by rosemary and lavender. Clove and thyme essential oils were the most effective food preservatives, when tested on an extract made of fish. The gelatin–chitosan-based edible films incorporated with clove essential oil were tested against six selected microorganisms: *Pseudomonas fluorescens*, *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Listeria innocua*, *Escherichia coli*, and *Lactobacillus acidophilus*. The clove-containing films inhibited all these microorganisms irrespectively of the film matrix or type of microorganism. When the complex gelatin–chitosan film incorporated with clove essential oil was applied to fish during chilled storage, the growth of microorganisms was drastically reduced in gram-negative bacteria, especially enterobacteria, while lactic acid bacteria remained practically constant for much of the storage period.

Oregano essential oil (OEO) dip was used in association with N, O-carboxymethyl chitosan (NOCC) to the control of *Listeria monocytogenes* and in extending the shelf life of chicken breast fillets. *L. monocytogenes* was inoculated

in chicken breast meat at 103 and 105 cfu/g and stored at 4 °C for a period up to 14 days. The results showed that total viable count (TVC) exceeded 7 log cfu/g after days 6 and 10 for control samples and samples treated with OEO, respectively. Samples treated with either NOCC or OEO plus NOCC never reached 7 log cfu/g throughout storage. NOCC had a substantially stronger antimicrobial effect as compared to OEO (Khanjari et al. 2013).

Chitosan and *Origanum vulgare* L. essential oil films were used to control *Rhizopus stolonifer* and *Aspergillus niger* in grapes (*Vitis labrusca* L.) (dos Santos et al. 2012). Oregano (*Origanum vulgare*, *Origanum onites*, *Origanum minutiflorum*, *Origanum vulgare*) is one of the most powerful and versatile essential oils. It contains strong immune-enhancing and antioxidant properties and supports the respiratory system. Oregano may also be used to enhance the flavor of food. Oregano is also a key oil used in the raindrop technique, a massage application of essential oils which is designed to bring about electrical alignment in the body. This study evaluated the efficacy of the combined application of chitosan (CH) and *Origanum vulgare* L. (OV) essential oil in the inhibition of *Rhizopus stolonifer* URM 3728 and *Aspergillus niger* URM 5842 on laboratory media and on grapes (*Vitis labrusca* L.) and its influence on the physical, physicochemical, and sensory characteristics of the fruits during storage (25 °C, 12 days and 12 °C, 24 days). The application of mixtures of different CH and OV concentrations inhibited the mycelial growth of the test fungi. The application of CH and OV at subinhibitory concentrations inhibited spore germination and caused morphological changes in fungal spores and mycelia, in addition to inhibiting the growth of the assayed fungi strains in artificially infected grapes as well as the autochthonous mycoflora of grapes stored at both room and cold temperatures. In general, the application of a coating composed of CH and OV at subinhibitory concentrations preserved the quality of grapes as measured by their physical and physicochemical attributes, while some of their sensory attributes improved throughout the assessed storage time. These results demonstrate the potential of the combination of CH and OV at subinhibitory concentrations to control postharvest pathogenic fungi in fruits (dos Santos et al. 2012). The antibacterial and antioxidant properties of chitosan edible films incorporated with *Thymus moroderi* or *Thymus piperella* essential oils have been investigated by Ruiz-Navajas et al. (2013). The essential oil has moderate antibacterial and antioxidant capacity. A similar information was given to edible film of chitosan with basil essential oil (Bonilla et al. 2011).

8 Chitosan and Clay

Natural polymers suffer from lower mechanical strength compared to synthetic polymers and high moisture barrier because of their hydrophilic nature. Many strategies have been explored to improve these problems of chitosan-based biodegradable packaging films. These include the addition of plasticizers such as glycerol which increase flexibility of the final product and addition of other biodegradable aliphatic polyesters (Correlo et al. 2005).

Other methods included the addition of layered silicates nanoparticles (e.g., sodium montmorillonite) to chitosan to improve its end-use properties such as barrier and mechanical properties (Xu et al. 2006). Montmorillonite (MMT) is the most studied nanoscale clay. It is a hydrated alumina–silicate layered clay made up of two silica tetrahedral sheets fused to an edge-shared octahedral sheet of aluminum hydroxide. Its advantages of high surface area and platelet thickness of 10 A make it suitable for reinforcement purposes.

Several reports deal with the preparation characterization of MMT/chitosan composites and films (Abdollahi et al. 2012; Hsu et al. 2012; Günister et al. 2007; Casariego et al. 2009; Lavorgna et al. 2010; Rhim et al. 2006). It has been shown that chitosan interacts with MMT and forms a homogenous film. Chitosan/MMT nanocomposites were prepared by an ion exchange reaction between water-soluble oligomeric chitosan and Na MMT. Chitosan showed high affinity to MMT clay host. Several studies have reported amelioration of mechanical properties (Lavorgna et al. 2010; Xu et al. 2006), thermal stability (Darder et al. 2003; Wang et al. 2005), functional properties (Rhim et al. 2006), barrier properties (Casariego et al. 2009; Rhim et al. 2006), and water solubility (Casariego et al. 2009) of chitosan films via incorporation of nanoclay into chitosan in the range of 1–5 wt%.

Abdollahi et al. (2012) showed that incorporating MMT and REO into chitosan improves water gain, water vapor permeability, and solubility of the chitosan film by more than 50 %. The combined effect of clay and REO improves significantly the tensile strength and elongation of chitosan ($p < 0.05$). Microstructure of chitosan/MMT–REO nanocomposites was characterized through X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The results showed that incorporating MMT leads to improvements of the composite films, and this in turn is due to the MMT exfoliation and good interaction between chitosan and MMT in the presence of REO. Antimicrobial properties of the films also improved by REO incorporation in 1.5 % v/v. The improvement of thermal stability is mainly due to the strong electrostatic interaction of cationic chitosan molecules with anionic silicate layers. It was also found that the nanocomposites showed a synergistic effect in the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Han et al. 2010). Delaminated MMT was found to be enriched on the surface of the nanocomposites when the amount of MMT was $>10^3$ ppm. This was accompanied by a decrease of the contact angle (Hsu et al. 2012). The proliferation of fibroblasts on MMT/CS 10^3 ppm was significantly greater than on other materials. The antimicrobial activity was enhanced markedly with the increased amount of MMT. These results suggest a potential application of chitosan–clay nanocomposites in the development of natural biopolymer-based biodegradable packaging materials with antimicrobial activity.

9 Antibacterial Activity of Chitosan and Chitosan Blends

In most fresh or processed foods, microbial contamination occurs at a higher intensity on the food surface, thus requiring an effective microbial growth control. Traditionally, antimicrobial agents are added directly to the foods, but their activity

may be inhibited by many substances in the food itself, diminishing their efficiency. In such cases, the use of antimicrobial films or coatings can be more efficient than adding antimicrobial agents directly to the food since these may selectively and gradually migrate from the package onto the surface of the food, thereby high concentrations being maintained when most necessary (Ouattara et al. 2000).

The molecular weight and amino group of chitosan have strong influence on its antibacterial activity (Liu et al. 2001). It has been found that chitosan activity against fungus to be less efficient as compared with its activity against bacteria (Sebti et al. 2005). On the other hand, results from Bautista-Banos et al. (2006) were much different than those of Guo-Jane et al. (2006) who emphasized on the efficiency of chitosan against fungi. Nevertheless, all these studies indicated that the polycationic nature of chitosan is the key to its antifungal properties in addition to the possible effect that chitosan might have on the synthesis of certain fungal enzymes and that the length of the polymer chain enhances that activity. Bautista-Banos et al. (2006) have shown that not only chitosan is effective in stopping the growth of the pathogen, but it also induces marked morphological changes, structural alterations, and molecular disorganization of the fungal cells.

No et al. (2002) studied the antibacterial activities of six chitosan samples and six chitosan oligomers with different molecular weights (Mws) against four gram-negative and seven gram-positive bacteria and found that chitosan markedly inhibited growth of most bacteria tested; however, the inhibitory effects differed with regard to the molecular weight of chitosan and the type of bacterium. Chitosan oligomers also inhibited bacterial growth by 1–5 log cycles at a 1.0 % concentration; however, effects were more limited than those of the chitosan. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria than gram-negative bacteria.

The antibacterial activity of chitosan film or chitosan–starch films could be enhanced by the action of irradiation (Zhai et al. 2004). The chitosan–starch blend films made by irradiation were tested against *E. coli*. The blend films exhibited significant antibacterial activities after irradiation by 30 kGy compared to the pure or un-irradiated blend films and that the antibacterial activity of blend films improved with increasing the dose (Zhai et al. 2004).

Devlieghere et al. (2004) investigated the antimicrobial effect of chitosan, with high deacetylation degree and low molecular weight, against several psychrotrophic food-borne pathogens and spoilage microorganisms and compared it to those known from the literature. They found that gram-negative bacteria were more susceptible, while the sensitivity of the gram-positive bacteria was highly variable: *Brochothrix thermosphacta* and *Bacillus cereus* were very sensitive to the applied chitosan, while *Listeria monocytogenes* and different lactic acid bacteria were less susceptible. Yeasts, represented by *Candida lambica* and *Cryptococcus humicolus*, showed an intermediate sensitivity, and the activity of chitosan was strongly decreased by high amounts (30 %, w/v) of starch, leading to a significantly shorter lag phase and a significantly higher growth rate.

The antibacterial activity of chitosan–starch film using microwave treatment has been carried out using agar plate diffusion method (Tripathi et al. 2008).

The antibacterial activity of the film and their same solution has been evaluated against three different test cultures, viz., gram-negative bacteria *E. coli*, gram-positive bacteria *S. aureus*, and gram-positive bacteria *Bacillus subtilis*. It was found that the solution of chitosan–starch showed inhibitory effect against the abovementioned test cultures, but the film proved to be negative.

Incorporating chitosan and lauric acid into starch-based film showed more effective antimicrobial ability against *B. subtilis* and *E. coli* (Salleh et al. 2007) the film had synergistic antimicrobial effect when chitosan and lauric acid were combined.

The application of electric fields has also been an important instrument among researchers in the area of edible films and coatings, and there are works showing that the application of electric fields promotes a significant improvement of several properties. Garcia et al. (2009) analyzed the effect of an electric field applied during drying on the microstructure and macroscopic properties of films obtained with different mixtures of chitosan (CS) and methylcellulose (MC). The analysis indicated that CS electrically treated film exhibited a more ordered structure lower WVP and higher Young's modulus values leading to stronger films. The authors concluded that electric field treatment would be a good alternative to improve film flexibility and water vapor barrier properties.

Lei et al. (2007) reported that using ohmic heating for the production of protein–lipid films improves the yield of the film formation rate and the rehydration capacity of the films. Souza et al. (2010) determined the effect of field strength on the functional properties of chitosan coatings. Chitosan films formed from solutions subjected to electric fields at 100 V cm^{-1} or higher were found to have lower values of oxygen and CO_2 permeability. Atomic force microscopy (AFM) observation of chitosan film surface treated at 100 V cm^{-1} or above showed smoother surface as opposed to a rougher surface of untreated films.

10 Increasing the Shelf Life of Foods

Edible coatings could reduce moisture transfer, restrict oxygen uptake, lower respiration, retard ethylene production, seal in flavor volatiles, and carry additional functional ingredients (such as antioxidants and antimicrobial agents) that retard microbial growth and potential discoloration. Many trials and investigations have been made to improve the performance of the edible films (Coma 2008; Rabea et al. 2003; Sathivel et al. 2007; Elsabee et al. 2008).

Pen and Jiang (2003) controlled the browning of fresh-cut Chinese water chestnut (CWC) by treating it with aqueous solution of 0.5, 1, or 2 g chitosan/100 mL, placed into trays over-wrapped with plastic films and then stored at 4 °C. The untreated samples develop surface discoloration of after 3 days of storage at 4 °C and became more serious after 6 days, while the eating quality decreased markedly. Treatment with chitosan coating delayed the development of the discoloration. Furthermore, increasing the concentrations (from 0 to 2 g) of chitosan/100 mL coating enhanced the inhibition of the discoloration and resulted in better appearance maintenance. Application of chitosan coating also inhibited effectively

disease development of fresh-cut CWC. The use of an antimicrobial coating consisting of chitosan-added yam starch helped in controlling the microbiota present in minimally processed carrot, inhibiting the growth of lactic acid bacteria, total coliforms, psychrotrophs, yeasts and molds, and mesophilic aerobes, thereby prolonging its shelf life (Peiyin and Barth 1998). In a different work, Durango et al. (2006) used coatings based on 4 % yam starch (w/w) + 2 % glycerol (w/w) and coatings based on 4 % yam starch (w/w) + 2 % glycerol (w/w) + chitosan in 0.5 % and 1.5 % concentrations, and carrot slices were immersed into these coatings. Starch + 1.5 % chitosan-coated samples after 14 days storage at 10°C showed reductions in mesophilic aerobes, mold, and yeast and psychrotrophic counting of 1.34, 2.50, and 1.30 log cycles, respectively, compared to the control. The presence of 1.5 % chitosan in the coatings inhibited the growth of total coliforms and lactic acid bacteria throughout the storage period. The use of edible antimicrobial yam starch and chitosan coating is recommended by the authors for controlling microbiological growth in minimally processed carrot (Durango et al. 2006).

Elsabee et al. (2008) used the modified polypropylene films by chitosan and chitosan/pectin multilayer as a packaging device for storing and increasing the shelf life of tomato, and they fabricated bags 20 × 20 cm using 12 multilayers (composed of chitosan/pectin) over corona-treated polypropylene (PP) films. Fresh tomato fruits of regular size (50 g) were collected, cleaned, and stored in bag (A). Another similar fruit was stored in regular PP bag (B), and a third one was kept in air (C). The three species were kept in a refrigerator at 4 °C. The above experiment was done in triplicate. The samples were investigated at intervals, and after 13 days the samples were compared, and it was found that samples B and C deteriorated completely, while the sample in the treated bag was kept almost intact with no apparent rotting infection.

Chien et al. (2007, 2013) used chitosan coating on mango and papaya slices which helped in increasing their shelf life by retarding water loss and drop in sensory quality, increasing the soluble solid content and the acidity and ascorbic acid content. It also inhibited the growth of microorganisms. Chitosan (CS1, 1 % w/w solution) was used for producing chitosan film and used for glazing skinless pink salmon fillets by Sathivel et al. (2007) which led to a delay in lipid oxidation in skinless pink salmon fillets after eight months of frozen storage. The chitosan film was a very good barrier to oxygen, while having low WVP. Chitosan-coated fillets had a higher thaw yield than that of the non-glazed control. Their study demonstrated the potential of chitosan solution as an edible glazing for pink salmon fillets (Sathivel et al. 2007).

Strawberries (*Fragaria x ananassa cv. Camarosa*) were treated with 1 % or 1.5 % chitosan acetate solution, with or without the addition of calcium gluconate (Hernández-Muñoz et al. 2006, 2008). The treatments consisted in immersing fruits for 5 min in (a) 1 % chitosan acetate, (b) 1.5 % chitosan acetate, (c) 1 % chitosan + 0.5 % calcium gluconate, and (d) 1.5 % chitosan + 0.75 % calcium gluconate solution. Fruits were allowed to dry for 2 h at 20 °C and were subsequently stored at 10 °C and 70 ± 5 % RH. Uncoated strawberries showed signs of fungal decay after the third day of storage at 10 °C. After six days of storage, 33.5 % of uncoated fruit

was infected by molds, while no sign of fungal decay could be detected by visual inspection of fruits coated with 1.5 % chitosan or 1.5 % chitosan + 0.75 % calcium gluconate. Of the fruit coated with 1 % chitosan, 12.5 % was observed to be infected on the sixth day of storage.

To extend the shelf life of strawberries, Vu et al. (2011) have used several essential oils together with chitosan as coating films. Red thyme (RT) and oregano extract (OR) were found as strong bioactive agents against molds and total flora isolated from strawberries, whereas limonene (LIM) and peppermint (PM) had lower antimicrobial properties. These essential oils were sprayed over the strawberries at 4 °C. RT, PM, and LIM were found to be more efficient preservative agents for strawberries during 14 days of storage. A bioactive coating has been formulated by mixing chitosan derivative with palmitoyl chloride which introduced a long hydrophobic group onto the chitosan backbone increasing thus its hydrophobic character. This modification ensured controlled release and improves its stability and adhesion to the fruit product. Formulations based on modified chitosan containing LIM and Tween®80 were shown to perform better than other formulations (Vu et al. 2011).

Duan et al. (2011) investigated different coatings on the postharvest quality of prewashed fresh blueberries. Both acid-soluble and water-soluble chitosan coatings showed potential for reducing rate of decay of blueberry during room temperature storage. The effects of edible coatings, Semperfresh™ (SF), acid-soluble chitosan (ACH), water-soluble chitosan (WCH), calcium caseinate (CC), and sodium alginate (SA) on the fruit quality of fresh blueberries during storage were studied. Semperfresh™ is a commercial coating product of sucrose fatty acid ester and was reported to effectively decrease weight loss of hardy kiwifruit, cherry, and summer squash and extend shelf life of pineapple (*Ananas comosus*) for up to 5 weeks by preventing moisture loss (Duan et al. 2011). Sodium alginate is a natural linear polysaccharide and has many attractive physical and biological properties, such as moisture retention, gel-forming capability, and good biocompatibility. The ACH, WCH, and WCH + SA coatings helped reduce the decay rate of "Duke" or "Elliott" fruit during room temperature storage. Their data (Duan et al. 2011) suggest that edible coatings have potential for retaining quality of prewashed, ready-to-eat fresh blueberries under commercial storage conditions, when appropriate coating material, container, and method of applying the coatings are used.

Edible coating with chitosan was effective in inhibiting bacterial growth and reduced the formation of volatile bases and oxidation products significantly of Indian oil sardine (Sardinella longiceps) in iced condition. Mohan et al. (2011) used edible chitosan coating (1 and 2 %) with degree of deacetylation of 83 %, and it was found that the coating was effective in inhibiting the bacterial growth and reduced the formation of volatile bases and oxidation products significantly. The chitosan coating improved the water holding capacity, drip loss, and textural properties significantly compared to untreated sample. The eating quality was maintained up to 8 and 10 days for 1 and 2 % chitosan-treated sardine, respectively, compared to only 5 days for untreated samples. Similar investigations were carried out by Fan et al. (2009) for the preservation of silver carp during frozen storage. Fish samples were treated with aqueous solution of 2 % chitosan and then stored

at −3 °C for 30 days. The control and the treated fish samples were analyzed periodically for microbiological (total viable count), chemical, and sensory characteristics. The results indicated that the fish samples retain their good quality characteristics with longer shelf life during frozen storage, which was supported by the results of microbiological, chemical, and sensory evaluation analyses.

Chitosan/whey protein film as active coating of ricotta cheese was studied by Di Pierro et al. (2010). The film had 35 % and 21 % lower oxygen and carbon dioxide permeability, respectively, and about three times higher WVP than film prepared with chitosan alone; they found that edible film reduced the growth of microbial contaminants and extended the shelf life of the product packed under modified atmosphere at 4 °C. Xing et al. (2010) investigated the effect of chitosan coating containing anti-browning agents and modified atmosphere packaging (MAP) on the browning and shelf life of fresh-cut lotus root stored at 4 °C for 10 days and showed that the proposed coating and modified atmosphere packaging could improve and extend the shelf life of fresh fruits. This information could be useful for the development of novel application to edible coating and MAP design for lightly processed lotus root. Maqbool et al. (2010) used a combination of 10 % Arabic gum with potato dextrose agar medium amended with 1.0 % chitosan to suppress the mycelial growth and conidial germination inhibition of the fungus *Colletotrichum musae* that results in major economic losses of banana during transportation and storage. This fungus causes the disease anthracnose, and the proposed treatment with chitosan and Arabic gum could be used as a biofungicide for controlling postharvest anthracnose in banana.

Enzymatic browning is the second largest cause of quality loss in fruits and vegetables. Methods to prevent browning are the subject of a great deal of research in the field of the food industry (Ioannou and Ghoul 2013).

The effect of coatings in combination with anti-browning agents (1 % chitosan, 2 % ascorbic acid + 0.5 % $CaCl_2$, and 2 % ascorbic acid + 0.5 % $CaCl_2$ + 1 % chitosan) on minimally processed apple slices was studied during storage by Haiping Qi et al. (2011). Their results show that chitosan coating treatments effectively retarded enzymatic browning on minimally processed apples during storage and they effectively retarded or avoided tissue softening and apple slices underwent a little loss of firmness. Chitosan coating did not perform very well as water vapor barriers in apple slices. The use of $CaCl_2$ as a firming agent helped apple slices maintain firmness. Apples treated with chitosan at 1 % content also showed constant firmness throughout 8 days of storage due to low level of Ca^{2+} contained in aqueous chitosan solution. In contrast with chitosan coatings, noncoated apples showed reduced firmness. After 2 days of storage, control apples lost around 19 % of their weight, while coated apples lost 15 % of their weight ($p > 0.05$). Chitosan coatings on apple slices did not prove to work effectively as water vapor barriers during the entire storage period.

The effects of edible coatings and mild heat shocks on quality aspects of refrigerated broccoli were studied (Ansorena et al. 2011). Minimally processed broccoli was coated with either chitosan or carboxymethyl cellulose with or without a previous application of a mild heat shock for 1.5 min at 50 °C. Product was

packaged in multilayered polyolefin bags and stored at 5 °C for 18 days. Edible coatings exhibited a beneficial impact on broccoli quality. The weight loss in uncoated broccoli was found to be between two and five times higher compared to coated samples. Chitosan coating always presented the lower ascorbic acid degradation rates (twofold lower compared with control samples). Broccoli texture for uncoated samples increased significantly during storage. However, for carboxymethyl cellulose-coated broccoli, a slight increase in texture was observed, while for chitosan-coated broccoli no significant changes in texture were observed throughout the storage period. After the edible coating application, the microbial broccoli load dropped by around 1.5 and 0.9 logarithmic units in chitosan and carboxymethyl cellulose films, respectively. During storage, the application of chitosan coating significantly reduced the total microbial counts in the thermally and nonthermally treated uncoated samples. Among the assayed edible coatings, chitosan effectively maintained quality attributes and extended shelf life of minimally processed broccoli (Ansorena et al. 2011). Another use of chitosan coating for reducing browning in pears was conducted by Xiao et al. (2011) in which a combined effect of sodium chlorite SC dip treatment and chitosan coatings on the quality of fresh-cut d'Anjou pears was investigated. Pear wedges were immersed in SC solution, followed by coating with CH or the water-soluble derivative carboxymethyl chitosan (CMCH) solutions. The effects of the SC and coating treatments on polyphenol oxidase (PPO) inhibition and microbial inactivation were also evaluated. Results indicated that SC exhibited significant ($p < 0.05$) inhibition of browning and PPO activity. The SC treatment was also strongly effective in inactivating *Escherichia coli* O157:H7 on pear slices. Coating SC-treated pear slices with CH adversely affected the quality of pear slices by accelerating the discoloration of cut surfaces and increasing the PPO activity. On the contrary, coating SC-treated samples with CMCH significantly prevented the browning reaction and inhibited PPO activity. In addition, SC and CH/CMCH coatings maintained tissue firmness and did not affect weight loss.

11 Conclusion

Chitosan, a natural polymer which can be derived from many renewable resources, is a biodegradable eco-friendly polymer with many fantastic properties, to name but few antibacterial and antifungal efficiency, and is also a film-forming material which can be used in making edible biodegradable films. The demand for biodegradable films for the packaging industry became so intense and presently has attracted the attention of a vast spectrum of researchers. The renewable biodegradability is becoming now a great salvation for humanity against the accumulation of garbage pollution from nondegradable plastics coming from fossil origin. Chitosan is proven to be a versatile material in protecting food product whether fresh fruits, vegetables, or meat and fish products. Chitosan alone or its blends with many other degradable polymers can also be used for food protection and extending the shelf life of fresh products. The combination of chitosan with essential oils and

antioxidants was also very successful in prolonging the lifetime of many fresh and other food products. The antimicrobial efficacy of chitosan and its combination with many other bioactive materials is now the focus of an intensive research conducted all over the world. The easy chemical modifications of chitosan impart into it additional properties, for example, increase hydrophobic capacity, making it a better coating for fresh food to reduce water vapor permeability and weight loss.

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Characterization of Psyllium (*Plantago ovata*) Polysaccharide and Its Uses

29

Ashwini R. Madgulkar, Monica R. P. Rao, and Deepa Warrier

Contents

Abstract

The *Plantago* is one of the genera in family Plantaginaceae, a large genus of herbs or sub-herbs distributed mostly in the temperate region and a few in the tropics. Psyllium has been in use as a medicinal agent since ancient times throughout the world. It is used for treatment of constipation, diarrhea, hemorrhoids, and high blood pressure. In olden days it was also used topically to treat skin irritations, such as poison ivy reactions and insect bites and stings. The husk of the seeds of various species of psyllium is used for its medicinal properties. The primary ingredient of the seeds and husk is a mucilaginous polysaccharide. Psyllium has been reported for the treatment of constipation, diarrhea, and irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis), colon cancer, diabetes, and hypercholesterolemia. When mixed with water, the therapeutic efficacy of the drug is due to the swelling of the mucilaginous seed coat which gives bulk and lubrication. Psyllium increases the volume of the feces by absorbing water in the gastrointestinal tract, which stimulates peristalsis.

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Modification of the polysaccharide by cross-linking or derivatization has been done to investigate its use as pharmaceutical excipient with multifarious roles. Various cross-linkers that have been studied include methacrylamide, *N*,*N*-methylenebisacrylamide, and polymethacrylamide.

Keywords
Psyllium • Ispaghula • Dietary fibers • Constipation • Irritable bowel syndrome • Cancer • Hyperlipidemia • Acrylamides • Excipients

Abbreviations

AAm	Acrylamide
APS	Ammonium persulfate
DNA	Deoxyribonucleic acid
GLUT-4	Glucose transporter protein
HEMA	Hydroxylethylmethacrylate
HLA-B2712	Human leukocyte antigen
IBS	Irritable bowel syndrome
IP	Indian Pharmacopoeia
LDL	Low-density lipoprotein
MAAm	Methacrylamide
N,NMBAAm	*N*,*N*-methylenebisacrylamide
NVP	*N*-vinylpyrrolidone
PAM	Polyacrylamide
PMA	Polymethacrylic acid
PVA	Polyvinyl alcohol
RAPD	Random amplified polymorphic DNA
SCFA	Short-chain fatty acids
SHP	Seed husk of psyllium

1 Introduction

Natural carbohydrates are the world's most abundant, renewable, and biodegradable polymers. They are the most basic source of fuel for all life forms on earth. The simplest carbohydrates are the monosaccharides such as glucose, fructose, galactose, xylose, and mannose. Polysaccharides are complex carbohydrate polymers consisting of two or more monosaccharides linked together covalently by glycosidic linkages in a condensation reaction. They are usually insoluble in water because of their large molecular size. Polysaccharides can be classified as homopolysaccharides and heteropolysaccharides. A homopolysaccharide is one with repeat units of a single type of monosaccharide, whereas a heteropolysaccharide is composed of two or more types of monosaccharides. In both types of polysaccharide, the monosaccharides are linked in a linear or branched fashion. Polysaccharides are extremely important in organisms for the purposes of energy storage and structural integrity (Berg 2007).

Starch is used as a storage polysaccharide in plants in the form of both amylose and the branched amylopectin. Cellulose and chitin are examples of structural polysaccharides. Cellulose is used in the cell walls of plants and other organisms and is said to be the most abundant organic molecule on earth. Chitin has a similar structure but has nitrogen-containing side branches, increasing its strength. Polysaccharides also include callose or laminarin, chrysolaminarin, xylan, arabinoxylan, mannan, fucoidan, and galactomannan (Campbell 1996).

Complex carbohydrates are important dietary elements for humans and are commonly called as dietary fibers. Dietary fibers are classified as soluble and insoluble fibers. Soluble fibers cause an increase in intestinal transit time of food and are readily fermented in the colon into gases. Insoluble fibers may be metabolically inert and provide bulk to intestinal contents or may undergo fermentation in the large intestine. Insoluble fibers tend to hasten movement of food through the intestine (Dietary Reference Intakes for Energy et al. 2005; Eastwood and Kritchevsky 2005). Soluble fibers reportedly bind to bile acids in the small intestine, thereby reducing their absorption. This consequently leads to lower cholesterol levels in the blood (Anderson et al. 2009). Soluble fibers also interfere with the absorption of sugar, regularize blood lipid levels, and produce short-chain fatty acids as by-products of fermentation in the colon with wide-ranging physiological activities. Although insoluble fibers are associated with reduced diabetes risk, the mechanism by which this occurs is unknown (Weickert and Pfeiffer 2008).

Modern lifestyle has given rise to a plethora of diseases including diabetes, cancer, and cardiovascular diseases. The short-term and long-term side effects associated with the therapeutic regimen used for control of these conditions have shifted the focus to alternative methods of prevention, cure, and control which can work complementarily with the treatment. This in turn has spawned a worldwide interest in the benefits of dietary fibers, the so-called wonder food of the new millennium. Dietary fiber is regarded as important for the diet, with regulatory authorities in many developed countries recommending increase in fiber intake (Dietary Reference Intakes for Energy et al. 2005; Eastwood and Kritchevsky 2005; European Food Safety Authority 2014; Jones and Varady 2008).

1.1 Polysaccharides as Pharmaceutical Excipients

Excipients are an integral part of pharmaceutical dosage forms and comprise the greatest proportion in dosage units. Knowledge of the composition, function, and behavior of excipients is a prerequisite for the successful design, development, and manufacture of pharmaceutical dosage forms. Interest in the physical effects and properties of the excipients used in pharmaceutical formulations has increased in recent years due to the awareness of the fundamental effect that excipients can exert on the bioavailability, bioequivalence, and stability of formulations.

Globally, there is a renewed interest in investigating drugs and excipients from natural sources, especially from plant and marine sources that include starches obtained from corn, wheat, rice, and tapioca (Andreev 2004) and different types of

essential and aromatic oils. A number of polysaccharides like chitosan (Park et al. 2002; Sakkinen et al. 2003; Thanou et al. 2000), tamarind seed polysaccharide (Gholardi et al. 2000; Kulkarni et al. 2005; Sumath and Ray 2002; Miyazaki et al. 1998), psyllium husk (Fischer et al. 2004a), guar gum (George and Abraham 2007), xanthum gum (Attama et al. 2006; Khourefieh et al. 2007), and rice bran wax (Dolz et al. 2007) are currently being used or investigated for varied roles as pharmaceutical excipients which include mucoadhesion, gel former, drug release retardant, plasticizer, thickener, and binder. The investigations to explore and investigate newer and newer plant sources continue unabated despite the availability of a large number of synthetic excipients.

2 Psyllium

Psyllium has been in use since ancient times throughout the world. In India the use of *P. ovata* dates back to 1500 BC in the Ayurvedic system of medicine, whereas the Chinese have used it in traditional medicine for thousands of years. The European use of the herb dates back centuries, and in North America, psyllium gained prominence in healing only near the end of the twentieth century (http://savoursomepsyllium.blogspot.in/2010/11/historytraditional-use.html 2014; Truestar Health: http://www.truestarhealth.com/Notes/2150006.html#Traditional-Use 2014). The Indian and the Chinese have used psyllium since 1500 BC for treatment of constipation, diarrhea, hemorrhoids, bladder problems, and high blood pressure. It was also used topically to treat skin irritations, such as poison ivy reactions and insect bites and stings. Europeans and North Americans began using psyllium for cholesterol and blood glucose-lowering effects (http://www.psylliums.com/psyllium.htm 2014). Typically, psyllium was administered in powder, flakes, or granular form. These would then be diluted in liquids and drank or sprinkled over meals. Only recently have they been made available in tablets, capsules, and liquid forms (http://savoursomepsyllium.blogspot.in/2010/11/historytraditional-use.html 2014; Bluementhal et al. 1998).

2.1 Biological Source

The dried, ripe seeds of *Plantago afra* (*Plantago psyllium*), *Plantago indica* (*Plantago arenaria*), and *Plantago ovata* (Plantaginaceae) are used in medicine. The *US National Formulary* includes all three species under the name "Plantago Seed" (Trease and Evans 2008). Psyllium husk is the cleaned, dried seed coat (epidermis) separated by winnowing and thrashing from the seeds of *Plantago ovata* Forskal. It is commercially referred to as blond psyllium or Indian psyllium or ispaghula. The husk may also be obtained from *Plantago psyllium* Linne or from *Plantago indica* Linne (*Plantago arenaria* Waldstein et Kitaibel) commonly known as Spanish or French psyllium (Plantaginaceae), in whole or in powdered form (United States Pharmacopoeia 2006). As per British Pharmacopoeia, psyllium seed

Table 1 Properties of psyllium seeds

Characters	*Plantago ovata*	*Plantago afra*	*Plantago indica*
Origin	India, Pakistan	Cuba, Spain, France	Egypt, Mediterranean, Europe
Color	Dull pinkish gray brown	Glossy deep brown	Dull blackish brown
Shape	Boat shaped; outline ovate	Boat shaped; outline elongated ovate	Boat shaped; outline elliptical
Length	1.8–3.3 mm	2.0–3.0 mm	2.0–2.5 mm
Weight of 100 seeds	0.15–0.19 g	0.09–0.10 g	0.12–0.14 g

consists of the ripe, whole, dry seeds of *Plantago afra* L. (*Plantago psyllium* L.) or *Plantago indica* L. (*Plantago arenaria* L. Waldstein and Kitaibel) (British pharmacopoeia 1968). According to IP, ispaghula husk (isabgol husk, *Plantago*) consists of the epidermis and collapsed adjacent layers removed from the dried ripe seeds of *Plantago ovata* Forsk (Indian Pharmacopoeia 2010).

2.2 Geographical Source

Ispaghula is an annual herb cultivated in India in the states of Gujarat, Maharashtra, Punjab, and in parts of Rajasthan and Sindh Province of Pakistan (Rangari 2008). *Plantago psyllium* is cultivated in France and Spain for the European market. *Plantago ovate* seeds are cultivated in Southern Europe, North Africa, and West Pakistan (Gupta 2005). India exports about 90 % of the gross production of ispaghula and nearly 93 % of the export being husk. Psyllium husks and industrial powders are exported in countries such as USA, UK, France, Germany, Japan, Indonesia, Canada, Mexico, Sweden, Spain, Norway, Italy, Australia, Denmark, Korea, Pakistan, and some Gulf countries. The USA is the largest buyer of ispaghula from India and accounts for about 75 % of the total husks exports from India (http://www.psylliums.com/psyllium.htm 2014) (Table 1).

2.3 Cultivation, Collection, and Treatment

The crop requires marginal, light, well-drained sandy-loam to loamy soils having pH between 7 and 8. The plant does not have any specific nutrient requirement. It requires a cool climate and dry sunny weather during harvesting. Mild dew, cloudy, or light showers cause seed shedding (Trivedi 2004). Seeds are sown by broadcasting method in sandy, loamy soil. Addition of farmyard manure or animal manure (prepared basically using cow dung, cow urine, waste straw, and other dairy wastes) has favorable effect but generally ammonium sulfate is added as a fertilizer. Irrigation is done regularly at an interval of 8–10 days. Ispaghula is not significantly affected by pests or diseases, but the annual harvest greatly suffers due to

Fig. 1 Morphology of psyllium seeds [*Plantago ovata*]

storm and rainfall. *Plantago* wilt "*Fusarium oxyspirum*" and downy mildew are the major diseases of ispaghula. White grubs and aphids are the major insect pests. The crop is harvested when the flower spikes turn reddish brown, lower leaves dry, and upper leaves turn yellow. Harvested seeds are dried below 12 % moisture to enable cleaning, milling, and storage (Rangari 2008). Ispaghula husk is separated from the seed by crushing in flat stone grinding mills by winnowing. It is marketed as a separate commodity. It is more in demand and fetches more price than that of seeds. Raw psyllium seeds are fumigated in their storage area to avoid any contamination. Psyllium products like husk and powder are treated in the fumigation chamber with approved chemical reagents like methyl bromide as per latest international guidelines. Psyllium products can also be sterilized by ethylene oxide or gamma radiation, if required (http://phytosanitorysolution.com/ 2014). Seeds and husks of *Plantago major* Linn, *Plantago lanceolata* Linn, *Plantago psyllium* Linn, as well as seeds of *Lepidium sativum* are used as adulterants in ispaghula (http://www.psylliums.com/psyllium.htm 2014).

2.4 Morphology

Psyllium seeds are ovoid-oblong in shape, about 2–3 mm long and 0.8–1.5 mm wide (Fig. 1). They are pinkish gray to brown in color with a convex dorsal surface and concave ventral surface with a deep groove running lengthwise along the center (http://www.psylliums.com/psyllium.htm 2014) (Figs. 2 and 3a, b).

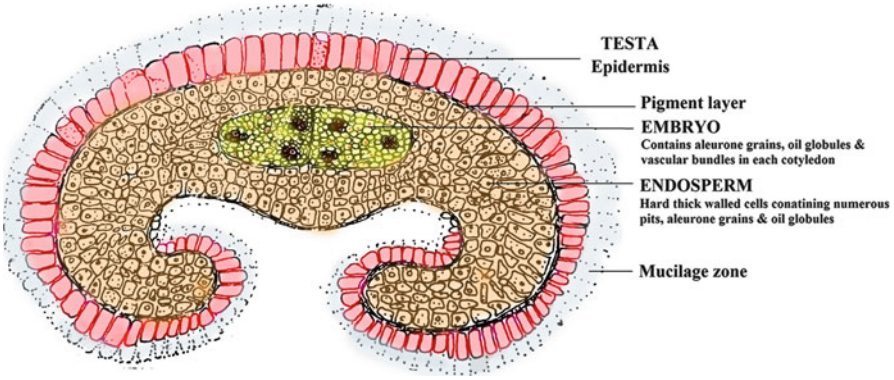

Fig. 2 Transverse section of seeds of *Psyllium ovate*

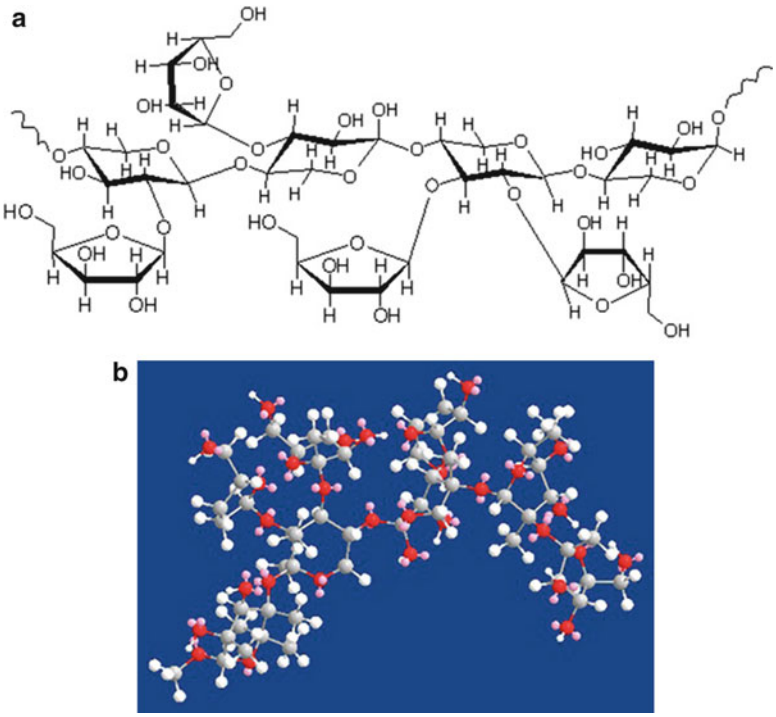

Fig. 3 (**a**) Chemical structure of arabinosyl (galactouronic acid) rhamnosylxylan. (**b**) Ball-and-stick model of arabinosyl (galactouronic acid) rhamnosylxylan

2.5 Microscopy

The transverse sections of the seed that cut through the central region possess a reniform outline and exhibit a spermoderm, endosperm, and embryo.

The spermoderm shows an outer epidermis of mucilaginous epidermal cells with obliterated walls in glycerine mounts; the radial and inner walls swell and disintegrate to form a clear mucilage upon irrigation of the mount with water, and a pigment layer with brown amorphous content. The endosperm is composed of irregular-shaped, thick-walled cells with walls of reserve cellulose. The outer layer of this region consists of palisade cells 15–40 μm in height. Aleurone grains and fixed oils are found in the endosperm cells (Bruneton 1995).

2.6 Chemical Constituents

Many elements have been identified in the seed which include proteins, lipids, sterols, triterpenes, and aucubin glycoside. The mucilage level is about 30 %. The principal constituent of the mucilage is 85 % of a soluble polysaccharide fraction dominated by D-xylose. The polymer backbone is a xylan with 1→3 and 1→4 linkages with no apparent regularity in their distribution. The monosaccharides in this primary chain are substituted on C-2 or C-3 by L-arabinose, D-xylose, and α-D-galactouronyl-(1→2)-L-rhamnose (Bruneton 1999). Psyllium seed mucilage contains 22.6 % arabinose, 74.65 % xylose and traces of other sugars, and 35 % nonreducing terminal residues (Fischer et al. 2004b).

2.7 Structure

The polysaccharide structure constitutes a densely substituted main chain of (1→4)-linked D-xylopyranosyl residues, some carrying single xylopyranosyl side chains at position 2, others bearing, at position 3, trisaccharide branches having the sequence 1-Araf- (1→3)-1-Araf. Chemically the polysaccharide is arabinosyl (galacturonic acid) rhamnosylxylan.

3 Clinical Indications

Psyllium has been reported for the treatment of constipation, diarrhea, and irritable bowel syndrome, inflammatory bowel disease (ulcerative colitis), colon cancer, diabetes, and hypercholesterolemia (Majmudar et al. 2008; Reynolds 1993).

3.1 Constipation

Psyllium husk is widely used for treatment of constipation due to its mucilaginous components.

When mixed with water, the therapeutic efficacy of the drug is due to the swelling of the mucilaginous seed coat which gives bulk and lubrication (Tyler et al. 1988). Psyllium increases the volume of the feces by absorbing water in the

gastrointestinal tract, which stimulates peristalsis (Read 1986). The intraluminal pressure is decreased, colon transit is increased, and the frequency of defecation is increased (Marteau et al. 1994; Sölter and Lorenz 1983). The effectiveness of fiber, and psyllium in particular, on constipation depends on the main cause of the constipation. In a study of 149 patients with chronic constipation, the consumption of 15–30 g daily of a psyllium seed preparation provided bowel relief in 85 % of participants who had no known pathological cause for their constipation. Only 20 % of individuals with slow transit responded to psyllium. A slightly greater percentage (37 %) of those with disorders of defecation – including rectocele, internal prolapse, anismus, and rectal hyposensitivity – found improvement (Alternative Medicine Review 2002; Voderholzer et al. 1997; Kumar et al. 1987; Qvitzau et al. 1988).

3.2 Fecal Incontinence

Psyllium has been shown to have the paradoxical property of both improving constipation by increasing stool weight and ameliorating chronic diarrhea. Because of its ability to retain water, psyllium has also been shown to benefit individuals with fecal incontinence from liquid stools or diarrhea. A placebo-controlled trial of persons with liquid stool fecal incontinence was performed in which supplementation with both gum arabic and psyllium showed approximately a 50 % decrease in the occurrence of incontinent stools. The psyllium group had the highest water-holding capacity of water-insoluble solids and total water-holding capacity of the stool (Alternative Medicine Review 2002; Bliss et al. 2001).

3.3 Hemorrhoids

With the known benefit of psyllium for both constipation and loose stools, psyllium was also reported to be beneficial for the treatment of hemorrhoids. It was found to have a significant improvement in reduction of bleeding and a dramatic reduction of congested hemorrhoidal cushions (Perez et al. 1996; Broader et al. 1974; Webster et al. 1978).

3.4 Irritable Bowel Syndrome (IBS)

Constipation is characterized by unsatisfactory defecation and infrequent stools, difficult stool passage, or both. On the other hand, the presence of clinically important abdominal discomfort or pain associated with constipation defines IBS with constipation. Intake of psyllium may be effective in alleviating chronic constipation in patients without slow colonic transit or disordered constipation. On the other hand, fiber with lactulose may improve stool consistency in patients with IBS with constipation. The easing of bowel dissatisfaction appears to be a major reason for the therapeutic success of psyllium in IBS (Degan and Phillips 1996; Bouchoucha et al. 2004; Koch et al. 1997).

3.5 Ulcerative Colitis (Crohn's Disease)

The two primary sites for Crohn's disease are the ileum, which is the last portion of the small bowel (ileitis, regional enteritis), and the colon (Crohn's colitis). A small number of studies have examined the ability of psyllium to maintain remission in ulcerative colitis. Dietary fiber has been proven to be beneficial in maintaining remission in human ulcerative colitis, an effect related with an increased luminal production of short-chain fatty acids (SCFA), i.e., acetate, propionate, and butyrate in the intestines. Dietary fiber supplementation ameliorated colonic damage in HLA-B2712. In an open label, randomized, multicenter trial of persons with ulcerative colitis, psyllium seed supplementation (10 g twice daily) was as effective as mesalamine in maintaining emission. It was inferred that this effect may possibly be due to increased levels of butyric acid with psyllium supplementation (Alternative Medicine Review 2002; Fernandez et al. 1999).

3.6 Appetite

Psyllium may also have an effect on appetite. A triple-blind study on 17 women looked at the effect of 20 g of psyllium seed 3 h pre-meal and again immediately post-meal during three 3-day study periods. The subjects reported significantly increased feelings of fullness 1 h after meals with the psyllium and exhibited a significantly lower fat intake with those meals (Turnbull and Thomas 1995).

3.7 Hyperlipidemia

Psyllium has been shown to reduce total cholesterol and low-density lipoprotein (LDL) cholesterol in animals and in humans (Fernandez 1995; Fernandez et al. 1995; Terpstra et al. 2000). Sprecher et al. demonstrated a 3.5 % reduction in total cholesterol and a 5.1 % reduction in LDL levels after consuming 5 g of psyllium husk twice daily for 8 weeks (Sprecher et al. 1993). Another study began with individuals on the American Heart Association Step 1 diet, followed by 8 weeks of psyllium, resulting in decreased total cholesterol (4.8 %) and LDL (8.8 %) (Bell et al. 1989). A meta-analysis was performed on eight trials of psyllium husk in conjunction with a low-fat diet in the treatment of hypercholesterolemia. After an initial 8-week, low-fat diet run-in, 10.2 g psyllium was given per day, resulting in a 4-percent reduction in serum total cholesterol and a 7-percent reduction in LDL cholesterol, compared to diet and placebo. A 6 % reduction in the ratio of apolipoprotein (apo) B to apo A-I was also noted (Anderson et al. 2000a; Levin et al. 1990; Anderson et al. 2000b; Olson et al. 1997). A meta-analysis of 12 studies of psyllium-enhanced cereal product consumption on total and LDL cholesterol in 404 adults with mild to moderate hypercholesterolemia demonstrated a reduction of 5 % of total cholesterol and 9 % of LDL cholesterol (Romero et al. 1998). Researchers studied the effect of fiber-enhanced cookies on blood

lipids in hypercholesterolemic men, using wheat bran-, psyllium-, or oat bran-containing cookies (wheat bran was used as the placebo since it has no demonstrated cholesterol-lowering effect). At the end of the 8-week study, plasma LDL cholesterol had decreased to 22.6 % in the psyllium group and 26 % in the oat bran group (Burton and Manninen 1982). A 4-month study of 12 elderly patients showed psyllium husk reduced total serum cholesterol by 20 %, a figure much higher than the abovementioned studies [(Stewart et al. 1991)]. In another study, a significant reduction in total serum cholesterol was noted in 176 elderly persons who used psyllium for 1 year (Vega-Lopez et al. 2001). The authors found that for every 1 g increase in daily psyllium dose, there was a 0.022-mmol/l (0.84 mg/dl) decrease in serum total cholesterol concentration. In a study to examine age and gender differences in the effect of psyllium on blood lipids, men and pre- and postmenopausal women were given psyllium (15 g daily) or placebo. Psyllium lowered plasma LDL cholesterol by 7–9 % in all groups. Triglyceride levels were lowered by 17 % in men, but were increased by 16 % in postmenopausal women. Premenopausal women displayed no significant shift in triglycerides (Agrawal and Pariddhavi 2012).

The mechanism of action of psyllium's hypocholesterolemic effects has not been fully expounded. Psyllium was shown to stimulate bile acid synthesis (7 α-hydroxylase activity) in animal models (Horton et al. 1994; Matheson et al. 1995) and in humans (Everson et al. 1992). The diversion of hepatic cholesterol for bile acid production is an established mechanism for reducing serum cholesterol. Psyllium's effect on the absorption of cholesterol (Everson et al. 1992; Turley et al. 1994) and fat (Ganji and Kies 1994) is negligible but may appear to contribute to cholesterol lowering. Additional mechanisms, such as inhibition of hepatic cholesterol synthesis by propionate (Anderson 1995) and secondary effects of slowing glucose absorption (Jenkins et al. 1990), may also play a role.

3.8 Diabetes Mellitus

Dietary fibers from psyllium have been used extensively in processed food to aid weight reduction, for glucose control in diabetic patients (Anderson et al. 1999). It has been shown to improve postprandial glycemic index and insulin sensitivity. In rats it was found to suppress sucrose and glucose absorption in the gastrointestinal tract. Studies also showed enhanced blood glucose disposal by regulating skeletal muscle plasma membrane GLUT-4 protein (glucose transporter) expression without phosphatidylinositol 3-kinase activation (Yu et al. 2009). The effect of psyllium husk was studied in 34 men with type 2 diabetes and hypercholesterolemia, given either placebo or 5 g psyllium twice daily for 8 weeks. Total cholesterol was lower by 8.9 % and LDL by 1 %. In addition, the postprandial rise of glucose was significantly reduced (Duke 1985). The mechanism for hypoglycemic effect in humans may be due to suppression of diffusion of glucose to small intestinal epithelium for absorption, delayed gastric emptying time, and reduced carbohydrate digestibility by retarding their access to digestive enzymes (Liangli and Wei Liu 2012).

3.9 Cancer

Seeds of *Plantago coronopus*, *P. lanceolata*, *P. ovata*, and *P. psyllium* were used by humans against cancer (Liangli and Wei Liu 2012; Hartwell 1982). Besides, the presence of luteolin-7-*O*-*b*-glucoside, a major flavonoid present in the leaves of *P. serraria*, *P. psyllium*, *P. coronopus*, and *P. lanceolata*, was able to strongly inhibit the proliferation of human cancer cell lines (Galvez et al. 2003). Butyric acid which is reported to exhibit antineoplastic activity against colorectal cancer is the preferred oxidative substrate for colonocytes and may be helpful in the treatment of ulcerative colitis (Whitkus et al. 1994). Butyrate may also dose-dependently suppress cancer cell proliferation, promote differentiation marker expression, and lead to reversion of cells from a neoplastic to nonneoplastic phenotype (Yu et al. 2009). In a study of patients with resected colorectal cancer, those given 20 g of psyllium seeds daily for 3 months exhibited an average increase of butyric acid production of 42 %, which decreased to pretreatment levels within 2 months of cessation of supplementation (Nordgaard et al. 1996).

Psyllium might also alter colonic sphingomyelin metabolism and apoptosis which may have an impact on colon tumorigenesis and inflammation in mice (Cheng et al. 2004).

4 Safety Data

4.1 Precautions

The recommended dose for psyllium varies as per the age and clinical indication. Nonetheless the precautions are common regarding safe use of psyllium. Psyllium should not be taken in case of suspected intestinal obstruction (ileus), diseases of the esophagus, and patients with difficulty in swallowing. It is also not recommended for patients with intestinal atresia and stenosis and for children below 6 years. It should be consumed with sufficient quantity of water, failing which may cause choking due to blockage of the throat or esophagus (New HMPC Commission 2014).

4.2 Drug Interactions

Bulking agents have been reported to reduce the absorption of some minerals (calcium, magnesium, copper, and zinc), vitamin B_{12}, cardiac glycosides, and coumarin derivatives (Gattuso and Kamm 1994; Hänsel et al. 1994a; Drews et al. 1981). The co-administration of psyllium with lithium salts has been reported to reduce the plasma concentrations of the lithium salts due to inhibition of their absorption from the gastrointestinal tract (Pearlman 1990). It has also been reported to decrease both the rate and extent of carbamazepine absorption, inducing subclinical levels of the drug. Therefore, ingestion of lithium salts or

carbamazepine and psyllium should not be concomitant (Etman 1995). Insulin-dependent diabetic people may require less insulin if they are concurrently taking psyllium (Bradley 1983).

4.3 Preclinical Safety Data

The material hydrates and swells to form mucilage in vivo as it is only partially solubilized. Less than 10 % of the mucilage gets hydrolyzed in the stomach. Preclinical studies in various animal species revealed pigmentation of various organs. Albino rats showed a dark pigmentation of the suprarenal gland, kidney, marrow, and liver after 125 days on a diet of 25 % of psyllium seeds. Dogs showed gray color of kidneys after a diet containing 25 % psyllium seeds for 30 days. Similar effects were not observed in humans. The pigmentation could be attributed to the dark pigment in the pericarp of the seeds (Hänsel et al. 1994b).

5 Preserving Genetic Integrity

Proper conservation and successful breeding of commercially viable crops is facilitated by appropriate identification of the plant. Conventionally, identification and classification of plant groups are solely based on differences in morphological features, especially the floral character which was considered to be consistent. It is well established that morphological characteristics are a result of the interaction between the environment and the genotype and can also be influenced by climatic and edaphic factors. Molecular techniques using DNA markers are therefore useful not only to identify the genotypes for authentication, but also in assessing and exploiting the genetic variability (Whitkus et al. 1994b). These techniques are suitable means for estimating genetic diversity because of their abundant polymorphism and the fact that they are independent of environment (Gepts 1993). DNA fingerprinting is a remarkable tool to create the genetic blueprint of all medicinal plants. Genetic diversity in five cultivars of *P. ovate* was estimated using molecular markers by Pal (Pal and Raychoudhuri 2004) and Raychoudhuri (Raychoudhuri and Pramanik 1997). Similarly, Vahabi et al. used molecular and morphological markers for the evaluation of genetic diversity between *P. ovate* (Vahabi et al. 2008). Wolff and Morgan-Richards used random amplified polymorphic DNA (RAPD) markers for differentiating subspecies of *Plantago major* (Wolff et al. 2000). Samantaray et al. used RAPD markers to assess genetic relationships and variance in seven species of Plantago, i.e., *P. ovata* (Forsk.), *P. indica* (L.), *P. arenaria* (Waldst.), *P. psyllium* (Linn.), *P. lanceolata* (Linn.), *P. serraria* (Linn.), and *P. coronopus* (Linn.) (Samantaray et al. 2010). In India, facilities have been developed for long-term storage of germplasm in the National Gene Bank in New Delhi, India, to avoid frequent regeneration and to check genetic drift (Trivedi 2004).

6 Chemical Modifications

A lot of work involving derivatization/cross-linking of polysaccharide obtained from psyllium seeds and husk is underway. The objective of these studies is to modify the properties of the polysaccharide in order to exploit it as a pharmaceutical excipient. Some of the pioneering works done in this field are enlisted below:

1. Singh and Sharma have developed psyllium–PVA–acrylic acid-based novel hydrogels for use in antibiotic drug delivery through graft copolymerization. The use of a very small amount of these petroleum products has developed low energy, cost-effective, biodegradable, and biocompatible material for potential biomedical applications. In this experiment, polyvinyl alcohol and acrylic acid were used as monomers; *N*,*N*-methylenebisacrylamide was used as the cross-linking agent, and ammonium persulfate was used as the thermal initiator (Singh and Sharma 2010).
2. Patil et al. have developed and evaluated psyllium seed husk polysaccharide-based wound-dressing films. In this study, wound-dressing films were fabricated using seed husk of psyllium (SHP) complexed with povidone iodine and were evaluated for various physicochemical properties as well as wound healing activity in albino rats (Basavaraj et al. 2011).
3. Singh and Chauhan have prepared psyllium- and acrylamide-/methacrylamide-based hydrogels, and the effects of pH on the release dynamics of insulin from drug-loaded hydrogels were studied to evaluate the drug release mechanism. Two types of hydrogels were prepared by using acrylamide and a mixture of acrylamide/methacrylamide. The hydrogels were named as psy-cl-poly (AAm) and psy-cl-poly (AAm-*co*-MAAm) hydrogels, respectively. *N*,*N*-methylenebisacrylamide was used as the cross-linking agent and ammonium persulfate was used as the thermal initiator (Singh and Chauhan 2010).
4. Sen et al. conducted research on a novel microwave-initiated method for synthesis of polyacrylamide-grafted psyllium (Psy-*g*-PAM). Psyllium was modified through grafting of polyacrylamide (PAM) chains on it using microwave radiations only, in the absence of any other free radical initiator. The synthesized grades of the graft copolymer were characterized through various physicochemical techniques. The flocculation efficacy of the synthesized graft copolymers was studied in kaolin and coal fine suspension through standard "Jar test" procedure. It was found that the high flocculation efficacy of Psy-*g*-PAM makes it a good candidate to be used as a flocculant for waste water treatment and treatment of effluents discharged from coal washeries (Sen et al. 2010).
5. Singh and Kumar prepared psyllium-*N*-vinylpyrrolidone (NVP)-based hydrogels by radiation-induced cross-linking. The reaction mixture was irradiated with gamma rays of a dose rate of 2.43 kGy/h, in 60 Co gamma chamber for a specific time. The cross-linking polymers thus formed were named as psy-cl-poly (NVP) hydrogels. These hydrogels were then studied for their release dynamics of anticancer model drug (5-fluorouracil). It was found that the release of the drug from the hydrogels occurred through non-Fickian diffusion mechanism (Singh and Kumar 2008).

6. Kumar and Sharma synthesized a new material (Psy-*g*-PMA) by grafting polymethacrylic acid onto the backbone of psyllium using a microwave-assisted method. Acetylsalicylic acid was incorporated in the various Psy-*g*-PMA samples and tablets were prepared to study the in vitro drug release. This biodegradable material is good for the fast release of medicine in acidic environments such as the human stomach. The material also demonstrated superabsorbent capacity for water and may be useful in diapers and feminine sanitary pads (Kumar and Sharma 2013).
7. Singh and Chauhan prepared psyllium 2-hydroxylethylmethacrylate (HEMA) and acrylamide (AAm)-based polymeric networks by using *N*,*N*-methylenebisacrylamide (*N*,*N*-MBAAm) as cross-linker and ammonium persulfate (APS) as initiator. It was concluded that the swelling of the modified psyllium-based hydrogels is affected by the composition of the hydrogels and pH of the swelling medium (Singh et al. 2008).

7 Conclusion

The surge in the development of newer drug molecules for the treatment of a plethora of diseases has definitely led to improved therapy, but it has also resulted in a concomitant rise in side effects and adverse reactions. This has led to a resurgence of plant-based traditional systems of medicine and nutraceuticals which either supplement or complement these therapeutic regimens. The impact of the role of dietary fibers, the "wonder food" of the new millennium, in the control and cure of many lifestyle diseases cannot be underestimated. Psyllium has been in use since ages for its various medicinal effects and continues to rule the roost for the treatment of constipation, diarrhea, irritable bowel syndrome, inflammatory bowel diseases, ulcerative colitis, colon cancer, diabetes, and hypercholesterolemia. The high mucilaginous content of psyllium seeds and husk has also spawned an interest in its use as pharmaceutical excipient. Chemical modifications have been done to improve its profile as multifunctional pharmaceutical excipient. Future studies could be aimed at exploring and discovering more uses of this remarkable but unassuming plant.

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Dietary Fiber and Prebiotics

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Contents

Abstract

Dietary fiber and prebiotics exert a great impact on health-promoting food for mankind. Under this aspect a general overview is given about the bioavailability of carbohydrates and their influence on dietary fiber intake and about the developing of the prebiotic concept and specific functional foods. Moreover, the occurrence and chemical composition of native dietary fiber such as resistant

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starch, pectin, hemicelluloses, ß-glucan, and fructan in context to their properties – in particular the prebiotic potential for human health – will be discussed. Important industrially produced bioactive carbohydrates from plant and seaweed sources with high prebiotic efficacy and increasing economic interest such as fructan – particularly inulin and fructooligosaccharides (FOS), heteropolysaccharides, xylooligosaccharides (XOS), and isomaltooligosaccharides (IMO) – will be presented. Additionally enzymatic processing of prebiotic-active oligosaccharides such as FOS, galactooligosaccharides (GOS), or nondigestible disaccharides such as isomaltulose and trehalulose derived from sucrose and lactose will be demonstrated and discussed.

Keywords

Nutrition values • Glycemic index • Functional food • Dietary fiber • Insoluble fiber • Soluble fiber • Prebiotics • Non-starch polysaccharides • Undigestible oligosaccharides • Fructan • Inulin • Resistant starch • β-Glucan • Heteropolysaccharides

Abbreviations

AACC	American Association of Cereal Chemists
AGO	Agarooligosaccharides
AOAC	Association of Official Analytical Chemists
dp	Degree of polymerization
FF	Functional food
FNB	Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences
FOS	Fructooligosaccharides
FUFOSE	Functional Food Science in Europe
GOS	Galactooligosaccharides
IOS	Inulooligosaccharides
IMO	Isomaltooligosaccharides
JNK	c-Jun NH_2-terminal kinase
NDO	Nondigestible oligosaccharides
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSO	Non-starch oligosaccharides
NSP	Non-starch polysaccharides
OF	Oligofructose
RS	Resistant starch
SCFA	Short-chain fatty acids
TCM	Traditional Chinese medicine
UCC	Unavailable complex carbohydrates
WHO	World Health Organization
XOS	Xylooligosaccharides

1 Introduction

1.1 Evolutionary and Historical Background of Human Nutrition

Two million years ago, at the evolutionary development of the *Homo sapiens* from ancient times, the herbivorous *Australopithecus* had a herbal diet that was the main basis of man's daily nutrition. Shortly after, animal diet, obtained by hunting or by consumption of carcasses (Cordain et al. 2000), delivered particular, long-chained polyunsaturated fatty acids enabling advancement of the human brain within evolutionary periods (Eaton and Cordain 2002). In the history of human development, this age is called the period of hunters and gatherers. Descendents of this era may still be found in semitropical regions of today's world, living on nutrition consisting of 60–80 % vegetable matter and about 20–40 % animal food (Eaton and Konner 1985).

Subsequently, starting about 10,000 years ago, cultivation of edible plants was performed intensely, and the era of farming was established (Gordon 1987). Based on agriculture and the production of fodder, it was possible to domesticate farm animals (Grupe 1992), a development followed by increasing consumption of animal food. At the same time, the utilization of milk from domesticated ruminants became an important food source, especially in the European region. The usage of milk, however, requires the genetic adaption of enzyme patterns for digestion of lactose, which took place with a large part of the European people. Yet in Asia and Africa, where milk is hardly used, lactose intolerance is highly pronounced and leads to serious symptoms of incompatibility. This fact proves convincingly the genetic adaption of distinct groups of humans to the intake of foods specific for certain regions in the course of evolution.

Reviewing the different stages of food development, humans can be classified as omnivores with the focus on plant nutrition. Today, a purely vegetarian diet is not a consequence of evolution but reflects certain social and cultural choices of the population. Based on the outstanding adaptability of humans to the availability of food, they possess sufficient genetic variability in order to enable their survival in different regions of the world. To this day, primitive populations with mainly plant and animal nutriment continue to exist their organism shows a fine adaption to the environment. Thus, the human organism, especially the intestinal tract and metabolism, is determined by the disposability of nourishment in many ways.

Only about 200 years ago, the personal living conditions of humans in the industrial regions changed because of a new working world (industrial democracy). Owing to the flight from land and the resulting increased accumulation of people in the cities, the profile of food consumption was totally changed and adapted to the new situation.

Dramatic changes of eating habits occurred: in Germany, for instance, the annual consumption of meat per capita rose from 34 lbs (pound) in the beginning of the eighteenth century to 200 lbs in 1990. Thus, the energy supply of food from animal fare increased to about one third of the total energy consumption. Likewise, the

consumption of animal proteins was raised from primarily 20 % to about two thirds of the total supply of proteins (Lemnitzer 1977). At the same time, drastic innovations happened in the food industry such as the production of foodstuffs with superfine flours, and refined sugars on one side and semifinished goods free from fiber at the other side. Particularly the portion of finely ground flours (superfine flour, refined grains, etc.) with a content of less than 4 % fiber was drastically enhanced and represents more than 80 % of the cereal consumption at present (Wiebke 2004). These changes of eating habits, happening generally in the highly developed industrial countries of the world, reflect the status of nutrition for the population of these countries.

1.2 Living Standard and Diseases of Civilization

Particularly after the Second World War which had caused a short-term undersupply of nutrition for certain groups of the population in many European countries, a targeted rehabilitation of the social system was undertaken in the following decades. Unfortunately, the policies implemented failed to propagate nutrition appropriate to the human species with a high proportion of plant food. On the contrary, the returning prosperity and the resulting living standard were provided with indices in no way compatible with the requirements of healthy nutrition. The consumption apiece of refined sugar and meat became indicators of improved living standards at the yearly statistical reports.

Only in the late 1980s it was recognized that the so-called civilization food with its huge portion of refined sugar, superfine flour, polished rice, animal fat, and protein does not represent a nutrition adequate for the prosperous, akinetic citizen. The problem is that the human body with its excellent adaptability copes for a long time with food extremely denatured and/or poor in fibers. Hitherto unknown disease patterns appeared, summarized under the term lifestyle diseases. Thus, because of inadequate eating habits combined with a deficit of exercise, diseases such as obesity, diabetes mellitus type 2, hypertension, hypercholesteremia, cardiovascular diseases, apoplexy, cancer, musculoskeletal disorders, tooth decay, and weakening of the immune system became commonplace, especially during the last decades (Chadwick et al. 2003; Schaafsma 2004).

1.3 Fast Food: Slow Food

The convenience-oriented marketing of fast-food chains has developed successful strategies that are at variance with the requirements of healthy eating. The fast-food industry is based on foods with long shelf-lives while preserving the valuable food ingredients. However, these foods usually contain significantly more calories than unprocessed, natural foods. Processed foods can be chewed and swallowed faster, and therefore, their saturation efficacy is lower although the consumption of calories is the same. This is one of the essential causes of hypercaloric nutrition.

Fast food only partly fulfills the requirements of the processing food industry. For enterprises marketing fast food, the preparation should preferably require little time and the consumption of the products should be as fast as possible; thus, the components of fast food are industrially prefabricated, minimizing the time of preparation. Finished and half-finished food products for domestic use, the so-called convenience food, also need little time of preparation. Typical examples of fast and convenience foods are precut French fries, deep frozen hamburgers and pizzas, and industrially produced sauces and dressings. These products stimulate substantial fat consumption, large portions of insulin-activating carbohydrates, and disproportionate protein consumption. Especially the rapid degradation of denatured starch products to glucose leads to a short-time elevation of blood sugar and to the cancelation of hungry feeling. This effect, however, vanishes quickly, resulting in new feelings of hunger and additional food consumption without real need. Exceeding consumption of such products is overly stressful to the human organism and frequently followed by uncontrolled obesity.

In 1986, the Slow Food organization was founded in Italy as a counterculture to the fast-food chains, espousing a cuisine based on pleasurable, regional, whole foods. This group counts presently more than 100,000 members in 150 countries around the world. In the light of a species-appropriate nutrition with a high proportion of plant food, the movement tries to give a positive input concerning healthy, tasteful, and satiable fare. Both animal and plant foods are represented in a well-balanced form. The dishes contain little or no fat; proteins are derived from animal as well as from plant sources. For the uptake of carbohydrates, the availability of vegetable raw materials is essential. Thus, whole-meal noodles, natural rice, whole-grain bread, vegetables (mainly in raw, uncooked form), and fruits are emphasized. Because of the constituent substances such as resistant starch and fiber, the level of blood sugar is kept constant and addephagia stay away.

As indicated above, human nutrition was and still is adapted to the regional conditions and may consist of different items of plant and animal food. Plant nutrition, scarcely processed, can be considered species appropriate and optimal for individual health. Additionally, the energy input of food should be controlled and attuned to the physical activity. With increased supply of animal food with high fat content on one side and little exercise on the other side, the risk to contract diabetes type 2 is clearly augmented (Van Dam et al. 2002; Aune et al. 2009).

2 Bioavailability of Carbohydrates

In principle, the nutrition of humans is formed by a mixed diet of about 55–60 % carbohydrates, 10–15 % proteins, and 30 % fat (Elmadfa and Leitzmann 2004). The proportion of digestible carbohydrates however is essentially determined by the ingested food and its processing. As already shown above, highly processed carbohydrates particularly plant starch, industrially produced sucrose, lactose from dairy products, or monosaccharides such as glucose and/or fructose occurring mainly in honey, vegetables, and fruits are converted to glucose by digestive

enzymes and delivered to the human body. Glucose is an essential energy provider for human cells besides fat and protein, the main energy provider, however, for blood and brain cells.

2.1 The Human Alimentary Tract

The digestion of glucose containing α-(1,4)-linked and α-(1,6)-linked polysaccharides, the so-called starches, is partly performed already in the mouth by α-amylase, contained in the saliva – a kind of predigestion into smaller fragments. After passing the stomach, these fragments are basically degraded to glucose by pancreatic and intestinal amylases and maltase in the upper small intestine and transported to the liver by cells of the intestinal mucosa using the portal circuit.

Likewise dietary fibers contained in food are channeled through this way and thus provided for fermentation by the microbiota of the colon. Such fibers are carbohydrates being not degraded by digestive enzymes of the human body. Depending on the kind and amount of plant food, the proportion of insoluble fiber, being very important for the peristalsis of the colon, is rather fluctuant and very different because of digestion of differing plant raw materials like grain. Generally high and sustainable saturation values have to be achieved at the uptake of carbohydrates consisting mainly of starch and fiber. The proportion of resistant starch should be held relatively high by appropriate food processing. A high portion of fibers and starch enhances the volume of nutrition (high water absorption) and makes the effect of saturation immediate. With plant food such as vegetables, potatoes, and whole-grain products, a decelerated transfer of glucose into the blood is achieved and the hungry feeling is delayed. High portions of fiber with high water-binding capacity cause expansion of the emptying time from the stomach to the small intestine, and thus, the feeling of satiety is ongoing for a longer time.

2.2 Glycemic Index and Glycemic Load

The use of finely ground flours and refined sugar produces dishes with high availability of glucose. At consumption of such dishes, the blood sugar load reaches peak values followed by an exceeding insulin secretion in order to lower the concentration of blood glucose back to the normal level. Nutrition rich in fiber causes an improved glucose tolerance which has a positive effect especially on the adjustment of the metabolism of persons suffering from diabetes type 2 (Meyer et al. 2002). For a better control of nutrients, the parameter “glycemic index” (GI) was established in the 1980s of the last century.

The glycemic index refers to the increase in blood glucose following the consumption of food containing 50 g of carbohydrate. The percentage compared to the increase following uptake of the same amount of glucose is calculated. The area corresponding to the glucose uptake is set equal to 100 (Jenkins and Jenkins 1987; Ludwig and Eckel 2002). The glycemic index of whole-grain products is

Table 1 Glycemic index and glycemic load of selected food items (Foster-Powell et al. 2002)

Food item	Glycemic index (glucose = 100)	Serving size (g)	Glycemic load per serving
With high starch content			
Long-grain rice	56	150	24
Jasmine rice	109	150	46
Basmati rice	58	150	22
Instant rice	87	150	36
Spaghetti, durum wheat	47	180	23
Baked potato	60	150	18
Boiled potato	70	150	19
French fries	75	150	22
Vegetable, fruit			
Raw carrot	16	80	1
Banana	42	120	11
Grapes	43	120	8
Apple	28	120	4
Mango	51	120	8
Orange	40	120	5

smaller than that of products from white flour. Pulse rich in dietary fiber need not be considered at the calculation of carbohydrates for diabetics; they hardly have any effect on blood sugar levels (Foster-Powell et al. 2002).

The glycemic index is standardized for carbohydrates and subject to strong fluctuations depending on the way and treatment of carbohydrates and on the composition of the respective food. For better comparison, the parameter of "glycemic load" was introduced. The dimension of the glycemic load is determined by the product of glycemic index and grams carbohydrate per serving of food (Table 1). Thus, a higher proportion of fat in a nutrient (e.g., chocolate) has a rather restraining effect on the rise of glucose levels due to retardation of glucose absorption. Also starch-containing foods (e.g., potatoes) may influence the availability of glucose very differently depending on its treatment.

2.3 The Influence of Dietary Fiber on the Enterohepatic Circulation of Bile Acids - Salts

Bile salts, synthesized from cholesterol only in the liver, are pooled in the gallbladder for their application. Due to the physiology of the gastrointestinal tract, bile salts and acids emulsify no polar compounds (cholesterol, lipids, fat) in order to be absorbed passively in the small intestine and in the jejunum, however actively in the terminal ileum. Bile salts return to the liver via the portal vein to be actively taken up and secreted once again. The rate of synthesis is determined by the availability of cholesterol and, more importantly, by the returning bile salt pool, regulating hepatic

synthesis of bile salts in a negative feedback manner. Only a small portion of bile acids/salts, regularly about 5 %, are lost from the intestine to the exit in the feces with each circuit (Hofmann 2011).

Due to its absorptive properties, dietary fiber manipulates the enterohepatic circulation. Depending on the structural composition of the dietary fiber, different amounts of free bile acids can be fixed and released into the feces. Lignin, arabinoxylan, and pectin show high, ß-glucan (oat bran), guar and alginate still distinct, native celluloses and hemicelluloses only low absorptive properties. Bile acids/salts, being absorbed more intensely in an acid environment than in a neutral one, are thus deprived of recirculation to the liver. To hold the bile acid pool at the same level, now synthesis from cholesterol is necessary anew. By the transformation of cholesterol to bile acids, the total cholesterol level in blood is lowered. At this process the level of undesirable LDL cholesterol is more reduced than the level of the requested HDL cholesterol. By enhanced uptake of dietary fiber from beans, oat bran, guar, and pectin, the cholesterol concentration in the serum could be reduced at about 5–18 % (Kasper 2000) because of absorption and excretion of bile acids and phospholipids.

3 Functional Foods

To ameliorate public health and to enhance personal well-being, great efforts were made worldwide in the last decenniums, developing different projects and programs. Thus, for interaction of healthy food, movement, and fun, the term "wellness" was created, causing an essential revaluation of the idea of health in the population. With the aid of the food industry, regional organizations, and the public health system, new marketing strategies were developed, granting access to a mature citizen responsible for a healthy nutrition, rich in dietary fiber. Thus, the concept of "functional food" had won recognition on the market enriching the fare of the individual more and more.

3.1 Classification of Intrinsic and Extrinsic Functional Food

Starting in Japan food having a certain healthy impact and exceeding its diet-related effect is marked as functional food (FF). The definition of FF is different depending on the producing country. Thus, the Japanese definition is relatively strict claiming natural origin for FF and an own licensing procedure for the name "food for specified health use." In Europe and the USA, the regulations for FF are not totally clear: Besides natural materials, also synthetically produced substances can be used in FF. According to Functional Food Science in Europe (FUFOSE), functional foods are basically foods consisting essentially of bioactive ingredients and their impact should be achieved by normal consumption habits; FF should be part of the daily food and not applied in capsules, in tablets, or in form of powder (Schaafsma 2004; Stehle 2000). There is demand of regulation especially relating to the statements about health, relating to the detectability, and relating to the patent

protection of FF. As one of the continuative expressions for FF, the term "designer food" got attention underlining particularly the effect of foods for tailor-made needs, e.g., nutrition for special groups of persons such as sportsmen, elderly people, or babies. The term "nutraceuticals" meaning bioactive molecules of plant origin is a synonym for FF as well.

Presently the specific value of bioactive molecules and element groups is only partly known. Also the synergistic interaction of these substances both isolated and in connection with nutrients is given only rudimentarily. Basically the knowledge about the biologic activity of single substances delivers additional parameters for a better design and for potential improvement at the processing of preventative food.

Two groups of FF can be distinguished: One group includes enough natural health-promoting components without any addition of bioactive substances marked as "*intrinsic* FF" containing, e.g., bioactive carbohydrates such as dietary fiber and resistant starch or secondary plant components such as phytochemicals and vitamins or health-promoting microorganisms such as probiotics. The second group of FF contains additions of health-promoting substances isolated from natural plant raw materials in order to receive *"extrinsic* FF" in terms of nutritional physiology mostly valuable products with tailor-made demand (Kiefer et al. 2002).

3.2 Human Health and Economic Interests

As already mentioned food should help to avoid or relieve diseases. The connections between diseases of civilization such as adiposities, coronary diseases, and cancer on one side and the daily nutrition on the other side are documented more and more; these diseases might be enhanced by genetic disposition, unlimited consumption of cigarettes, or aging structure. Thus, the target of health policy moves away from the relief of deficiency diseases toward a preventive nutrition strategy optimizing the health condition of the individual and coming along with prolongation of life and improvement of life quality. On behalf of the food industry and trade, this approach is supported vigorously and communicated with different strategies and campaigns to the consumer. The large developing potential of production and trade of FF in the USA, Europe, and Asia verifies the growing economical interest. Supported by research initiative, a considerable increase of sales and profit can still be expected in this market segment.

4 Dietary Fibers: Prebiotic Concept

4.1 Dietary Fiber

4.1.1 Definition and Determination

Dietary fibers are effective bioactive substances in terms of nutritional physiology. Because of its positive impact on the human organism, they gain more and more importance both in the daily nutrition and in the production of intrinsic and extrinsic FF.

The positive effects of dietary fiber in food were already taught by Hippocrates in the school of nutrition in Greece 400 years B.C. These findings were known as well in Asian cultures such as China and India and are the pillars of their traditional medicine till today. Recently the application of practice of Traditional Chinese Medicine (TCM) and of the Indian Ayurvedic attitude toward life gain more and more acceptance in highly industrialized countries and are protagonists for a healthy way of life.

In 1940 the term "dietary fiber" was defined for the first time in literature relevant for food and nutrition, and the responding methods of determination were published. First studies about the composition of dietary fiber concerning the attenuation of risks of lifestyle diseases were published in 1953 (Hipsley 1953). Cleave demonstrated in 1970 the positive impact of plant cell carbohydrates on human health and developed the first scheme of dietary fiber (Cleave 1974). This definition was revised in 1972 and in 1986 (Trowel 1972; Trowel and Burkitt 1986). Trowell established a hypothesis about dietary fiber, indicating that a too small proportion of dietary fiber in food might be a possible reason for diseases of civilization. Further modified definitions were used in 2001 and 2002 by the American Association of Cereal Chemist (AACC) and by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences (FNB). AACC defined dietary fiber as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine; dietary fiber including polysaccharides, oligosaccharides, lignin and associated plant substances promotes beneficial physiological effects causing laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (Jones 2000). This description includes non-starch polysaccharides, resistant poly- and oligosaccharides, and analogous carbohydrates. In highly developed countries, the definition and the analysis of dietary fiber are often different, leading to mistakes at comparison of proportions of dietary fiber in food. Therefore, indication of the analytical methods is important in tables of and at further information about nutrients.

The detection of the crude fiber is the oldest method for the determination of dietary fiber; the results represent only 20–50 % of the real physiologically effective amount of dietary fiber because of neglect of soluble dietary fiber. Nowadays the recommended official AOAC method 985.29 for a total dietary fiber determination in foods (Prosky et al. 1985) is a gravimetric analysis after enzymatic digestion imitating the human digestion. Low-molecular soluble substances such as oligosaccharides are not caught in this process. Because of upgrading of the definition for dietary fiber – the differentiation into indigestible insoluble and soluble carbohydrates – an adaption of analysis methods was made for determination and distinction of these components including oligosaccharides starting with a degree of polymerization (dp) of 3 (Prosky 1999).

4.1.2 Classification: Soluble and Insoluble Fibers

Dietary fiber can be classified either according to its chemical structure and constituents or according to its solubility or dispersibility in aqueous systems.

Usually dietary fibers are non-starch polysaccharides, resistant oligosaccharides, analogous carbohydrates (e.g., indigestible dextrins, synthesized carbohydrate compounds), lignin and substances associated with the non-starch polysaccharide, and lignin complex in plants (e.g., waxes, phytates, tannins, saponins).

Polysaccharides, such as cellulose, high polymer hemicelluloses, and ß-glucans, and the polyphenylpropan lignin are insoluble fibers. These fibers, however, possess a high water-binding capacity and thus deliver a greater fecal bulk and increase the rate of transit of material through the large intestine called a "decrease in the transit time." Vegetables, wheat, rye, and most grain products contain a relatively high part of insoluble fibers.

Pectins, gums, mucilages, and low polymer hemicelluloses possess a high dispersibility and viscosity and are partially soluble in aqueous systems. Together with water-soluble hemicelluloses and fructans, these carbohydrates can be classified as soluble fibers. Soluble fibers tend to have little effect on the fecal bulk. But as an available fodder for the microflora, they deliver indirectly additional fecal bulk for growing up of bacterial organisms. They decrease the rate of transit of material through the upper gastrointestinal tract and tend to delay the rate of absorption of nutrients, probably because of their ability to form viscous solutions. They may decrease the rate of absorption of glucose by the small intestine as well and may reduce the rate of increase of plasma glucose following the intake of food, and they also may reduce plasma cholesterol levels. Fruits, fructan-containing vegetables, oat, barley, and legumes have a relatively high content of soluble fibers (Kasper 2000; Table 2).

4.1.3 Daily Consumption: Recommended Intake and Their Application

Dietary fiber is collectively acknowledged to be one of the key factors in a health-promoting diet. Today the intake of dietary fibers is not adequate in many developed countries. The intake of fibers per head in Europe amounts to 18–22 g day^{-1} and in the USA even only 13–15 g day^{-1}. This intake does neither meet the recommendations of the World Health Organization (WHO) nor those of the country itself. The WHO recommends more than 25 g day^{-1} (WHO 2003) for dietary fiber intake. Comparatively in rural areas of developing countries, the intake of dietary fiber amounts still to 50–120 g day^{-1}. This proves that generally the supply of dietary fiber decreases with higher material prosperity. At a targeted diet (e.g., vegetarians), however, the intake may rise up to about 40 g day^{-1} (Elmadfa and Leitzmann 2004).

Nutrition high in dietary fiber does not only have a lower energy content but also is able to absorb harmful substances from the food such as saturated fat, a fact that partly may explain the effect of dietary fiber on health. It is unlikely that a diet for adults could have too high fiber content; however it may cause flatulencies being rather inconvenient. Foods containing traditionally no dietary fiber, but being consumed more and more in the current diet of many countries could be enriched by addition of fiber – a new way for the production of healthy food. Consumer's interest in ready-to-eat foods and functional beverages is increasing, and thus, their dietary fiber content could possibly be modified (Elleuch et al. 2011).

Table 2 Dry matter and content of total and soluble dietary fiber in selected vegetable, fruit, and cereals

		Content in fresh matter	
Food item	Dry matter%	Total dietary fiber g.100 g^{-1}	Soluble dietary fiber g.100 g^{-1}
Vegetable			
Bean[a]	9.8	3.0	0.2
Cabbage[a]	9.8	2.3	0.3
Carrot[a]	11.0	3.7	1.2
Potato[a]	20.0	1.8	0.2
Spinach[a]	10.0	2.6	0.4
Tomato	5.9	1.0	0.15
Fruit			
Banana	18.9	1.8	0.7
Mango	20.1	2.0	1.0
Watermelon	5.6	0.6	0.3
Apple	13.4	3.2	0.9
Cereals[b]			
Wheat	88.5	14.9	2.2
Rye	88.0	20.5	4.8
Oat	87.5	11.5	2.9
Barley	88.5	17.6	4.1

[a]Cooked
[b]Grain

4.1.4 Energy Efficiency

The uncertainty about the energy availability of the indigestible part of foods has centered on dietary carbohydrates, in particular non-starch polysaccharides (NSP) and oligosaccharides, resistant starch, and sugar alcohols (Livesey 1992).

From the side of legislative, energy calculations of foods will be extensively unified in different countries or regions of the world. The European Council Directives for current energy values are 10 kJ (2.4 kcal) g^{-1} for all sugar alcohols; for dietary fiber no values have been assigned yet.

Basically the variation in the availability of dietary energy has been related formally to the occurrence, utilization, and effects of those dietary carbohydrates escaping small intestinal digestion. For unavailable carbohydrates found in conventional foods as energy provider, the term unavailable complex carbohydrates (UCC) has been applied. Both NSP and nondigestible resistant starch (RS) contain UCC. There are few published studies about the energy value of NSP alone and few about the starch fraction that escapes digestion in the small intestine. Where studies are undertaken with NSP, carbohydrate is often defined more precisely, for example, as guar gum, crystalline cellulose, inulin, or FOS. Studies about RS deliver results with different energy values depending on quality characteristics.

Generally the combustion heat of UCC equals the energy content of digestible carbohydrates with 17 kJ (4.3 kcal) g^{-1}. Assuming a possible digestibility with a factor of 0.7 for UCC in mixed diets, and a conversion energy for the fecal bacteria of 0.3 kJ per kJ fermented carbohydrate, a value of 8.4 kJ g^{-1} or 2 kcal g^{-1} can be calculated valid for every intake of the UCC. Energy values of UCC related to its apparently small digestibility allow to assign specific energy values to individual carbohydrates and to consider energy losses due to fermentation, thus giving net energy values.

Specific energy values for digestible NSP – e.g., of wheat bran with 4.2 kJ (1.0 kcal) or of apple with 8.2 kJ (2.0 kcal) g^{-1} – prove the different availability of dietary fiber by the microbiota in the large intestine. Similar effects can be shown for water-soluble carbohydrates such as fructan. Inulin, a high polymer fructan, possesses an energy value of approx. 8.4–9.2 $kJ.g^{-1}$. FOS (dp 3–10) with a good accessibility for fermentation deliver values between 12 and 13 kJ g^{-1}. But the effective net energy values of these dietary fibers are about 30–40 % lower than the calculated values in the diet of the human (Roberfroid et al. 1993; FAO/WHO 2003).

4.2 Prebiotic Concept: Influence of Gut Flora and Health Benefits

Generally dietary fiber promotes bioavailability of nutrients, participates in regulation of transit time and intestinal motility of nutrition in the gastrointestinal tract, and supports the formation of a healthy colonic microflora. The specific health-promoting effect of nondigestible food ingredients in particular soluble carbohydrates takes place in the large intestine. Carbohydrates being not digested in the small intestine reach the colon, where they can be partly or totally fermented by the microflora. The efficiency of fermentation is influenced by the monomer composition, the type of glycosidic linkages, the dp, and the structural arrangement of the molecule. Thus carbohydrates of different sources with different structure and properties stimulate the growth and colonization of various bacteria with properties healthy for the human well-being.

Based on this knowledge, the prebiotic term and definition was published (Roberfroid 1993; Gibson and Roberfroid 1995): A prebiotic is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host's health." This prebiotic concept supports the assumption of the efficacy of selected carbohydrates as growth substrate for beneficial bacteria already resident in the colon.

Thus, fructan, for instance, such as both inulin and FOS in the diet or as supplement in foods stimulates the growth of bifidobacteria selectively, maintaining populations of potential pathogens at relatively low level. These findings were successively confirmed in diet-controlled human studies (Roberfroid 1998). Further studies about NSO and RS oligomers as substrate for the microflora confirm this positive effect of the soluble carbohydrates for different beneficial bacteria such as lactobacilli in the colon.

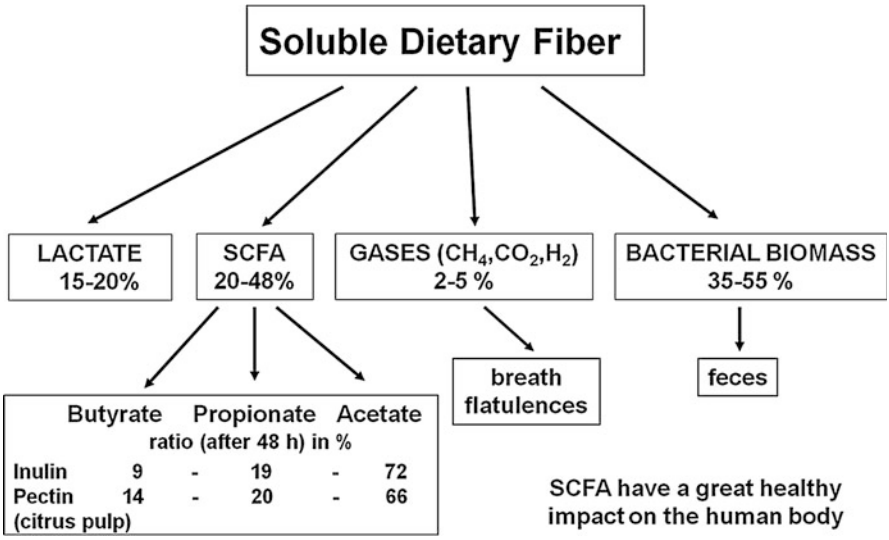

Fig. 1 Fermentation of soluble dietary fiber in the colon by the gut microflora

The major products of prebiotic metabolism are short-chain fatty acids (SCFA), the gases hydrogen, carbon dioxide and methane (in ca. 50 % of persons) and bacterial cell mass. The proportions of SCFA between acetate, propionate, butyrate and lactate are related to the carbohydrate sources in the colon (Fig. 1). In vivo animal studies showed that supplementing a diet with nondigestible oligosaccharides (NDO) decreases the fecal pH and increases the size of the fecal pool of SCFA, with acetate the primary acid followed by butyrate and propionate (Roberfroid and Slavin 2000). When incubated in vitro with a human fecal flora, inulin and FOS produce SCFA. In both cases the butyrate concentration compared to acetate, propionate and lactate was positively stimulated.

Acetate is the energy source; it is transported to the muscle tissues in the body and is a potential precursor for cholesterol synthesis. Contrariwise propionate transported into the liver inhibits the cholesterol synthetase, thus reducing the cholesterol, triglyceride, and LDL level in the blood. Butyrate plays an essential role in the maintenance of colonic mucosa integrity, by acting on metabolism, proliferation, and differentiation of epithelial cell types. It prevents distal ulcerative colitis and reduces the risk of intestinal diseases such as inflammation or certain forms of colon cancer.

Additionally SCFA liberated during the fermentation of prebiotics increase the mineral absorption of calcium in the intestine. Low pH promotes solubility of minerals, and fatty acid absorption is directly accompanied by mineral absorption. The growth of the epithelial cells in the colon stimulated by butyrate might increase their capacity of absorbing minerals. The higher absorption of calcium reduces the risk of osteoporosis and promotes better bone structure and density (Cieślik et al. 2009).

Some studies report about the positive stimulation of the associated immune system of colon by prebiotics. Especially the production of a number of proinflammatory cytokines (e.g., tumor necrosis factor alpha) and the expression of a number of receptors on T and B lymphocytes and macrophages were reported (Sotnikova et al. 2002).

4.3 Probiotics and Their Combination with Prebiotics: Synergistic Effects

The term probiotics for living bacteria was created in the 1960s of the last century for the first time in the case of animal feed supplements in order to reduce antibiotics in the animal husbandry. Living bacteria in pharmaceutical products have been on the market for a long time for different medical applications without being named probiotics.

Probiotic microorganisms affecting the intestinal flora do not act exclusively in the large intestine. They also affect other organs of the human modulating immunological parameters, intestinal permeability, and bacterial location or provide bioactive or regulatory metabolites. Under this aspect the definition of probiotics is "a preparation of or a product containing viable, defined microorganisms in sufficient number, which alter the micro flora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in the host" (Schrezenmeir and De Vrese 2001).

The older definition of probiotics refers to cultures of defined viable microorganisms which when applied to man or animal reaching the large intestine in an active state may beneficially improve the properties of indigenous flora.

Both pro- and prebiotics are based on the concept of multiplying those bacteria in the large intestine, which are health promoting or at least not pathogenic or toxic. The combination of pro- and prebiotics is called symbiotic meaning that a synergistic interaction is happening between a specific probiotic and a particular prebiotic (Topping et al. 2003).

For application in the daily diet, probiotic (and prebiotic) foods contain living probiotic microorganisms in an adequate matrix and in sufficient concentration. Today, most of probiotic foods are fermented milk products. Yogurt products with probiotic cultures have a market share of over 15 % with increasing tendency. There are shortcomings with using probiotics for gut health: Only a small proportion of ingested organisms reach the colon unbrokenly; probiotic organisms are washed out of the gastrointestinal tract if probiotic consumption decreases. Prebiotics may safeguard against these losses by providing physical protection and by slowing the rate at which bacteria are lost after cessation of probiotic consumption. In such symbiotic foods, the concentration of prebiotics is often below 10 %. However, the term "symbiotic" should only be used in case of a true synergistic mutual reinforcement (Roberfroid 1998).

5 Occurrence, Chemical Composition, Classification, Properties, and Prebiotic Efficacy of Essential Dietary Fibers

5.1 Bioavailability of Starch: Resistant Starch

Starch is the reserve polysaccharide of a lot of plants being applied as starchy foods. In the vegetal reproduction of annual and biennial plants, starch is transferred into granules being stored in roots, tubers, and bulb organelles such as in potatoes or cassava roots (manioc). During the generative propagation of plants, a continuous storage of starch takes place while simultaneously granules are developed in the amyloplasts of endosperm cells in the seeds. At the same time, the cell body is dehydrated and the water content of seeds reduced. Finally, starch granules in the endosperm of cereals such as wheat, rye, rice, and corn contain about 10–13 % of water. Generally starch granules are insoluble and tightly packed. Size and shape of these granules vary among plant species and even among cultivars of the same species.

Chemically, starch is a glucan being composed of α-(1,4)-linked and/or α-(1,6)-linked D-glucopyranosyl residues. There exist two main structural types of starch: highly branched amylopectin being represented 70–80 % in the granules and the low/non-branched amylose with helical structure. X-ray diffraction pattern confirms crystalline structure of starches being identified as A and B types. Starch granules of cereals with water content between 10 % and 13 % deliver the A-type spectrum. Granules of potatoes and amylose-rich starches with water content between 15 % and 16 % deliver the B-type spectrum. C-type starches, being a mixture of A and B types, are found in legumes. At cooking or hot-water treatment, starch granules swell and disintegrate, resulting in viscous starch solutions being ready for digestion: Thus, starch can be successfully hydrolyzed by enzymes in the small intestine to yield free glucose being then absorbed.

In 1992 while the development of an in vitro assay of non-starch polysaccharides it was found that some part of starch could not be removed by human amylases – it was resistant against these enzymatic attacks. Following in vivo studies with ileostomy subjects, the presence of starch components being resistant against digestion in the stomach and small intestine could be confirmed. These starch components, however, could be fermented in the colon. The term resistant starch (RS) was formed and used to describe starches with such properties (Englyst et al. 1992).

The term RS is not defined by any government agency. The definition was done in a concerted action by the European Union (EU) in the EURESTA (1994) program. RS is defined as the total amount of starch (and the parts of degraded starch) resisting digestion in the small intestine of healthy people (Asp 1992). In the large intestine, RS can act as a substrate for microbial fermentation, the end products being hydrogen, carbon dioxide, methane, and SCFA. This definition described RS only concerning its physiological functionality.

As to the aspect of resistance against enzymatic attack in the small intestine, the knowledge of physical and chemical characteristics of RS as healthy properties is essential (Nugent 2005). Starches being amorphous or crystalline with B- and C-type patterns, high-amylose level, and high disposition for retrogradation appear to be more resistant to digestion. Retrograded starches are referred to certain structural forms of RS. Retrogradation occurs when starch with preferably high content of amylose is cooked in water beyond its gelatinization temperature followed by cooling. Amylose retrogrades and adopts amorphous structure, whereas amylopectin rather shows crystalline structure – both delivering a more complex insoluble structure – retrograded starch with high stability against enzymatic attack.

Resistant starch has been classified into four general subtype groups called RS1–RS4:

The term RS1 is given to RS with physical inaccessibility to digestion, e.g., due to the presence of intact cell walls in grains, seeds, or tubers. In this case the factor of milling and chewing influences the amount of RS content. Whole or partly milled grains, legumes, and pasta products deliver mainly RS of subtype RS1.

RS2 describes ungelatinized native starch granules with crystallinity of B type as potato starch being only slowly hydrolyzed by α-amylases. Digestion is prohibited by conformation or structure of the starch granules as in partly raw potatoes, green bananas, and some legumes. One particular RS2 type is unique – it retains its structure and resistance even during processing and preparation of many foods – these RS2 starches are called high-amylose starches being found in corn, pea, or cassava.

RS3 refers to nongranular starch-derived materials resisting digestion. These RS3 types are generally formed during the retrogradation of starches. Examples for this type are cooked and cooled potatoes, processed cornflakes, different kinds of bread especially after longer storage, and food products produced under specific process conditions at prolonged and/or repeated moist heat treatment.

RS4 describes a group of starches having been chemically modified, e.g., etherized, esterified, or cross-linked with chemicals in such a manner as to decrease their digestibility. At present it is not known if and how the various modifications of RS4 are affected by digestion in vivo.

Thirty to seventy percent of RS under consideration with these different profiles can only be absorbed in the large intestine and deliver high prebiotic efficacy similar to non-starch polysaccharides.

5.2 Pectin

Pectin is found in the primary cell wall of plants such as fruits and vegetables, particularly citrus and apple fruits and in unripe plants. The structure of pectin shows variation with botanical origin, cell wall type and cell wall development. Extracted pectin can be used in the cuisine or in the food industry as gelling, thickening, and film-forming agent. The sources of commercial pectin are apple

pulp or citrus peels. In daily food apples, pears, kiwis, plums, grapefruits, lemons, and oranges are some common sources of pectin.

From the point of chemical composition, pectin is a polysaccharide consisting basically of D-galacturonic residues (main chain) with different kinds of sugar such as L-rhamnose, L-arabinose, D-galactose, and marginally D-xylose in the side chains of the molecules. It is partly esterified with methanol and possesses non-branched and highly branched regions. Pectin forms network structures such as gels, where the chains are interconnected to form a three-dimensional network. This network can be solved at higher temperature; the gel however reforms after cooling (MacDougall and Ring 2004).

The free carboxyl groups of the galacturonic residues enable absorption of calcium and magnesium ions influencing the mineral balance in the intestinal tract. However, the application of pectin amid produced from citrus pectin, being nontoxic and approved as food additive, may avoid this problem.

Pectin is a soluble fiber and effective as prebiotic carbohydrate. Recent studies have shown that pectin promotes beneficial bacteria, lowers blood cholesterol levels, and speeds up recovery from infection due to its ability to promote anti-inflammatory healing cells (Brouns et al. 2012).

5.3 Cellulose, Lignin, and Hemicelluloses

Structure-giving substances of plants are cellulose, lignin, and hemicelluloses located in the cell wall. Normally cellulose microfibrils are linked via hemicellulose links to build up the cellulose-hemicellulose network, being enclosed in the pectin matrix. During ripening or because of stress in plant organs, a lignification process to woody material can take place. In vegetables the content of cellulose and lignin accounts for around 30 % and 3 %, in fruits 20 % and 17 %, and in cereals 17 % and 7 %, respectively, relating to the total polysaccharide fiber content in plant organs.

Cellulose, the most abundant organic molecule in the nature, is a non-branched polymer of several thousand glucose units connected by β(1,4) linkages. Cellulose, an insoluble fiber, occurs in plants in tightly packed aggregates and tends to resist microbial hydrolysis.

Lignin, a three-dimensional network built of units of phenylpropane, is the component of the tough or wood-like portions of plants and seeds. Bran and other cereal products are the main sources of dietary lignin. This fiber is insoluble and differs from others by the fact that it is degraded neither by human enzymes nor by the gut microflora.

Hemicelluloses are heterogeneous polymers occurring in various chemical structures. Different plant sources deliver heteropolysaccharides such as arabinoxylan, glucomannan, glucuronoxylan, or xyloglucan. Most of these polysaccharides contain pentose sugars such as D-xylose and L-arabinose linked with hexose sugars such as D-mannose, D-galactose, D-glucose, and D-rhamnose, and sometimes include acid sugars such as glucuronic or galacturonic acid (Elleuch et al. 2011). At the investigation of plant material, water-insoluble and

water-soluble fractions have been found. Soluble fractions in particular xylan and arabinoxylan deliver high prebiotic efficiency.

5.4 ß-Glucan

High amount of β-glucan is found in cereal grains, in particular in the bran of wheat, rye, oat, and barley. Furthermore, fungi from different provenience possess a high content of β-glucan. The β-glucan content of oat and barley varies between 3 % and 11 % making these grains useful natural sources of β-glucan in nutrition. Isolated β-glucan can also be added to cereal based or other foods, thus expanding the selection of possible sources of fiber in the diet. Commercial food products containing β-glucan are, e.g., pasta, oat flakes, cereals, and bakery products.

β-Glucan is a non-branched polysaccharide consisting only of β-D-glucopyranosyl units. These units are linked by either (1,3)- or (1,4)-β-D-linkages; hence, its exact name is mixed-linked (1,3),-(1,4)-β-D-glucan. Building blocks of 1,3-linked cellotriosyl and cellotetraosyl units constitute over 90 % of the molecule. The (1,3)-linkage prevents close packing of the molecule and makes it partly soluble in water unlike cellulose; 20 % of oat and 40 % of barley β-glucan are insoluble (Åman and Graham 1987).

Soluble β-glucans deliver viscous and shear-thickening solutions even at quite low concentrations. Therefore, they are suitable alternatives for thickening agents in foods, e.g., beverages, sauces, salad dressings, and ice creams. Barley β-glucan, for instance, was found to produce a higher viscosity than pectin at a concentration of about 0.5 %.

In principle the efficacy of β-glucan in the human intestinal tract is dependent on its dose, viscosity, molecular weight, and solubility. The potential physiological mechanisms causing the efficacy of β-glucan are suggested to be its ability to retard the absorption rate of food in the intestine due to increased viscosity, in this way balancing the postprandial glucose and insulin response (Wood 2001). Additionally, high viscosity in the small intestine interferes with cholesterol absorption or reabsorption, in this way affecting the cholesterol balance and synthesis in the body (Sayar et al. 2005; Drzikova et al. 2005). β-Glucan has been shown to reduce elevated blood cholesterol levels and balance blood glucose and insulin response after meals; thus, it possesses a high prebiotic efficacy.

5.5 Fructan

About 15 % of plants, monocotyledons as well as dicotyledons, contain fructan as reserve polysaccharide instead of (sometimes besides) starch. In contrast to starch fructan possesses excellent water solubility and is stored in cells of plant-specific organs as roots, tubers, bulbs, blades, stems, or in the bottom of flower buds. In all cases the biosynthesis and storage of fructan takes place only in particular compartments of cells – in the vacuoles.

Table 3 Dry matter and content of total fructan and fructooligosaccharides (FOS) from plant organs

		Content in fresh matter		
Plant organ	Dry matter %	Total fructan g.100 g^{-1}	FOS dp 3–10 g.100 g^{-1}	Linkages type
Composites				
Chicory, root harvest autumn	25	16.2	6.5	Inulin β-2,1
Jerusalem artichoke, tubers harvest fall	24	15.1	7.5	
Harvest spring	18	9.7	7.2	
Globe artichoke	18.2	9.0	1.3	
Liliaceae				
Onion, bulb	13.5	5.3	4.6	β-2,1 and β-2,6 linked
Leek	12.0	6.6	3.0	
Chives	11.5	6.5	n.d.	
Garlic, bulb	32.5	19.1	0.2	
Asparagus	6.5	1.8	1.0	
Agave, stem	31.5	14.3	9.9	
Banana	25.7	1.4	n.d.	
Poaceae (cereals)				
Wheat, grain	88.5	2.2	n.d.	Levan β-2,6
Rye, grain	88.0	4.8	n.d.	
Oat, grain	87.5	0.4	n.d.	
Barley, grain	88.5	2.7	n.d.	

A lot of fructan plants have been applied in the daily diet of humans preferring more plant food with high level of dietary fiber and prebiotic efficiency. Table 3 shows the fructan content of plants being applied in the cuisine and/or consumed as vegetable or fruit, raw or cooked (Praznik et al. 2004).

Fructan-containing vegetables from roots such as salsify, murnong (yam daisy) and chicory; from tubers such as yacón and Jerusalem artichoke; from bulbs such as onion, garlic and leek; from shoots of asparagus or chicory; or from the stem of agave are delivered by the organs of annual, biennial, or perennial plants cultivated by vegetal propagation. The flower buds of globe artichoke, banana, and the grains of cereals, also applied as vegetable and fruits, are components of the generative propagation of plant. Further fructan sources are flour and products of cereals containing between 1 % and 7 % fructan depending on the degree of milling and food processing.

As to the chemical composition of fructan, three types were found in plants: the inulin-type with β(2,1)-linked fructofuranosyl residues starting from the trisaccharide 1-kestose, the levan type with β(2,6) linked fructan starting from 6-kestose, and the mixed-type fructan containing both β(2,1)-linked and β(2,6)-linked fructofuranosyl residues. Fructans are nonreducing carbohydrates due to their

origin from the sucrose pool of plants. The high variability in elongation, polymerization, and degradation of fructan molecules during their vegetation period delivers polymers with different dp depending on harvest time. Under this aspect the amount of fructooligosaccharides (FOS) in plants – per chemical definition fructans from dp 3 up to 10 – can differ more or less. Mixed-type fructan from garlic or agave with a significant branching structure delivers molecules with random coil characteristic and therefore offers an extremely better solubility in water compared to inulin with mainly helical structure (French and Waterhouse 1993; Praznik et al. 2007).

From the aspect of the physiological functionality in the human body, all three fructan structures deliver the same effect in the intestinal tract: The β-configuration in the linkages of fructan cannot be attacked by the digestion enzymes of the small intestine, which are more specific for α-glucosidic linkages of the starch polymers. In vivo studies with volunteers have demonstrated that fructans such as inulin and FOS are practically indigestible in the small intestine of the human (Andersson et al. 1999). Thus, fructans pass through the small intestine without degradation and without influencing the absorption of nutrients or minerals, only increasing the bulk, directly to the colon where they will be fermented by the microflora to healthy components such as SCFA, thus helping to establish the proper balance of a beneficial intestinal flora. No parts of fructan were ever recovered in the feces. Because of these healthy properties of fructan, its prebiotic effect is excellent and it may easily support the well-being of the human.

At the intake of fructan-containing food in both intrinsic and extrinsic way, the bowel peristalsis is mobilized because of the high water-binding capacity of fructan; the mineral absorption particularly of calcium is enhanced and the energy input is low.

5.5.1 Fructan Plants for Daily Use and Industrial Application

Composites

Chicory (*Cichorium intybus* L.*)* roots contain 18–20 % soluble carbohydrates with a content of inulin-type fructan between 14 % and 18 % (Praznik et al. 2004, Table 3). In several regions, in particular in Belgium, France, and the Netherlands besides the roots even the sprouts of chicory (chicoree) are used as salad and vegetable of light bitter taste due to the taste-dominating bitter compound intybine (lactucopicrin) being contained in the roots as well as in the sprouts of chicory.

The first use of chicory roots was the production of a "healthy" and compliant coffee surrogate: Sliced chicory roots containing fructan in combination with the bitter compounds were roasted together with additional ingredients such as malt from barley and figs.

After the finding that inulin was nondigestible in the human intestinal tract, it became a perfect tool to test renal clearance.

For the last two decades, the interest for inulin as source of soluble dietary fibers has been ongoing. Consumer request for healthy food and consequently "tailored" FOS and inulin matches perfectly the demand of application profiles of prebiotic

foods in various market segments. Of course the predominance of large-scale inulin production from chicory is supported by the relatively simple possibility of cultivating this crop, easy harvesting of the roots, and the utilization of the already existing sugar beet processing technology. The most crucial point in isolation and purification of inulin is the quantitative elimination of salts and bitter compounds.

Jerusalem artichoke (*Helianthus tuberosus* L.) tubers are another source of inulin-type fructan and FOS. In some US areas (California, Washington, Minnesota), Canada, and Europe (Belgium, France, Germany, Ukraine), Jerusalem artichoke tubers are popular vegetables known as sunchoke or topinambur. Recently the interest for Jerusalem artichoke as a vegetable and for industrial application is increasing also in Asia particularly China. Different varieties and different time of harvesting – both autumn and spring in one vegetation period deliver different carbohydrate profiles in the tubers with more or less FOS concentrations. Tubers are consumed crude as well as boiled, baked, or fried. In particular goods made from autumn tubers are a perfect basic food for diabetics because of very low sucrose content.

The tubers of Jerusalem artichoke contain no bitter compounds and can be processed without pretreatment. Moreover, the FOS of Jerusalem artichoke tubers are nonreducing; due to this fact, they form no Maillard products and do not become brownish in low-temperature processing.

However, enzymatically catalyzed oxidation may be observed in juice production if phenoloxidases are not deactivated. Powders of dried and milled slices of Jerusalem artichoke tubers can be utilized at the manufacture of bread and bakery products (Praznik et al. 2002). Utilizable total carbohydrates typically range between 18 % and 20 % containing approx. 90 % fructan with dp >3.

The vegetable **globe artichoke** (*Cynara scolymus* L.) contains about 10–12 % inulin-type fructan in the bottom of the flower bud. The origin of this plant traces back to Ethiopia and it entered South European countries via Egypt. Currently globe artichoke is cultivated in France, Italy, Romania, Bulgaria, and California/USA. The crop grows to a height between 60 and 200 cm and forms pronounced sprouts (flower buds) being utilized as vegetables. For use in many different ways, the sprouts typically are boiled. For therapeutic purposes, extracts of globe artichoke roots and leaves are known for more than 400 years. The effect of such extracts is caused by phenol carbon acids (cynarin, caffeic acid, chlorogenic acid), leteolines (luteolin, cynarosid, scolymosid), and the bitter compound sesquiterpene lactone (cynaropicrin). The composition of inulin and FOS strongly depends on the status of the shoots. Globe artichoke before flowering contains inulin in its flower bottoms with a dp between 60 and 120 being nearly five- to tenfold compared to inulin of other composites.

Liliaceae

Allium L. species, such as **onion**, **leek**, **garlic,** and **chives**, are worldwide applied in the cuisine of any cultures as vegetables, spices, and tasty components. Much of the well-known health preserving and even therapeutic effects of onion and garlic most probably are due to their sulfur-containing compounds. However, even the aqueous

soluble low and high molecular mixed-type fructans, being present in onion and garlic in high percentages, are jointly responsible for the positive image of stimulating the intestinal tract and supporting consumer's well-being. Onion, leek, and chives contain only 3–7 % FOS and fructan, however, predominantly FOS with dp between 3 and 10. Garlic differs significantly with about 19–24 % of fructan with a mean dp of 26.

Sprouts of **asparagus** (*Asparagus officinalis* L.) are a worldwide popular vegetable containing 2–4 % fructan, similar to onion, with mixed-type structure, typical for Liliaceae.

Agave (*Agave tequila* Weber L. var. Azul) from the family of *Agavaceae* has been applied traditionally for the tequila production in Mexico. The content of fructan is 20–23 % being a mixed-type fructan with low content of FOS. Due to the negligible content of monosaccharides (glucose, fructose <0.2 %) and sucrose (0.5–1.5 %), this agave is a perfect raw material to obtain highly pure fructan with branching structure and high solubility in water.

From the Amaryllidaceae family, the most important species are **bananas**, in particular plantain (*Musa sapientum* L.) and banana fruit (*Musa paradisiaca* L.) used as vegetable and as fruit. Besides starch, glucose, fructose, and sucrose, banana fruits contain more than 5 % fructan, corresponding to approx. 1.4 % in fresh weight and approx. 26 % in dry matter, respectively.

Poaceae

Cereals (grasses) such as **wheat**, **rye**, **oat, barley,** and **rice** play an important role in the human nutrition. In the growth period of roots, leaves, and stems, grasses utilize fructan as storage metabolites which finally will be found as co-storage polysaccharides besides starch in the grains (caryopses). In early development states, the caryopses contain a dry matter fructan content of 2–4 %; in adult grains this content is reduced to approx. 1.3–2.5 % (e.g., wheat grains). The content in flour is reduced to 1.0–1.3 % and in the bran at approx. 3 %. Adult rye grains contain 4–6 % fructan with 2.5–4 % in the flour and 7 % in the bran after milling. Barley grains contain 0.5–1.5 % and oat grains approx. 0.5 % fructan.

6 Polysaccharides and Derived Oligosaccharides from Natural Sources for Prebiotic Application

6.1 Fructans from Chicory, Jerusalem Artichoke, and Agave

6.1.1 Inulin from chicory and Jerusalem artichoke; FOS produced by endoinulinase action

For the worldwide production of inulin and FOS, predominantly chicory and partly Jerusalem artichoke are applied. Chicory roots or tubers of Jerusalem artichoke are washed, sliced, milled, and subjected to hot-water extraction. The extracts are decolorized, demineralized, filtered, and concentrated. The concentrated extracts (40 %) are converted to inulin powder by means of spray drying (Orafti 1999).

Native inulin products from chicory mainly include molecules with a dp between 3 and 60. Companies in Belgium and the Netherlands produce inulin in different grade of purity and dps with the trade names Raftiline®, Frutafit®, and Fibruline®.

For the large-scale production of mixtures of FOS from native inulin, *endo*inulinase-catalyzed hydrolysis is applied. This enzymatic inulin hydrolysis yields two types of FOS – FOS with α-(1,2)-linked glucose at one end of the molecules, designated $G_{py}F_n$ (glucopyranosyl-(fructofuranosyl)$_n$), and FOS with a reducing fructopyranosyl residue, designated inulooligosaccharides (IOS) or oligofructose (OF), and having the general formula $F_{py}F_n$ (fructopyranosyl-(fructofuranosyl)$_n$ (Roberfroid 2005). In this manner, IOS of dp ranging from 2 to 7 are produced. A commercial inulinase preparation is the purified endoinulinase from *Aspergillus ficuum*, Novozyme 230, offered by NOVO AS of Denmark. Commercial products of FOS with high amount of IOS are, e.g., Raftilose®, Frutalose®, and Fibrulose®.

Roberfroid demonstrated the important health-promoting effects of inulin and FOS being a selective substrate for the fermentation of bifidobacteria, which are considered, together with lactobacilli, the principal health-promoting bacteria of the human colon (Roberfroid 2003). It was shown that with 15 g of FOS or inulin per day for 15 days bifidobacteria became the preponderant genus in the human feces (Gibson et al. 1995). Inulin and FOS enhance the mineral absorption, especially calcium and magnesium (Coudray et al. 1997), reduce triglyceridemia and cholesterol level (Beylot 2005), and improve stool consistency and microbial balance. This suggests the option of beneficial application of inulin-type fructans in conditions related to hypercaloric nutrition and the metabolic syndrome.

6.1.2 Fructan from Agave

For the production of fructan from agave (*A. tequilana* Weber), pines (5–8 years old) are harvested before flowering and the tough peel is removed by peeling and scraping. The pines are sliced and the slices subjected to water extraction in a counter flow extractor at ca. 75 °C. The crude water extract is decolorized over charcoal/diatomaceous earth, filtered with the aid of filter panels, and further purified by ion-exchange chromatography. The resulting solution is evaporated under vacuum to form a syrup (containing 70 % dry matter). Spray drying of the syrup affords a white, amorphous powder of agave fructan (Loeppert et al. 2009). Agave fructan and Agave FOS from a Mexican company can be obtained with the trade names Metlin® and Metlos®.

The structure of native agave fructan belonging to the mixed-type fructan combines three well-known structural features of fructans, namely, (2,1)- and (2,6)-linkages of fructofuranosyl residues and a chain-internal α-linked glucose residue substituted with a fructofuranosyl residue at C-6. Derivatives containing the chain-internal glucose residue are referred to as neoinuloglycans (Praznik et al. 2013).

Application of fructan and fructan-derived oligosaccharides isolated from agave delivers similar successful health-promoting effects in the human as inulin products from chicory or Jerusalem artichoke (Allsopp et al. 2013).

6.2 Heteropolysaccharides from Plants (Seeds, Exudates, Tubers) and from Seaweeds

The application of exopolysaccharides isolated from seeds, exudates, and tubers of plants and from seaweed constituents as healthy components in food and therapeutic drugs has high reputation and acceptance in the indigenous population of Asia, America, Australia, and partly Europe. "Traditional medicines" deliver hints for application particularly for bowel diseases and the well-being of humans. For a long time the main interest for these polysaccharides was only in the field of hydrocolloid texture modifiers and viscosity enhancers in the food production. Nowadays the interest is changing more and more toward functional food and pharmaceutical products containing partly soluble dietary fiber with prebiotic effect. And additionally the high water-binding capacity, viscosity, and swelling characteristic support the peristalsis of the bowel and the fecal bulk production.

The properties of these polysaccharides depend on monosaccharide composition, organization of moieties, kind of linkages, and molecular weight distribution.

One group of important gums are galactomannans derived from legume seeds. These heteropolysaccharides consist of β(1,4)-linked D-mannopyranosyl residues in the main chain with varying degrees of substitution with α(1,6)-linked D-galactopyranosyl residues in the side chain. Ratios of galactose/mannose determine the physical properties of the galactomannans and are listed, together with an overview of diverse structural studies, in a comprehensive review by Srivastava and Kapoor (2005). Guar gum (also called guaran) comes from the seeds of *Cyamopsis tetragonoloba*, locust bean gum from seeds of *Ceratonia siliqua* (carob tree), and cassia gum from seeds of *Caesalpinia spinosa* (Ramsden 2004).

Galactomannans are used extensively in the food industry for water retention, gelling, binding, suspending, emulsifying, and thickening applications. However, the polysaccharides pass the human digestive tract essentially unchanged, with very little fermentation in the colon (EFSA 2005), and may therefore be considered dietary fiber but not prebiotic. Galactomannans have been reported to have interesting biological effects. For example, galactomannan from *Caesalpinia spinosa* (Tara gum) induces phenotypic and functional maturation of human dendritic cells (Santander et al. 2011).

Structural features of Plantago-mucilage, a representative mucous polysaccharide from the seeds of *Plantago asiatica* L. (psyllium), were reported by Tomoda et al. (1984). The main polysaccharide chain is a highly branched polymer of β-1,4-linked D-xylopyranose units. Present as branches in position 3 of xylopyranose units are β-D-xylopyranose residues and two acidic disaccharide branches, *O*-α-(D-glucopyranosyluronic acid)-(1,3)-α-L-arabinofuranose and *O*-α-(D-galactopyranosyluronic acid)-(1,3)-α-L-arabinofuranose.

Anderson et al. (1988) reported on the cholesterol-lowering effects of psyllium mucous polysaccharide in hypercholesterolemic men. Serum total cholesterol levels were reduced by 14.8 %, LDL cholesterol by 20.2 %, and the ratio of LDL cholesterol to HDL cholesterol by 14.8 %. The results were taken to indicate that psyllium is an effective and well-tolerated therapy for mild to moderate hypercholesterolemia.

Flaxseed or linseed mucilage is a mixture of rhamnogalacturonan I and arabinoxylan with unusual branches (Naran et al. 2008). The backbone of rhamnogalacturonan I is a polymer of the disaccharide repeating unit (1,4)-α-D-galacturonic acid-(1,2)-α-L-rhamnose (Lau et al. 1985) with the rare L-galactopyranosyl units linked to *O*-3 of many of the L-rhamnosyl residues. The arabinoxylan has a backbone of β-(1,4)-linked xylopyranose units, with the unusual feature of nonreducing arabinofuranosyl residues attached simultaneously to *O*-2 and *O*-3 of xylopyranose to form doubly branched units. Flaxseeds are traditionally used whole for a variety of health benefits, with the mucilage considered beneficial for gut health.

Chia seeds from *Salvia* species have been traditionally used as food by Native Americans and rural Mexicans. Besides oil and protein, they contain a polysaccharide that is exuded in water and forms a mucilaginous gel. Lin et al. (1994) have reported on a structure study of this polysaccharide, concluding that is likely a glucoxylan with the repeat unit -4)-β-D-xylopyranose-(1,4)-α-D-glucopyranose-(1,4)-β-D-xylopyranose-(1, with an α-4-*O*-methyl-D-glucuronic acid residue linked to *O*-2 of one of the xylose residues. The composition of D-xylopyranosyl-, D-glucopyranosyl-, and 4-*O*-methyl-glucopyranosyluronic acid residues is in the ratio of 2:1:1 (Lin et al. 1994; Popa and Spiridon 1998).

The seeds of *Sinapis alba* (yellow mustard) release mucilage when they are swelling in water. This mucilage contains two water-soluble polysaccharides: an acidic heteropolymer containing galacturonic acid and rhamnose and a neutral fraction with a ß-glucan characteristic (Ramsden 2004).

Glucomannans originated from tubers of orchid species applied in India in the Ayurveda treatment are gums with non-branched chains of D-mannopyranosyl and D-glucopyranosyl residues linked β(1,4) with some side groups of D-glucopyranosyl and D-mannopyranosyl residues linked β(1,3) (Ramsden 2004).

Soybean oligosaccharides extracted from defatted soybean whey and treated by de-salting, decolorization, and drying (Hayakawa et al. 1990) deliver products with high content of stachyose (α-D-galactopyranosyl-(1,6)-α-D-galactopyranosyl-(1,6)-α-D-glucopyranosyl-(1,2)-β-fructofuranose) and raffinose (α-D-galactopyranosyl-(1,6)-α-D-glucopyranosyl-(1,2)-β-fructofuranose).

The refined oligosaccharides were subjected to in vitro growth studies and were shown to be fermented by most species of bifidobacteria more effectively than FOS. Saito et al. (1992) demonstrated that the administration of the refined oligosaccharide extract decreases the activity of azoreductase, β-glucuronidase and β-glucosidase, enzymes of potential toxicological importance.

The seaweeds or marine algae are excellent sources for polysaccharides being applied as thickeners, stabilizers, and as functional food products as well.

Agar is produced mainly in Asia by hot-water extraction of red seaweeds (Rhodophyta) such as *Gelidium amansii*, *Porphyra yezoensis*, and *Gracilaria verrucosa* (Higashimura et al. 2014). Agarose is the main component of polysaccharides in agar, containing linear chains of alternating 3-*O*-linked β-D-galactopyranose and 4-*O*-linked α-L-3,6-*O*-anhydro-galactopyranose residues (Araki and Arai 1967). Of the two types of glycosidic linkage in the polysaccharide,

the α-L-anhydrogalactose linkage is hydrolyzed more easily, so that agarooligosaccharides (AGO) can be produced by mild-acid treatment (Enoki et al. 2010).

AGO were shown to have antioxidant, hepatoprotective, and antitumor activities (Chen and Yan 2005; Chen et al. 2006; Enoki et al. 2012). An interesting application was reported by Higashimura et al. (2014) who reported on the prevention of anti-inflammatory drug that induced small intestinal injury in mice. According to their interpretation, cytoprotection against NSAIDs (nonsteroidal anti-inflammatory drugs) is achieved via phosphorylation of the JNK pathway, upregulation of heme-oxidase 1, and acceleration of the phenotypic shift to M2 macrophages.

Alginate present in brown seaweeds (*Phaeophyta*) is an acidic polysaccharide, non-branched with regions of preferably alternating (1,4)-linked β-D-mannuronic and α-L-guluronic acid and regions with blocks of only D-mannuronic or L-guluronic acid residues (Ramsden 2004).

Carrageenans are a family of sulfated polymers with non-branched chains of D-galactosyl residues linked alternately α(1,3) and β(1,4). The sulfate groups are present at 2-, 4-, or 6-positions of the hexose units. Also furcellarans are sulfated non-branched polysaccharides with D-galactosyl and D-3,6-anhydrogalactosyl residues linked in α(1,3) and β(1,4) positions and partly sulfated in position 4. Both heteropolysaccharides are isolated from different varieties of red seaweed (*Rhodophyta*) (Ramsden 2004).

6.3 Xylooligosaccharides from Wood and Other Lignocellulosic Materials

Xylooligosaccharides (XOS) are β-1,4-linked oligomers of xylopyranosyl units and have found applications in functional foods, in feed formulations, for agricultural purposes, and as pharmaceuticals. Production and applications of XOS have been comprehensively reviewed by Vazquez et al. (2000). The authors list three strategies for the production of XOS from lignocellulosic materials, namely, direct enzymatic treatment of feedstocks, chemical fractionation mostly with alkali hydroxides or aqueous ammonia to remove lignin and solubilize xylan, followed by enzyme-catalyzed degradation (Kim and Lee 2007), and hydrolytic degradation of xylan by water, steam, or dilute mineral acids (Vazquez et al. 2000). Hydrothermal processing generates relatively high concentrations of undesirable by-products, the removal of which poses complex technical challenges (Vazquez et al. 2005).

The biological activities of XOS were investigated in a semicontinuous, anaerobic colon simulator with four vessels mimicking the conditions of the human colon. Three XOS compounds and a xylan preparation were fermented for 48 h by human colonic microbes (Mäkeläinen et al. 2010). The numbers of bifidobacteria increased in all XOS and xylan fermentations. Similarly increased were concentrations of SCFA, especially butyrate and acetate. The authors concluded that the combination of XOS and *Bifidobacterium lactis* would be a suitable candidate for an effective synbiotic product.

XOS and FOS markedly reduced the number of aberrant crypt foci in the colon of 1,2-dimethylhydrazine treated rats. Aberrant crypt foci are clusters of aberrant

cells in the lining of the colon or rectum. They may develop into colorectal polyps and thus may constitute an early stage of cancer (Takayama et al. 2005). Other parameters beneficially influenced by XOS were decreased cecal pH and serum triglycerides and increased cecal weight and bifidobacteria population. The results were taken to suggest beneficial effects of XOS and FOS on gastrointestinal health, with XOS more effective than FOS.

6.4 Isomaltooligosaccharides from Starch

Commercially isomaltooligosaccharides (IMO) are produced from starches of different sources. In the first stage, starch is converted into high-maltose syrup under catalysis by amylases. By the action of transglucosidase (α-D-glucoside glucohydrolase, E. C. 3.2.1.20), the nonreducing α-(1,4)-linked glucose unit of maltose is released and attached to the 6-position of glucose, maltose, or other saccharides present in the reaction mixture. In this manner, maltose is converted into a complex mixture of IMO and related oligosaccharides. At the turn of the last century, a commercially produced IMO preparation was the market leader in the prebiotic oligosaccharide sector in Japan with annual sales of 10,000 tons (Japanscan 1998). The typical composition of an IMO preparation is described as isomaltose 23 %, isomaltotriose (α-glucose-(1,6)-α-glucose-(1,6)-glucose) 17 %, non-isomaltooligosaccharides as panose (α-glucose-(1,6)-α-glucose-(1,4)-glucose), maltose (α-glucose-(1,4)-glucose), maltotriose (α-glucose-(1,4)-α-glucose-(1,4)-glucose), nigerose (α-glucose-(1,3)-glucose) and kojibiose (α-glucose-(1,2)-glucose), together 30 % and higher oligosaccharides of dp 4 and 6, 26 %.

In a comparative study of fermentation properties of oligosaccharides (Rycroft et al. 2001), IMO and GOS were found to qualify as effective prebiotics as they increased numbers of bifidobacteria with little effect on the other bacterial groups. Furthermore, they increased the production of lactate and produced the lowest gas volumes.

In an interesting application, the enzyme transglucosidase has been administered to humans and has been shown to decrease blood glucose levels and prevent body weight gain in patients with type 2 diabetes mellitus, apparently through the production of oligosaccharides in the alimentary tract and modulation of the gut flora composition (Sasaki et al. 2013).

7 Prebiotics and Dietary Fiber Derived from Disaccharides

7.1 Fructooligosaccharides Derived from Sucrose

Three alternative approaches for FOS production from sucrose have been described by Yun and Song (1999). Crude fructosyltransferase is prepared by lysozyme treatment of *Aureobasidium pullulans* cells grown in submerged culture. The enzyme may be used directly for batch production of FOS from sucrose.

Alternatively, the enzyme may be immobilized on a highly porous ion-exchange resin and used for continuous production in a column reactor. The industrial use of immobilized enzymes has been reviewed by DiCosimo et al. (2013). Immobilization of whole *A. pullulans* cells is another alternative for catalysis in continuous production of FOS (Yun et al. 1992). These authors found the physiological functionalities of FOS as well and confirmed their prebiotic qualities as mentioned above. Commercial products with the application of fructosyltransferase from *Aspergillus niger* are Neosugar® and Actilight®.

7.2 Isomaltulose and Trehalulose from Sucrose

Several bacterial strains have been reported to produce the enzyme sucrose isomerase (sucrose mutase or α-glucosyl transferase, E. C. 5.4.99.11). This enzyme converts the nonreducing disaccharide sucrose (α-glucose-(1,2)-β-fructose) into the reducing disaccharides isomaltulose or palatinose (α-glucopyranosyl-(1,6)-fructose) and trehalulose (α-glucopyranosyl-(1,1)-fructose.

Cheetham et al. (1982) describe the production of isomaltulose using column reactors containing *Erwinia rhapontici* cells immobilized in alginate beads (for a recent discussion of immobilized enzymes see DiCosimo et al. 2013).

Isomaltulose (palatinose) has found extensive use as a sugar substitute. It is only half as sweet as sucrose. With a glycemic index of 32, isomaltulose is metabolically degraded much more slowly than sucrose (Yamada et al. 1985), so that rapid postprandial increases of blood glucose and insulin levels are avoided. Trehalulose has similar properties and is essentially noncarious (Ooshima et al. 1991).

7.3 Galactooligosaccharides Derived from Lactose

Galactooligosaccharides (GOS), also referred to as *trans*-GOS, are produced from lactose (β-D-galactopyranosyl-(1,4)-D-glucose) by the reversed (synthetic) action of certain β-galactosidase enzymes. A β-D-galactopyranosyl residue is cleaved from a lactose molecule and transferred to the 3′, 4′, or 6′-position of the nonreducing galactose unit of another lactose molecule or of the growing oligosaccharide chain. To assure preference of oligosaccharide synthesis over hydrolysis, high concentrations of lactose are required. In this manner, GOS mixtures of dp between 2 and 8 are produced. Transgalactosidase enzymes from different sources will catalyze the preferential formation of different interglycosidic linkages: Thus, the enzymes from *Aspergillus oryzae* (Vera et al. 2012) catalyze the formation of mainly β-(1,6)-linkages, enzymes from *Bacillus circulans* or *Cryptococcus laurentii* favor β-(1,4)-linkages, whereas β-(1,3)-linkages are preferentially formed when enzymes from *Bifidobacterium bifidum* (Rabiu et al. 2001; Tzortzis et al. 2005) are used. The use of microbial β-galactosidases including enzymes from thermophilic and hyperthermophilic organisms in the production of GOS from lactose has been reviewed (Park and Oh 2010).

GOS have been amply demonstrated to have many beneficial effects. In a study in healthy humans, GOS have been shown to have a strong bifidogenic effect, together with FOS, soybean oligosaccharides, and RS3 (Bouhnik et al. 2004). Most of the use of GOS has been in infant formulas. For example, in a study with preterm infants, an oligosaccharide mixture containing 90 % GOS and 10 % FOS was used to supplement a standard preterm formula at a concentration of 10 g/l (Boehm et al. 2002). The supplemented formula was found to stimulate the growth of bifidobacteria and to improve stool characteristics to the level found with preterm infants fed human milk. Mineral absorption was also found improved by GOS in young girls (Whisner et al. 2013) and in postmenopausal women (van den Heuvel et al. 2000).

8 Conclusion

Dietary fiber is collectively acknowledged to be one of the key factors in a health-promoting diet. Foods containing traditionally not enough dietary fiber such as meat are consumed more and more in the current diet of many developed countries. Hence, nowadays the intake does meet the recommendations of neither the WHO (25 g day^{-1} $person^{-1}$) nor those of the country itself and is around 30–50 % under this level. However, consumer's interest in fiber-rich ready-to-eat foods and functional beverages is increasing, and thus, their dietary fiber intake could possibly be modified toward higher doses in the future.

To enhance public health and the personal well-being, great efforts were made worldwide in the last decenniums, developing different projects and programs. For the interaction of healthy food, movement, and fun, the term "wellness" was created, causing an essential revaluation of the idea of health in the population. Predominantly plant nutrition, scarcely processed, can be considered species appropriate and therefore optimal for individual health. Additionally, the energy input of food should be controlled and attuned to the physical activity.

The interest for functional food is increasing more and more. The target for FF is the global population in general – in particular, however, the elderly, pregnant women, diabetics, sportsmen, or obese people. FF products, raw materials for FF, and process technology to produce FF are of enormous economical interest.

In the field of *intrinsic* FF containing enough bioactive carbohydrates with prebiotic efficacy, fructan plants play an important role, especially in the traditional Chinese and Indian health systems. Additionally the overwhelming interest for healthy food in the USA and in Europe paves the way for a bright future of food products with nondigestible carbohydrates such as fructan.

For *extrinsic* FF with tailor-made profiles containing additions of health-promoting substances, a lot of industrially proceeded carbohydrates mostly isolated from natural raw plant materials will be used. Besides fructan such as inulin and FOS, different bioactive carbohydrates are applied for *extrinsic* FF such as heteropolysaccharides isolated from mucilages of different plant seeds and their hydrolyzed oligosaccharides, gums from plant exudates, or specific

polysaccharides collected from seaweeds. These carbohydrates possess a traditional utilization for foods in the countries of origin and gain worldwide acceptance due to excellent prebiotic functionality. Additional prebiotic active oligosaccharides such as FOS and GOS or nondigestible disaccharides such as isomaltulose and trehalulose can be obtained by modification of sucrose and lactose in enzymatic reactors. Prebiotically effective XOS processed from sawdust or pulp of cellulose production and isomaltooligosaccharides from starch are excellent examples for innovative waste utilization. The developments of new patents for tailor-made products, especially the combinations of different bioactive carbohydrates for particular groups of persons (elderly, infants, sportsmen, etc.), are in progress and have high potential for the future.

Furthermore, the health policy of developed countries moves away from relief of deficiency diseases toward a preventive nutrition strategy with the idea of life prolongation and improvement of life quality. The large developing potential of production and trade of FF in the USA, Europe, and Asia verifies the growing economical interest. Supported by research initiative, a considerable increase of sales and profit can still be expected in this market segment.

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Polysaccharide-Protein Complexes from Edible Fungi and Applications

31

Jian-Yong Wu

Contents

Abstract

Edible and medicinal fungi (mushrooms) have found wide and increasing applications in functional foods and nutraceutical products because of their proven nutritive and medicinal properties. Polysaccharides (PS) and PS-protein (PSP) complexes represent a major class of constituents of the edible fungi with notable bioactivities such as immunomodulation, antitumor, antioxidant, antiviral, and prebiotic. Commercial mushroom materials including PS and PS-rich hot-water extracts are mainly (>80 %) derived from cultivated mushrooms (in fruit form) and a smaller amount (~15 %) from mycelial fermentation. Although the most common immunobioactive PS structures have a β-D-glucan main chain such as (1→3)-β-D-glucans and (1→6)-β-D-glucans with side chains, various other bioactive PS structures have also been documented such as α-D-glucans, glucomannans, and glycoproteins. In addition to functional food and therapeutic uses, mushroom PS and PSPs have also been applied as cosmeceutical ingredients.

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Mushrooms • Polysaccharide • Submerged fermentation • Extraction • Functional food • Cosmetics

Abbreviations

DEAE	Diethylaminoethyl cellulose
DI	Deionized water
EPS	Exopolysaccharide
HWE	Hot-water extraction
IEC	Ion-exchange chromatography
IPS	Intracellular polysaccharide
MW	Molecular weight
PS	Polysaccharide
PSP	Polysaccharide–protein complex

1 Introduction

Edible fungi or mushrooms provide a rich and favorable source of natural and bioactive polysaccharides (PS) for health food and nutraceutical applications. Over the last few decades, considerable research effort has been devoted to investigating the molecular structures and biological activities of PS and PSPs extracted from edible and medicinal fungi as summarized in several reviews (Borchers et al. 1999; Misuno 1999; Wasser 2002; Zhang et al. 2007; Stachowiak and Reguła 2012). In addition to their well-known immunomodulation and antitumor functions, fungal PS/PSP have shown many other health benefits such as antioxidation, anticoagulation, antimicrobial, anti-inflammation, antidiabetic, hypolipidemic, hypoglycemic, prebiotic, and wound healing.

Mushrooms have been used as favorable food and tonic for thousands of years since the early human civilization, and their health benefits and medicinal values have been widely recognized for a long time. Recent years have seen a notable interest in research effort and commercial application of mushroom extracts and PS. This chapter describes the sources, preparation methods, and functional food applications of mushroom PS.

2 Sources, Preparation Methods, and Product Properties

2.1 Sources

Commercial mushroom polysaccharides are mainly extracted from two sources, fruit bodies and fungal mycelia. Currently, 80–85 % of mushroom products has been derived from the fruiting bodies, which are either cultivated or wild, and 15 % from cultivated mycelia from solid or liquid fermentation, such as PSK and PSP

from *Trametes versicolor* and tremellastin from *Tremella mesenterica* (golden jelly fungus). A much smaller percentage of mushroom PS or PSP has been obtained from mycelial culture liquid (as EPS), such as schizophyllan (β-glucan) from *Schizophyllum commune* and scleroglucans from *Sclerotium glucanicum*, *S. rolfsii*, and *Sclerotinia sclerotiorum* (Lindequist et al. 2005; Schmid et al. 2011; Zhang et al. 2013).

2.1.1 Cultivated Mushrooms as a Major Source of PS

Mushroom is a general name referring to the fruiting body of a macrofungus, which is large enough to be picked with hand (Chang and Miles 2004). Mushrooms belong to two major subdivisions of fungi, basidiomycetes for most mushrooms and ascomycetes for relatively few. According to their relationship with the substrate/host, fungi are classified into saprophytic (decomposing nonliving organic matters), parasitic (to other living organisms, e.g., fungi, plants and animals, harmful, or pathogenic), and mutualistic or symbiotic (on an interdependent and beneficial relationship with other living organisms such as plants. The known mushroom species in the world are over 14,000, of which about 2,000 are edible and 700 showing significant medicinal properties (Wassor 2002; Chang and Miles 2004; Stachowiak and Reguła 2012).

Because of the limited supply and high price of wild mushrooms, artificial cultivation has become the major source of most edible mushrooms on the market (Borchers et al. 1999; Smith et al. 2002). World production of edible mushrooms has been increasing constantly at an exponential rate over the last 40 years at a much faster rate than world population (Aida et al. 2009). The most significant growth has been recorded in China and India, due partially to the rapid growth of their economy and wealth. For example, the global mushroom production in 2012 was 7.96 million tons and the production in China was 5.16 million tons, accounting for 65 % of the world production (Factfish http://www.factfish.com). *Agaricus bisporus* (button mushroom) is the most widely cultivated edible mushrooms worldwide, followed by *Lentinus edodes* (shiitake), *Pleurotus spp.* (oyster mushrooms), *Auricularia auricula* (wood ear mushroom), *Flammulina velutipes* (winter mushroom), and *Volvariella volvacea* (straw mushroom).

2.1.2 Submerged Fermentation of Fungal Mycelia

Compared with solid-state fermentation (SSF) systems, liquid or submerged fermentation systems allow for more effective control and operation but also require higher equipment and operating costs and produce a large amount of wastewater (Smith et al. 2002; Kim et al. 2007; Wu et al. 2014). Mechanically agitated fermenters are commonly used for submerged fermentation. The growth of mycelium biomass and the productivity of EPS in the fermentation process are strongly influenced by the liquid medium composition and several operating conditions, such as pH, temperature, oxygen transfer, and agitation power. In most cases, optimum values of temperature and pH for biomass formation and EPS production differ considerably. Moreover, considerable changes in the rheological properties of fermentation liquid occur during the course of fermentation due to biomass and

1. **Fungal species and stock culture.** The fungus Cs-HK1 strain was isolated from a natural Cordyceps fruiting body. The stock culture of Cs-HK1 mycelia was maintained on potato dextrose agar (PDA) medium at 4°C.

2. **Inoculum preparation and liquid fermentation.** For initiation of liquid culture, the stock culture on a Petri dish was incubated at 20°C for about 3 weeks until sporulation. The fungal mycelia/spores were then inoculated into a 250 mL Erlenmeyer flask containing 50 mL of liquid medium and incubated at 25°C on a rotary shaker at 150 rpm for 7 days. The liquid medium was composed of 40 g/L glucose, 15 g/L yeast extract, 5 g/L peptone plus three inorganic salts (K, P, Mg).

3. **Liquid fermentation in a 15-L fermenter.** The mycelial suspension from the shake-flasks was transferred into a 15 L stirred and aerated fermenter filled with ~8 L of liquid medium, and operated for 6 days at 20-25°C, an air flow rate of 1 vvm and dissolved oxygen above 20% air saturation.

4. **Large-scale fermentation.** Inoculum for industrial fermenters was prepared in a 300-L seed fermenter and the inoculum size was about 1% (v/v). The fermentation were operated at an pH 7.0, 20-25 °C, agitation speed 150 rpm, and aeration rate 0.5-1 vvm for an overall period of 6 days. The nutrient chemicals and antifoams were all of food grade for large-scale fermentation.

5. **Separation of mycelium biomass and liquid medium.** The mycelial suspension from the shake-flasks or fermenters was centrifuged at 6000-8000 rpm for 20 min, the biomass pellet was washed thoroughly with distilled water, and dried at 60-80 °C for ~12 h to constant weight, and the supernatant liquid medium was collected for isolation of EPS.

Scheme 1 Liquid fermentation of Cs-HK1 fungal mycelia (Yan et al. 2014; Wu et al. 2014)

EPS production. This results in a highly viscous and non-Newtonian broth which in turn may cause serious problems for mixing, heat transfer, and oxygen supply. Scheme 1 illustrates the procedure for liquid fermentation and EPS production with the Cs-HK1 fungus originated from the Chinese caterpillar fungus *Ophiocordyceps sinensis* (=*Cordyceps sinensis* Berks Sacc.)

2.2 Preparation

2.2.1 Extraction of PS

In both research studies and commercial processes, the first and an essential step in the isolation of bioactive natural products and PS from the source plant and microbial materials is extraction (liquid–solid). The extraction method can strongly affect the quantity (yield) and also the quality (composition and bioactivities) of the isolated products. Since most of the bioactive mushroom polysaccharides are present in the fungal cell wall, the selection of extraction solvents and conditions should be able to effectively dissolve and decompose the cell wall to release the PS into the extracting solvent. Many of the bioactive PS are water soluble and isolated from mushroom fruiting bodies or fungal mycelium through hot-water extraction

(HWE), which is also a common method for the preparation of herbal decoctions in Chinese and other folk medicines and for the isolation of water-soluble bioactive compounds from natural products. Aqueous alkaline has been applied to extract PS that are less soluble in water. In addition to the conventional HWE method, various means have been applied to enhance extraction rate, such as the application of ultrasound, microwave and enzymes, and high pressure.

2.2.2 Purification of PS

PS in the liquid extract solution is usually isolated by ethanol precipitation. In general, 4–5 volumes of ethanol to the extract solution (70–80 % v/v) are required for complete precipitation of PS (Yan et al. 2014). Many high- and low MW impurities such as proteins, fats, and pigments are precipitated together with PS and cause difficulty to the subsequent purification process. Some of the low MW fats and pigments can be removed from the mushroom or mycelium powder prior to water extraction by extraction with an organic solvent such as acetone and ethanol. Crude PS can be further fractionated and purified through column chromatography. Figure 1 shows the common process for extraction of PS from various sources including extracts of mushroom or mycelium biomass and EPS from liquid fermentation medium, and for further isolation and purification of the PS. Scheme 2 illustrates the procedure with conditions for extraction and purification of PS from mycelial fermentation of a medicinal fungus Cs-HK1.

3 Commercial Applications

3.1 Functional Foods and Pharmaceuticals

Functional food refers to natural or formulated food that enhances a physiological performance or prevents or treats a particular disease; nutraceutical is a term referring to a medicinal or nutritional component of food, plant, or naturally occurring material that is used for the improvement of health, by preventing or treating a disease (Doyon and Labrecque 2008). Edible and medicinal fungi have found wide applications in functional foods and nutraceutical products because of their well-known nutritive and medicinal properties (Chang and Buswell 1996; Lindequist et al. 2005; Stachowiak and Reguła 2012; De Silva et al. 2012). Fresh mushrooms have about 90 % water and the remaining 10 % dry matter is composed of 10–40 % protein, 3–28 % carbohydrate, 3–32 % fiber, 2–8 % fat, and 8–10 % ash, varying with the mushroom species and other factors. Mushrooms contain a large array of mineral elements (K, Ca, P, Mg, Fe, Zn, and Cu) and vitamins (niacin, thiamin, riboflavin, biotin, and vitamin C and provitamins A and D), which are beneficial to nutrition and health. Several groups of bioactive low molecular weight (MW) organic molecules have been found in certain mushroom species, including terpenoids, steroids, phenols, nucleotides, and amino acids (Mattila et al. 2001; Smith et al. 2002; Wasser 2002). Among the most popular mushroom dietary

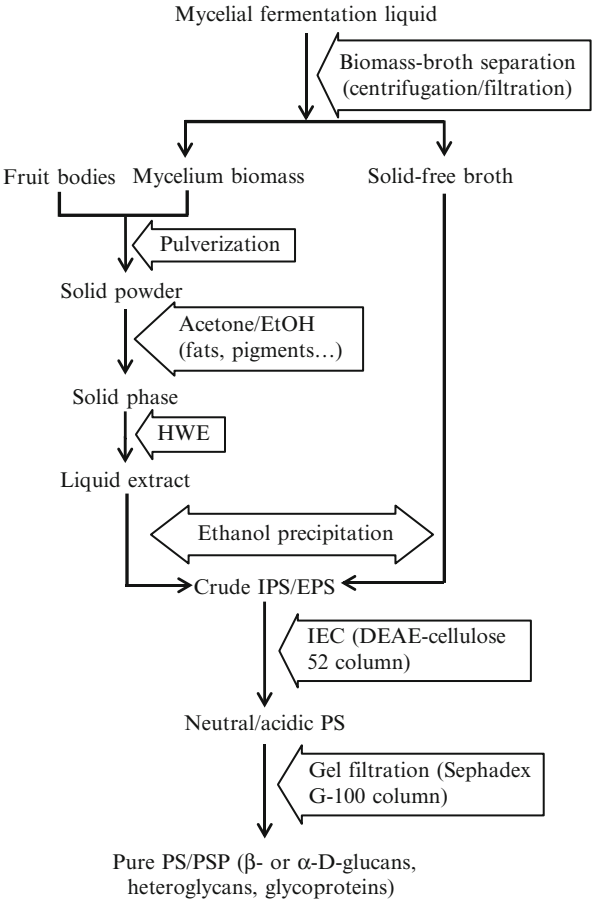

Fig. 1 A general procedure for isolation and purification of fungal polysaccharides from various sources, IPS from extracts of fungal mycelium and fruit bodies, and exopolysaccharides (EPS) from the mycelial fermentation broth

supplements are lyophilized whole and extracts of a few common and specialty mushrooms, *Lentinus edodes*, *Grifola frondosa*, and *Ganoderma lucidum*.

PS represent a major class of high-MW bioactive constituents of edible and medicinal fungi with several health benefits including immunomodulatory, anticancer, anti-inflammatory, antioxidant, hypoglycemic, hypolipidemic, antiviral, and hepatoprotective (Lindequiset al. 2005; Stachowiak and Reguła 2012). Mushroom PS also have a therapeutic function including anticancer and immunotherapy. Some purified PS fractions such as β-glucans and PS-protein (PSP) complexes from edible and medicinal fungi have found clinical applications for immunotherapy and cancer treatment and as the adjuvant for chemotherapy/radiotherapy (Mizuno 1999; Borchers et al. 1999; Ramberg et al. 2010; De Silva et al. 2012). In addition to structure, the bioactivity of PS can be affected by solubility and molecular weight. Table 1 shows the sources and bioactivities of

1. **Separation of mycelium biomass and liquid medium.** The mycelial fermentation broth was centrifuged at 6000 rpm for 20-30 min. The mycelium pellet was washed with distilled water and dried at 60 °C till constant weight.

2. **Extraction of water soluble PS from mycelium biomass.** Mycelium biomass was extracted in hot water at 90 °C under reflux (e.g. 40 g dry mycelium in 600 mL distilled water), followed by centrifugation (6000 rpm, 30 min). The extraction was repeated once and the liquid extract was collected and concentrated by evaporation under vacuum.

3. **Ethanol precipitation of PS from aqueous solution.** The concentrated liquid extract was mixed well with 4-5 volumes of ethanol (~80% v/v), and then left overnight in a refrigerator at 4 °C for PS precipitation. The liquid mixture was centrifuged (6000 rpm, 30 min), and the precipitate was collected and redissolved in distilled water, and then lyophilized to give the crude PS.

4. **Extraction of alkaline-soluble PS.** The mycelium powder was extracted with aqueous solution of 1.25M NaOH and 0.04% NaBH4 for three times (each at 10:1 v/w). The liquid extract was collected after centrifugation and precipitated with 36% acetic acid (final concentration). After centrifugation, the supernatant was collected and concentrated by evaporation, and then precipitated with 3 volumes of cold acetone, followed by centrifugation (8000 rpm, 30 min). The precipitate was collected, redissolved in distilled water and lyophilized, yielding the crude alkaline-soluble PS.

5. **Purification of PS from mycelium biomass**. The crude PS isolated from water or alkaline extract was deproteinized by Sevag reagent and decolorized with 30% H_2O_2. The PS solution was then dialyzed against DI water for 3 days with 12–14 kDa MWCO membrane, and then concentrated, and lyophilized.

6. **Fractionation of PS.** The purified PS was redissovled in water, and loaded into a DEAE-52 cellulose (anion) ion-exchange (IEC) column and eluted with de-ionized water containing 0–0.5M NaCl at a flow rate of 1.0 mL/min. The eluate was monitored by the phenol–sulfuric acid method and the major PS fractions detected were collected and concentrated by evaporation under reduced pressure, followed by dialysis against DI water for 3 days with 12–14 kDa MWCO membrane. The PS was concentrated and lyophilized.

Scheme 2 Extraction and purification of polysaccharides from Cs-HK1 fungal mycelia (Huang et al. 2013; Yan et al. 2014)

several well-known mushroom PS. Beta-1, 3-D-glucans are most well-known antitumor and immunobioactive PS from medicinal fungi. Table 2 presents example commercial products of functional foods and cosmetics made or supplemented with mushroom extracts or PS products.

Mushrooms have also been recognized as a potential and promising source of prebiotics (Aida et al. 2009; Stachowiak and Reguła 2012). The term "prebiotics" refers to a nondigestible food component that stimulates the activity or the growth of gastrointestinal microbiota or the bifidobacteria and lactic acid bacteria in the digestive system (Gibson et al. 2004). The beneficial effects of prebiotics for human health may include the enhancement of the immune

Table 1 Sources, structures, and bioactivities of well-known PS and PSPs from edible and medicinal fungi (Zhang et al. 2007; Yan and Zhang 2009; Mizuno and Nishitani 2013; Yan et al. 2014)

Fungal species (general name)	Raw material/ extraction	PS structure[a]	Main activities
Agaricus blazei Murill. (Almond Portobello, Himematsutake)	Fruit body, alkaline extract	(1→3)-β-D-glucan	Antitumor
	Fruit body, HWE	FA-1b, branched (1→2)-β-D-glucomannan, PSP	Antitumor
	Mycelium, HWE/ alkaline	β-(1→2)- and β-(1→3)-glucomannan	Antitumor
Cordyceps sinensis (Chinese caterpillar fungus, Dong-chong-xia-cao)	Mycelium, HWE	CS-81002, Man:Gal: Glc=10.3:3.6:1	Immunomodulating
	Culture broth (EPS)	Poly-N-hexNAc, $ManNH_2$: $GalNH_2$:Gal=1.0:1.1:0.2	Antioxidant activity
Ganoderma lucidum (Reishi, Lingzhi)	Fruit body, HWE	GL-1, hot-water extract	Antitumor
	Culture broth (EPS)	GLP-2, branched (1→4)-α-D-glucan	Immunomodulating
Grifola frondosa (Maitake, Hui-shu-hua)	Fruit body, HWE	D-Fraction, branched (1→3)-β-D-glucan	Antitumor
Lentinus edodes (Shiitake, Xiang-gu)	Fruit body, HWE	Lentinan, branched (1→3)-β-D-glucan	Anticancer
	Mycelium, HWE	Mannan with protein	Antitumor
Sclerotinia sclerotiorum (White mold)	Mycelial culture broth (EPS)	Scleroglucan (SSG), branched (1→3)-β-D-glucan	Antitumor, antiviral, antimicrobial
Trametes (*Coriolus*) *versicolor* (Turkey tail, Yun-zhi)	Mycelium, HWE	Krestin (PSK), a PSP with 25–38 % protein, branched (1→4)-α-D-glucan	Anticancer

[a]The pure PS/PSPs are usually isolated from the crude and purified with procedure shown in Fig. 1

function, improvement of colonic integrity, reduction of intestinal infections, and suppression of allergic responses. Oligosaccharides such as inulin, fructo-, and galacto-oligosaccharides have been recognized as the most promising candidates for prebiotics (Aida et al. 2009). Therefore, the high-MW PS from mushrooms including chitin, glucans, mannans, and galactans can be digested within the human gut or degraded externally into low MW PS and oligosaccharides to act as prebiotics.

Table 2 Commercial health-care and cosmetic products with extracts and PS of medicinal fungi

Component	Products	
Maitake extracts (Grifron, D-Fraction)	Functional food: Grifron Maitake Caplet; Perricone MD Maitake Mushroom Extract SX-fraction	 
Lentinan	Therapeutic: Lentinan™ for injection; Immune-Assist™	 
	Cosmetic: Aveeno Positively Ageless Night Cream; teeth whitening	
Krestin (PSK) Yunzhi extract	Pharmaceutical: Krestin™	 
	Dietary supplement: Bio Essence™	
Schizophyllan	Cosmetic: Murad's Sleep Reform Serum; Moisture Emulsion: Cuskin™	
Tremella fuciformis (white jelly fungus, Silver ear) water extract	Cosmetic: Beauty Diy Aqua Circulation Hydrating gel; Dietary supplement: Tremella™	 
Agaricus blazei water extract	Functional food: Agaricus Extract veggie capsules	

3.2 Cosmeceuticals

PS from mushrooms with various bioactivities can be used not only in functional food and pharmaceutical products but also in supportive human health-care products, especially functional cosmetics or cosmeceuticals. PS with a high capacity of hydration and water retention are required for skin repair and skin renewal. PS have become the important ingredients of cosmetic products for revitalizing the skin and anti-skin aging (Hyde et al. 2010). In recent years, we have seen an increasing number of cosmetic and skin care products that are incorporated with mushroom extracts or PS, with a few examples shown in Table 2. Mushroom PS and extracts may be attributable to their antioxidant and cytoprotective activities against skin damage, skin aging, and cancer caused by reactive oxygen species and ultraviolet (UV) radiation. In a reported study (Kim et al. 2007), for example, the EPS as well as the mycelial extract of *G. frondosa* produced by submerged fermentation exhibited significant activities for radical scavenging, stimulation of collagen biosynthesis, and cell proliferation and inhibition of melanogenesis without significant cytotoxicity, which are beneficial and useful for skin care.

4 Conclusions

Edible and medicinal mushrooms are valuable and promising sources of bioactive PS and PSPs with diverse structures and properties. Mushroom PS have remarkable antitumor, immunomodulatory, and other useful functions and activities. The bioactive properties of PS are associated with the structures, molecular weight, water solubility, and other molecular and solution properties. In addition to the bioactive functions, fungal PS can be used as thickening and gelling agents in foods and cosmetic products because of their high-MW and hydration properties. Mushroom PS have been applied to a wide range of health-promoting products including foods, pharmaceutical, and cosmetic on the market for public use. Many new products and applications can be developed to improve human health and life quality.

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Oligosaccharides from *Botrytis cinerea* and Elicitation of Grapevine Defense

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Contents

Abstract

An extract from *Botrytis cinerea* culture filtrate was sprayed on grapevine plants (*Vitis vinifera*) to investigate its potential to stimulate defense reactions. The extract triggered the induction of genes encoding pathogenesis-related (PR) proteins as chitinases (*CHIT*), polygalacturonase-inhibiting protein (*PGIP*), serine proteinase inhibitor (*PIN*), and enzymes involved in phytoalexin synthesis as phenylalanine ammonia-lyase (*PAL*) and stilbene synthase (*STS*). Correlated to the up-regulation of these latter genes, stilbene content increased in treated leaves. Consequently, treatment of grapevine leaves with the fungal extract triggered protection toward *Plasmopara viticola* and *Erysiphe necator*,

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the causal agents of grapevine downy and powdery mildews, respectively. Disease severity was significantly reduced in elicited plants, approximately 61 % for downy mildew and 83 % for powdery mildew. This approach could represent a valuable strategy to protect grapevine from diseases as an alternative or complementary method to the use of pesticides.

Keywords
Elicitor • *Vitis vinifera* L. • Gray mold • *Plasmopara viticola* • *Erysiphe necator* • Stilbenes • Protection

Abbreviations

Bc	*Botrytis cinerea*
BTH	Benzothiadiazole
CHIT	Chitinase
Ct	Cycle threshold
DP	Degree of polymerization
DW	Dry weight
HPLC	High-performance liquid chromatography
INA	2,6-Dichloroisonicotinic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OGs	Oligogalacturonides
PAL	Phenylalanine ammonia-lyase
PG	Endopolygalacturonase
PGIP	Polygalacturonase-inhibiting protein
PIN	Serine proteinase inhibitor
PR	Pathogenesis-related
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SA	Salicylic acid
STS	Stilbene synthase
TFA	Trifluoroacetic acid

1 Introduction

Grapevine (*Vitis vinifera* L.) is susceptible to many diseases, especially fungal ones such as gray mold (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), and dieback (*Eutypa lata*). To defend themselves against pathogens, plants have evolved several mechanisms. Passive defenses as structural barriers and preformed antifungal compounds help to delay the infection process but are insufficient. So plants have also developed active defense mechanisms leading to the accumulation of antimicrobial compounds such as phytoalexins (stilbenes in *V. vinifera*) and PR proteins (Jeandet et al. 1995; Adrian et al. 1996; Dufour et al. 2013; Rivière et al. 2012).

These active mechanisms are induced only if the plant has recognized the attack by the perception of signal molecules also called elicitors. Elicitors can be abiotic or biotic (Sticher et al. 1997; Mauch-Mani and Métraux 1998; Pieterse et al. 1998; Zimmerli et al. 2000; Ton et al. 2005; Delaunois et al. 2014). "Abiotic elicitors" can be physical stimuli like wounding or UV light exposure (Gus-Mayer et al. 1998; Douillet-Breuil et al. 1999; Colas et al. 2012) or chemicals like aluminum chloride or phosphite (Saindrenan et al. 1988; Borie et al. 2004; Bock et al. 2012). "Biotic elicitor" usually refers to molecules secreted by microorganisms, derived from the cell walls of fungi, bacteria, and host plants (Côté and Hahn 1994; Ebel and Cosio 1994; Guo et al. 2011) or from seaweed (Bouarab et al. 1999; Klarzynski et al. 2000; Cluzet et al. 2004; Trouvelot et al. 2008; Jaulneau et al. 2011). Oligosaccharides were among the earliest elicitors that have been characterized (Ebel 1998), and many cell wall poly- or oligosaccharides, such as microbial β-glucans and chitin-derived oligomers or plant pectin-derived oligogalacturonides (Ebel 1998; Côté et al. 1998; Aziz et al. 2007), exhibit elicitor activities on defense responses across different plant species (Cardinale et al. 2000; Mithofer et al. 2000; Inui et al. 1997; De León and Montesano 2013). Moreover, β-glucans from fungal origin have been reported to enhance phytoalexin production in soybean (Sharp et al. 1984) and protection against viruses in tobacco (Rouhier et al. 1995; Fu et al. 2011). As the intensive use of phytochemicals to protect plants triggers the emergence of pesticide-resistant strains (Leroux et al. 1999; Gressel 2011; Corio-Costet 2012) and as a current attention was paid to the environment, alternative treatments have to be developed. Application of elicitors could be an attractive approach to control plant diseases (Lyon et al. 1992; Benhamou and Nicole 1999; Mishra et al. 2012; Dufour and Corio-Costet 2013).

In a search of new elicitor compounds of grapevine defenses, a preparation of *Botrytis cinerea* extract was performed. *B. cinerea* is one of the most prevalent fungi that damage plants, and in reaction, plants have developed an ability to recognize its elicitors and to react to them strongly by inducing defense responses, as in *Vitis* spp. (Langcake and Pryce 1976). Eliciting properties could be attributed to saccharidic and/or proteic compounds secreted by the fungus. An endopolygalacturonase (BcPG1), purified from culture filtrates of *B. cinerea* and known as a virulence factor participating in this fungus pathogenicity, exhibited elicitor activity on defense responses in grapevine (*V. vinifera* cv. Gamay) (Ten Have et al. 1998; Poinssot et al. 2003). The authors demonstrated that the protein itself, rather than its enzyme activity, was responsible for defense response activation. Another hypothesis of this elicitor activity could be the presence in the filtrate of extracellular polysaccharides such as glucans and rhamno-galacto-mannans (Fanizza et al. 1995). Oligosaccharide fragments have previously been shown to elicit resistance reactions in several plants (Guo et al. 2011; Aziz et al. 2007), like chitooligosaccharides in wheat and grapevine leaves (Vander et al. 1998; Aziz et al. 2006).

The aim of this study was to determine whether exogenous application of a crude preparation of *B. cinerea* (*Bc*) culture filtrate on susceptible grapevine plants (*V. vinifera* L. cv. Cabernet Sauvignon) was able to induce protection toward

major pathogens. After treatment of plants by *Bc* extract, the expression of defense-related genes encoding enzymes involved in the phenylpropanoid pathway (*PAL* and *STS*) and PR proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PIN*, and *PGIP*) was monitored by real-time quantitative RT-PCR. Stilbene content in leaves was evaluated by high-performance liquid chromatography (HPLC) analysis. Protection experiments toward downy (*P. viticola*) and powdery (*E. necator*) mildew infections were undertaken on detached leaves of *Bc* extract-treated plants.

2 Sources, Preparation, Usage

2.1 *Botrytis cinerea* Extract Preparation

The ascomycete pathogen *B. cinerea* (strain 163, virulence factor 6.19) was friendly provided by Dr. M. Fermaud (INRA, Villenave d'Ornon, France). The fungus was grown on malt agar medium at 20 ± 2 °C with a 12 h photoperiod to obtain conidia. For elicitor preparation, the fungus was grown in liquid medium as previously described (Fanizza et al. 1995). Conidia from 10-day-old fungal cultures on solid medium were scrapped from the surface of cultures and suspended in water containing 0.05 % Tween 20®. Aliquots of the conidial suspension were added to 2 L Erlenmeyer flasks containing 800 mL of Czapek-Dox medium to give a final concentration of $2.5.10^4$ conidia.mL^{-1} medium. The fungus was grown at 20 ± 2 °C in still cultures in darkness for 4 weeks. Culture filtrate was collected after mycelium removal by filtration and autoclaved for 1 h at 120 °C. This autoclaved filtrate contained 8.5 g of β-glucan.L^{-1} including DP6 (50 %), DP5 (32 %), and DP2–3 (18 %).

Concentration of the extract was expressed in g of glucose equivalents.L^{-1} according to the phenol-sulfuric method (Dubois et al. 1951). Before plant treatment, the solution was diluted in sterile water and the wetting agent Triton X-100® was added at 0.1 % (v/v). The extract was sprayed on plants at a final concentration of 2 g of glucose equivalents.L^{-1}.

3 Biological Properties

3.1 Plant Material, Treatment, and Infection Procedures

Plants of cultivated grapevine (*V. vinifera* L. cv. Cabernet Sauvignon), kindly supplied by Dr. M. F. Corio-Costet (INRA, UMR Santé Végétale, Villenave d'Ornon, France), were propagated from woodcuttings in a greenhouse. Plants were grown under controlled conditions at 25/20 °C day/night temperature, with 75 % relative humidity and a 16 h photoperiod (350 µmol.m^{-2}.s^{-1}). Two-month-old plants with 10–12 leaves were used, and a solution containing either water or the *B. cinerea* extract preparation, both supplied with the wetting agent Triton X-100® at 0.1 % (v/v), was sprayed on all the leaves. In the case of further inoculation tests, a negative control (water) and a positive one (commercial product Aliette® (Bayer)

containing fosetyl-Al) were introduced in the experiment. Twelve plants were used per treatment and each experiment has been repeated three times. At several times after treatment, leaves were collected for analysis. Fungal inoculation tests were performed on 48 h treated detached leaves from the upper part of the shoots. *P. viticola* and *E. necator* fungal strains were kindly provided by the UMR Santé Végétale of the INRA, Villenave d'Ornon, France. *P. viticola* was maintained on grapevine leaf-disk and subcultured two times before the assay. Sporangia were collected and suspended in demineralized water. Thoroughly rinsed, cleaned and dried leaves were placed upside down on moist paper filter in Petri dishes. Lower surfaces of the leaves were inoculated with the fresh prepared sporangia suspension and incubated for seven more days at 22 °C under a 16 h photoperiod. For infection with *E. necator*, detached leaves were cleaned, decontaminated with NaOCl, rinsed, and dried. Leaf disks were deposited lower side down on sterile agar plates and placed at the bottom of a Plexiglas settling tower (Délye and Corio-Costet 1998). Conidia were blown in at the top from leaves displaying spores. Inoculated leaves were incubated for 14 days at 22 °C under a 16 h photoperiod.

3.2 Defense-Related Gene Expression in Grapevine Plants

Most elicitor- and/or pathogen-induced genes that have been characterized in grapevine correspond to genes encoding PR proteins (Dufour et al. 2013; Busam et al. 1997; Davies and Robinson 2000; Jacobs et al. 1999; Robert et al. 2001; 2002; Belhadj et al. 2006, 2008a, 2008b; Bavaresco et al. 2012; Lambert et al. 2013) or enzymes involved in the synthesis of stilbene phytoalexins (Dufour et al. 2013; Belhadj et al. 2006, 2008a, 2008b; Bavaresco et al. 2012; Lambert et al. 2013; Melchior and Kindl 1990, 1991; Sparvoli et al. 1994; Wiese et al. 1994).

In this study, grapevine foliar cuttings of a susceptible cultivar (Cabernet Sauvignon) were sprayed with a fungal preparation derived from *B. cinerea* at the previously determined optimal concentration of 2 g of glucose eq L^{-1}. During the 48 h following plant treatment, expression of several defense-related genes encoding enzymes implicated in the synthesis of stilbenes (*PAL*, *STS*) and genes encoding PR proteins (*CHIT1a*, *CHIT3*, *CHIT4c*, *PGIP*, *PIN*) was analyzed in leaves. This expression was monitored by RT-qPCR using specific primers (Table 1) with an actin gene as internal standard. The quantification of mRNA expression levels was carried out as follows: total RNA was extracted from frozen leaves as described by Chang et al. (1993). Contaminating DNA in the RNA preparation was removed by DNase I (Promega Corp.) and a phenol/chloroform/isoamylic alcohol mixture was done to remove the DNase. Total RNA was checked for its integrity by electrophoresis and 2 µg was reverse-transcribed with oligo (dT) (ImProm-II™ reverse transcription System, Promega Corp.). For the determination of the mRNA copy number of the genes of interest, real-time quantitative RT-PCR (RT-qPCR) was performed using the detection system MyiQ (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). PCR reactions were carried out in triplicates in 96-well plates by using SYBR Green I dye and the appropriate primers

Table 1 Gene accession numbers and corresponding primer sequences used for real-time quantitative polymerase chain reaction

Names	Accession numbers	Forward primer	Reverse primer
PAL	X75967	TGCTGACTGGTGAAAAGGTG	CGTTCCAAGCACTGAGACAA
STS	AF274281	GTGGGGCTCACCTTTCATT	CTGGGTGAGCAATCCAAAAT
CHIT1a	AJ291505	TTTTGTCCACTCTGCTATGGTG	CACAGAAAGATTTGGATGCTCA
CHIT3	AJ291507	ATAAGTTCATGGGCACTGCTCT	AGGTTAGTGGTGTTGCCAGAAG
CHIT4C	AY137377	GGCGACGAATCCATTTATGTT	CGGAACAAGGGTTTCATAATTC
PGIP	AF305093	ACGGAACTTGTTCCAGTTTGAT	CGATTGTAACTCACGTTCAGGA
PIN	AY156047	GCAGAAACCATTAAGAGGGAGA	TCTATCCGATGGTAGGGACACT
ACT	TC30205	TCAGCACTTTCCAGCAGATG	TAGGGCAGGGCTTTCTTTCT

couple. To check the specificity of the PCR reaction, melting curves were analyzed for each data point. Transcripts level was calculated as described by Arrieta-Montiel et al. (2001) with the use of a standard curve of known copy number for target sequence. The copy number of the sample was estimated by plotting the threshold cycle (Ct values) against the logarithm of the starting copy number. The absolute copy number for each sample was calculated from standard curves using the Ct value and normalized against grapevine actin gene as an internal control (Bézier et al. 2002) and control leaves as reference sample. The gene-specific primers are indicated in Table 1. Relative gene expression was obtained with the formula: fold induction $= 2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [CtGI where ΔΔCt = [CtGI(unknown sample) – Ct VACT(unknown sample)] – [Ct GI (reference sample) – Ct VATC (reference sample)]. GI is the gene of interest and VACT is the grapevine actin gene used as internal control. The calibrator sample is the sample chosen to represent 1 × expression of the gene of interest (e.g., control leaves) (Winer et al. 1999).

In control leaves, no significant transcript accumulation of the different genes was detected during the 48 h incubation period.

Phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, is involved in the biosynthesis of various defense-related compounds (phenolics, lignin, salicylic acid). In this pathway, downstream of PAL, stilbene synthase (STS) catalyzes the synthesis of resveratrol, the main phytoalexin produced by grapevine in response to biotic or abiotic stresses (Adrian et al. 1997; Coutos-Thévenot et al. 2001; Langcake and Pryce 1977a; Langcake and Pryce 1977b; Shen et al. 2012). Expression of *PAL* and *STS* was highly induced in response to *Bc* extract treatment. In elicited grapevine leaves, *PAL* and *STS* mRNA accumulation was transient and followed the same expression profile. Transcripts were detected at least 3 h after treatment, reaching a maximum around 12 h (300-fold increase for *PAL*, -600 for *STS*), and then rapidly returned to a basal level 24 h after treatment (Fig. 1a).

Bc extract treatment also led to the accumulation of mRNA transcripts of genes encoding PR proteins. The chitinase genes *CHIT1a*, *CHIT3*, and *CHIT4c* showed different expression patterns after treatment (Fig. 1b). Chitinases play a direct role in plant defense by degrading chitin, a major component of fungal cell walls, and thus inhibit hyphal growth (Collinge et al. 1993). An increased pathogen resistance was observed in transgenic plants overexpressing chitinases (Grison et al. 1996; Prasad et al. 2013; Chen et al. 2014). Moreover, chitinolytic breakdown products induce the production of phytoalexins and systemic acquired resistance (Brunner et al. 1998; Van Loon and Van Strien 1999). *CHIT1a* transcripts accumulated during the 48 h incubation with a sixfold maximum increase. *CHIT4c* mRNA transcripts accumulated the first. Induction of this gene started immediately after treatment, with two maxima (40-fold increase each) at 3 and 18 h, then decreased slowly until 48 h. *CHIT3* transcripts accumulation started later, with a peak around 18 h and a 60-fold increased level (Fig. 1b). *CHIT3* is inducible by pathogens such as *P. viticola* and *E. necator* or by chemicals such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), or benzothiadiazole (BTH) (Busam et al. 1997; Jacobs et al. 1999; Robert et al. 2001, 2002).

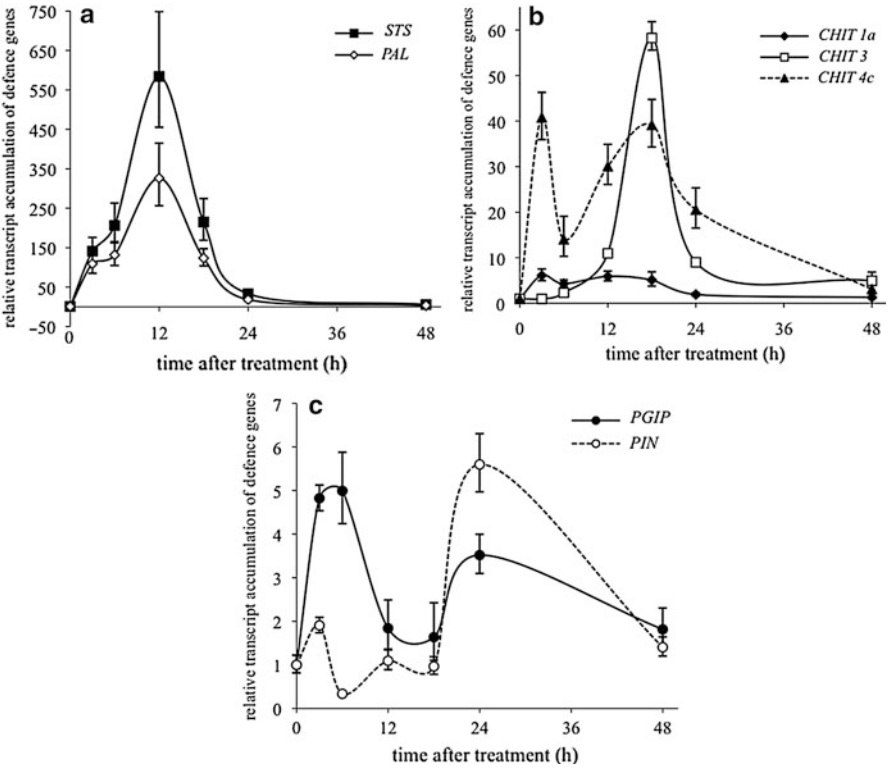

Fig. 1 Transcript accumulation of defense genes in grapevine leaves after treatment by *B. cinerea* extract. Expression profiles of genes encoding (**a**) a phenylalanine ammonia-lyase (*PAL*) and a stilbene synthase (*STS*), (**b**) chitinases (CHIT1a, CHIT3, CHIT4c), (**c**) a serine proteinase inhibitor (*PIN*), and a polygalacturonase-inhibiting protein (*PGIP*). Analyses were performed by RT-qPCR. Levels of transcripts were calculated using the standard curve method from triplicate data, with grapevine actin gene as internal control and no treated leaves (at time zero) as reference sample. Results represent the mean fold increase of mRNA level over no treated leaves, referred as the 1× expression level. In control leaves, the transcript level of defense genes was very low. Results represented are means of triplicate data ± SD of one representative experiment out of three

Bc extract treatment also induced a slight accumulation of polygalacturonase-inhibiting protein (*PGIP*) gene. PGIPs are plant defense proteins which reduce the hydrolytic activity of fungal endopolygalacturonases (PGs), preventing thus plant cell wall degradation and favoring the accumulation of oligogalacturonides (OGs) known to be elicitors of a variety of defense responses (Caprari et al. 1996; Rasul et al. 2012). In *Bc* extract-treated leaves, *PGIP* transcript accumulation increased from 3 h after the treatment with a first maximum (5-fold increase) as soon as 6 h followed by a second peak of lower intensity around 24 h (Fig. 1c).

Inhibitors of serine proteinases (*PIN*) have potent activity against plant and animal pathogens (Van Loon and Van Strien 1999; Revina et al. 2008). The RT-qPCR analysis revealed that the *PIN* gene was up-regulated in grapevine leaves

in response to fungal extract treatment. Accumulation began 5 h after treatment, peaked within 24 h (6-fold increase), and then decreased slowly (Fig. 1c).

3.3 Induction of Phytoalexin Biosynthesis

During the 2 days following *Bc* extract treatment, phytoalexin production in the upper leaves of the plant was monitored. In *V. vinifera*, the best characterized phytoalexins are stilbenes. These phenolic compounds are synthesized by stilbene synthase (STS), which catalyzes the synthesis of resveratrol (3,5,4′-trihydroxystilbene) (Wiese et al. 1994; Schöppner and Kindl 1984; Schröder et al. 1988; Richter et al. 2005). Resveratrol could be metabolized in piceid (glucosylation), viniferins (dimerization), or pterostilbene (methylation) (Bavaresco et al. 2012; Coutos-Thévenot et al. 2001; Pezet et al. 2004a). Identification and quantification of stilbenes were obtained by HPLC with fluorimetric detection, according to calibration curves of pure standards.

The *trans*-form content, which is the main form found in leaves, of five major stilbenic phytoalexins – resveratrol, piceid, ε-viniferin, δ-viniferin, and pterostilbene – was analyzed. Concerning standards, *trans*-resveratrol and *trans*-pterostilbene were purchased, whereas *trans*-piceid (*trans*-resveratrol 3-*O*-β glucoside) was purified from *V. vinifera* L. cell cultures as previously described (Waffo Teguo et al. 1996). *trans*-δ-Viniferin was synthesized by horseradish peroxidase from *trans*-resveratrol (Langcake and Pryce 1977b). *trans*-ε-Viniferin was purified from woody material and characterized by NMR and MS, as previously described (Pezet et al. 2003; Pawlus et al. 2012).

Except piceid, phytoalexins were undetectable in control leaves (Fig. 2a). *B. cinerea* extract treatment of grapevine leaves induced phytoalexin production with different time courses and levels of accumulation (Fig. 2).

Resveratrol was chronologically the first stilbene detected in leaves, 6 h after elicitor treatment. Accumulation of this compound during the 48 h analysis was quantitatively the most important among stilbenes produced. It accumulated transiently, peaked around 14 h after treatment (500 nmol g^{-1} dry weight (DW)), decreased until 24 h, and then remained stable to the end of the analysis (250 nmol g^{-1} DW) (Fig. 2a).

When resveratrol levels began to rise in elicited leaves, the other stilbenes were produced quite at the same time and peaked around 24 h. The level of piceid slowly increased to reach a maximum of approximately 200 nmol g^{-1} DW (fivefold level compared to control leaves) 24 h after treatment and then stabilized at a plateau. Both ε- and δ-viniferin syntheses were induced in elicited leaves. ε-Viniferin appeared earlier (around 6 h) and accumulated quantitatively to higher levels, a maximum around 350 nmol g^{-1} DW for ε-viniferin and 40 nmol g^{-1}DW for δ-viniferin. The levels of δ-viniferin rapidly decreased until 48 h (10 nmol g^{-1}DW), whereas ε-viniferin decreased slower (180 nmol g^{-1}DW at 48 h). Pterostilbene was also detected in treated leaves, but at relatively low levels (10 nmol g^{-1}DW). After reaching a maximum around

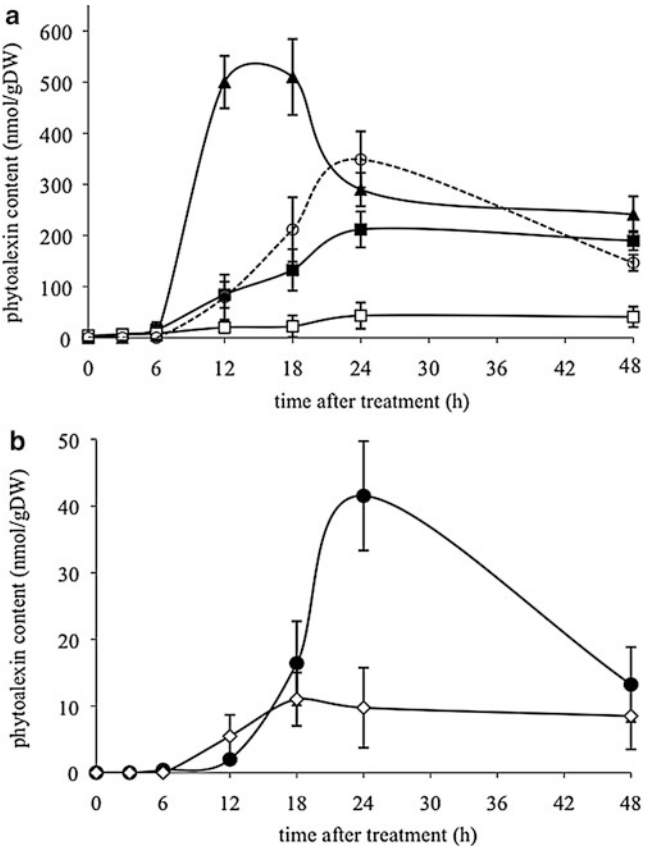

Fig. 2 Time course of several *trans*-stilbenes accumulation in *V. vinifera* (L.) cv. Cabernet Sauvignon leaves in response to *B. cinerea* extract treatment: (**a**) resveratrol (▲), piceid (■), and ε-viniferin (○). (**b**) δ-viniferin (●) and pterostilbene (◊). No stilbene except piceid was detected in untreated leaves (□). Values represent the mean ± SD of triplicate assays of one representative experiment out of three. Stilbenes from leaves were extracted by methanol, then pre-purified on a Sep-Pak® C18 cartridge to remove chlorophylls. Analysis of stilbenes was performed by HPLC on a C18 (5 µm) reverse-phase column (4 mm i.d. × 250 mm). Solvents used for the separation were (**a**) water with 2.5 % TFA and (**b**) 20 % A with 80 % acetonitrile. The elution program at 1 ml/min was as follows: 0–13 min, from 14 % B to 18 % B; 13–15 min, 18 % B; 15–34 min, from 18 % B to 32 % B; 34–36 min, 32 % B; 36–40 min, from 32 % B to 40 % B; 40–49 min, from 40 % B to 80 % B; 49–50 min, from 80 % B to 100 % B; 50–56 min, 100 % B. Fluorimetric detection was recorded at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 300$ nm

20 h, the level of pterostilbene remained constant until the end of the analysis (Fig. 2b).

Forty-eight hours after treatment, the amount of stilbenes in leaves has decreased for resveratrol and ε- and δ-viniferins, compared to the maximal levels they reached during the incubation period, whereas piceid and pterostilbene levels stabilized into a plateau from 24 h to the end of the analysis.

3.4 Protection Against *Plasmopara viticola* and *Erysiphe necator* by Bc Extract

To determine whether our extract induces disease protection in grapevine, *V. vinifera* cv. Cabernet Sauvignon leaves were treated with *Bc* extract.

An aqueous solution of the extract added with a wetting agent to improve penetration into the plant was sprayed on all parts of plants. Two days later, treated leaves were detached from plants and inoculated by *P. viticola* (downy mildew) and *E. necato*r (powdery mildew). As previous authors observed that older leaves from the bottom of the shoots are more resistant (Reuveni 1998), inoculation experiments were performed on young leaves from the upper part of the plant. Disease intensity was estimated 7 and 15 days post-inoculation for downy and powdery mildew, respectively, by measuring the infection rate.

As shown on Fig. 3, the validity of the test was checked according to the results obtained in Aliette® (fosetyl-Al)-treated leaves (3 g L^{-1}). Pretreatment with the fungal elicitor 2 days before inoculation induced a significantly strong reduction of the infection by both fungi, *P. viticola* and *E. necator*. The development of the pathogens was respectively reduced for about 61 and 83 % in leaves of plants pretreated by fungal elicitor extract compared to untreated plants. Aliette® protection rates were almost 100 % against *P. viticola* and about 86 % for *E. necator*.

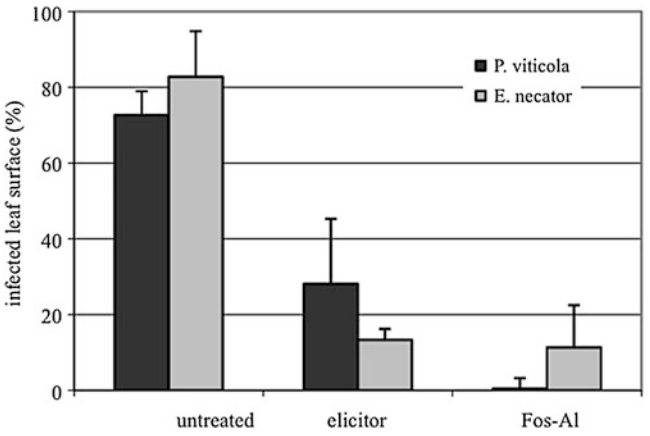

Fig. 3 Protection of grapevine detached leaves pretreated by *B. cinerea* extract against *P. viticola* (*black bars*) or *E. necator* (*gray bars*). Plants were sprayed with Bc extract or the known active Aliette® (fosetyl-Al) 2 days before inoculation, and disease assessment was done 7 days postinoculation for *P. viticola* and 14 days for *E. necator* and expressed as a percent infected leaf surface. Twelve plants were used per treatment. The experience was repeated twice with similar results. Values represent the mean ± SD of triplicate assays of one representative experiment out of two. Disease intensity was estimated by measuring the level of growth and intensity of fungal mycelium and sporulation on leaves. The contamination level was visually evaluated and expressed as percentage of total leaf area according to a 0–100 % scale with steps from 0 to 5 (the note zero corresponding to the absence of pathogen development). The intensity and aspect of spores formation was observed with microscope

4 Discussion and Conclusion

4.1 *Botrytis cinerea* Extract Induces Defense Responses in Grapevine

In *Bc*-treated plants, the expression of two of the three analyzed chitinase genes – *CHIT3* and *CHIT4c* – was rapidly detected after treatment and increased significantly, whereas *CHIT1a* expression remained relatively low during the 48 h incubation period. Indeed, grapevine chitinases are known to be inducible by various biotic stresses but differentially, according to the applied stress, the studied organ, and the grapevine cultivar (Busam et al. 1997; Jacobs et al. 1999; Robert et al. 2002; Aziz et al. 2003). *PGIP* and *PIN* mRNA transcripts accumulated in treated leaves. Levels of *PGIP* have been shown to correlate in several cases with an increased resistance of plants to fungi (De Lorenzo and Ferrari 2002; D'Ovidio et al. 2004; Wang et al. 2013). Increased expression of *PIN* gene was also observed. The prevailing role of serine proteinase inhibitors seems to be the control of endogenous proteinases during seed dormancy and protection against pathogens (Pautot et al. 1991). *PIN* mRNA and protein synthesis accumulation has been shown to occur in tomato leaves after treatment by plant-derived oligogalacturonides and fungal-derived chitosan oligosaccharides (Doares et al. 1995; Akagi et al. 2010).

PAL and *STS* genes were the two most rapidly and intensively up-regulated genes upon *Bc* extract treatment. Indeed, activation of phenylpropanoid metabolism is one of the most important resistance reactions in many plants (Dixon and Paiva 1995; Dixon 2011). In grapevine, previous studies showed that both *PAL* and *STS* genes were induced in leaves infected by *B. cinerea* (Belhadj et al. 2008a; Lambert et al. 2013; Bézier et al. 2002). Moreover, *PAL* and *STS* showed coordinated gene expression.

Consistent with the up-regulation of *PAL* and *STS* genes, we noticed an increased production of resveratrol and its derivatives – piceid, viniferins, and pterostilbene – in leaves of elicited plants. Resveratrol and its dimer ε-viniferin were quantitatively the major stilbenes produced in grapevine leaves in response to the fungal elicitor treatment. This result is in accordance with previous observations made in grapevine treated by biotic elicitors such as laminarin (Lambert et al. 2013; Aziz et al. 2003).

Resveratrol is the primary phytoalexin produced by a stilbene synthase after a stress (Jeandet et al. 2002, 2013). Once resveratrol synthesis began in leaves, consistently with the up-regulation of *STS* gene, its metabolization into the other compounds started. In our experiment, compared kinetics of accumulation of all compounds support the idea that resveratrol is the precursor of the phytoalexin grapevine phytoalexins. Furthermore, the highest resveratrol levels are reached around 12 h, whereas derivatives peaked later, at around 24 h.

The accumulation of ε- and δ-viniferins rapidly occurred in elicited leaves. Both compounds are highly fungitoxic and the presence of both viniferins in stressed

grapevine leaves has been correlated with enhanced protection against downy mildew (Pezet et al. 2004a, 2003, 2004b). The fungitoxic activity of δ-viniferin against zoospores of *P. viticola* is quite similar to the one of pterostilbene, the most toxic known stilbene. Our data showed that this latter compound was also detected in leaves of pretreated plants, even if the amounts seemed low compared to the other analyzed stilbenes. In the majority of grapevine cultivars, pterostilbene levels remain very low or undetectable and few studies reported its presence in response to elicitation. Resveratrol could be also glycosylated as piceid, which is protected from enzymatic oxidation (Regev-Shoshani et al. 2003). In our experiment, the accumulation of piceid was lower to the ones of resveratrol and ε viniferin. This could be explained by the fact that the plant does not need its presence because of its lower fungitoxicity. This stilbene could also represent a form of reserve or transport of resveratrol in the plant (Douillet-Breuil et al. 1999; Belhadj et al. 2008a). If the plant is stressed, the presence of basal piceid levels could constitute a pool of immediately usable resveratrol, which can rapidly be mobilized as a primary defense response.

4.2 *B. cinerea* Extract Treatment Led to an Increased Protection of Grapevine Against Downy and Powdery Mildews

Correlated to the induction of defense-related genes, *Bc* extract treatment of grapevine plants triggered enhanced protection toward two of the most deleterious grapevine fungal diseases. Indeed, pretreatment of plants reduced the development of *P. viticola* and *E. necator* by approximately 61 % and 83 %, respectively, compared to control leaves. In the case of *E. necator*, the protection obtained with *Bc* extract treatment was comparable to the one induced by Aliette®. We observed less difference in the efficiency against both pathogens in Aliette-treated plants than in *Bc* extract-treated ones. This probably could be explained by the fact that fosetyl-Al exhibits at the same time a direct antifungal activity (Dercks and Creasy 1989) and an indirect potentiation of phytoalexin biosynthesis (Adrian et al. 1996).

Bc extract triggers induction of PR genes. Activities of chitinases have been correlated in many grapevine cultivars with their observed field resistance to powdery mildew (Busam et al. 1997; Renault et al. 1996; Giannakis et al. 1998; Nirala et al. 2010), and constitutive accumulation of PR proteins in grape berries after veraison confers increased resistance to fungi such as downy and powdery mildews (Jacobs et al. 1999; Giannakis et al. 1998; Robinson et al. 1997; Tattersall et al. 1997; Salzman et al. 1998; Derckel et al. 1998; Kambiranda et al. 2014).

The protection acquired by grapevine leaves treated by the extract could be due to the up-regulation of *PAL* and *STS* gene expression and the consequent accumulation of the two quantitatively most produced stilbenes, resveratrol and its dimer ε-viniferin. Indeed, a higher tolerance to both powdery and downy mildews was observed in grapevine producing high levels of resveratrol (Dai et al. 1995; Malacarne et al. 2009). ε- and δ-viniferins have been shown to be the major

stilbenes produced in grapevine leaves infected by *P. viticola* (Pezet et al. 2003; Kortekamp and Zyprian 2003) and their synthesis has previously been shown to correlate to grapevine resistance to *B. cinerea* or *P. viticola* (Douillet-Breuil et al. 1999; Pezet et al. 2004a). Moreover, some studies suggest that both *P. viticola* and *E. necator* cannot detoxify resveratrol or suppress phytoalexin production in *Vitis* spp. (Romero-Pérez et al. 2001). In a recent work, Dufour et al. (2013) showed that the treatment of grapevine leaves with benzothiadiazole (BTH) led to a significant reduction in the development of these latter two fungi correlated with a significant increase only in pterostilbene contents (Dufour et al. 2013).

The enhanced tolerance of the treated grapevine leaves could thus be due to both the accumulation of PR proteins and phytoalexins (Belhadj et al. 2006, 2008a; Corio-Costet et al. 2012).

We noticed that the major stilbenes biologically active against pathogens were not present at sufficient levels in our experiments at the moment of the inoculation to counteract pathogen penetration. However, two of the most grapevine-destructive fungi, *P. viticola* and *E. necator*, exhibited a reduced development in treated plants. We can hypothesize that pretreatment of plants with the *Bc* extract certainly triggered a faster and stronger activation of defense responses to subsequent fungal attack as a potentiation.

We have shown the efficiency of an extract derived from *B. cinerea* culture filtrate. This fungal extract certainly contains elicitor compounds: *Bc* extract treatment showed a direct stimulating effect on the expression of defense-related genes which was correlated with the enhanced production of antimicrobial compounds. Consequently, induced plants exhibited a more efficient response to pathogen attack, with highly reduced infection rates. Experiments are in progress with purified fractions of *B. cinerea* filtrate in order to determine which molecules are responsible for the eliciting properties. Exploiting this strategy to control diseases and pests clearly meets with the current need toward sustainable agriculture at a lower environmental cost.

A few oligosaccharide products have been approved in many European countries, in the USA, and in several other countries for their application in agriculture. To our knowledge Iodus^{R} (a laminarin-based product acting as elicitor) approved as plant protection agent against several diseases and Elexa^{R} (a chitosan-based product) available as biological elicitors are the two main used products.

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Physical Properties of Inulin and Technological Applications

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Contents

Abstract

This chapter deals about a carbohydrate polymer highly used as an ingredient in food products and commonly consumed by humans, but very often unnoticed: inulin. The chapter provides an overall view about the inulin, its benefits in human's health, introducing the physical properties, providing a deeper understanding about the relation between its structure and properties, and discussing some technological applications. The chapter is divided into three parts. Section 1 provides an overall view about the inulin and its benefits in human's health and overview some applications. Section 2 introduces the physical properties of inulin,

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providing a deeper understanding about the relation between its structure and properties. The importance of the amorphous state in inulin is explained, some undesired characteristics presented during the processing of inulin are revealed, state diagrams are introduced, including a complex state diagram for inulin, and the effect of the polymerization degree on the properties is disused as well. Section 3 presents the technological application of inulin, that is, the performance of inulin in food products; a complex state diagram is presented and discussed for the system inulin-orange juice. In general, the chapter is based on the reviewed literature, which is a very recent research topic, and the presentation of some new experimental results. The aim of the work is to present the inulin in such a way that the reader interested in this topic may recognize this material as a very important ingredient in the formulation of food products. Likewise, the reader who is beginning to learn about this material may realize about the importance of the structure of inulin on the final properties of the food product. Ultimately, the expectation of the authors is that the reader after finishing the chapter be motivated to take a food product from his/her refrigerator and look for the presence of inulin in the nutritional information of the product.

Keywords

Fructans • Inulin • Polymerization degree • Physical properties • Glass transition temperature • Technological applications

Abbreviations

a_w	Water activity
Da	Dalton
DP	Degree of polymerization
DSC	Differential scanning calorimetry
FOS	Fructooligosaccharides
LCI	Long chain inulin
MDSC	Modulated differential scanning calorimetry
M_n	Number average molar mass
M_w	Mass average molar mass
MWD	Molecular weight distribution
NI	Native inulin
OM	Optical microscopy
SEM	Scanning electron microscopy
Tg	Glass transition temperature
XRD	X-ray diffraction

1 Introduction

Inulin is the name given to a heterogeneous blend of fructose polymers found widely distributed in nature as plant storage carbohydrates in more than 30,000 vegetable species. Due to their wide distribution in nature and technological

applications, the extraction, isolation and characterization of this polysaccharide have gained attention in recent years. Inulin is a storage carbohydrate naturally found in plants, vegetables, fruits, and cereals. The chemical and physical properties impart several advantages such as a wide technological adaptability and health benefits in humans. For this reason, inulin is mainly used as ingredient in food products like dairy, bakery, and candies. When inulin is added to the food product, this is converted into a functional product (Glibowski and Pikus 2011), that is, presenting a beneficial selective activity, providing an additional physiological effect to the nutritional value of food components or nutrients. The benefits of inulin are largely related to its chemical structure because the chemical bonds are not hydrolyzed by human digestive enzymes, remaining intact in their journey through the gastrointestinal tract until the colon, where it is totally hydrolyzed and fermented by the bifidobacterium and lactobacillus (Warchol et al. 2002). For this reason, inulin is considered as a prebiotic in addition to contributing in the dietary fiber. The beneficial effect in human's health is related with the improvement of body functions and the decreased risk of disease (Kawai et al. 2011; Silveira et al. 2003).

Among the main plant species from which inulin is produced are chicory root (*Cichorium intybus*), artichoke (*Helianthus tuberosus*), asparagus (*Asparagus officinalis*), American agave (*Agave* spp.), root of dahlia (*Dahlia* spp.), onion (*Allium cepa L*), yacon (*Smallanthus sonchifolius*), and rye (*Secale cereale*). Industrially inulin is mainly extracted from the chicory root because this root contains 79 g of inulin per 100 g of dried material, unlike other species as the root of dahlia and onion containing only 59/100 g and 48/100 g, respectively.

There are no accurate data for the total area harvested with chicory root for the industrial use, however, is possible to estimate the existence of about 120,000,000 m^2 of cultivated land with this specie. Globally, the exportation of inulin is basically dominated by three countries, Belgium, Netherlands, and Chile, with a contribution per country of 50.4 %, 24.1 %, and 18.7 %, respectively. In 2013, these countries together produced 93 % of the total production of inulin in the world.

The main uses of inulin in food industry are as fat substitutes, stabilizer in ice cream, sauces, desserts, and more. It is presented as an odorless withe powder with neutral flavor. The majority of its physical properties are established from a system in the solid state, where molecules have a restricted mobility (Liu et al. 2006). In nature, solids may exist in the crystalline state or in the amorphous (glassy) state. In the former, the molecules or atoms have a long-range order, while in the latter the molecules are accommodated in a disordered fashion or with short-range order. The physical state of inulin, stability, and molecular mobility are closely related to first-order and second-order phase transitions, which in turn depends on the initial arrangement of molecules in the food product. In polysaccharides as inulin, phase transitions play an important role in the physical properties of the employed food product (Kasapis 2006). These phase transitions determine some properties such as the melting of crystals (melting temperature) and the change from the glassy into the rubbery state (glass transition temperature) (Miquel and Hall 2002). Other authors have noted that the most important properties of inulin are its high

thermal stability, resistance to sticking, agglomeration, and crystallization (André et al. 1996; Dan et al. 2009; Glibowski and Pikus 2011; Kawai et al. 2011; Pitarresi et al. 2012; Ronkart et al. 2009; Zimeri and Kokini 2003). These properties are directly influenced by the structure of the polysaccharide, i.e., thermal stability is related to the structure of the molecule, and stickiness, agglomeration, and crystallization are related to the collapse of the structure, defined according to Slade and Levine (1988) as the phenomenon where several time-dependent phase transformations are included. All these properties will be discussed more in detail in the following sections.

2 Physical Properties

2.1 Structure

Fructans are extracted from dicotyledonous and monocotyledonous plants; from the former linear chain, inulin is synthesized, while the latter produces more complex fructans (Barclay et al. 2010). Fructans are natural polymers composed of fructose and derived from sucrose. The length of the polymer chain varies from three to a few hundreds of fructose units. In nature, according to the type of bonding, fructans are classified in five distinct groups: inulin, levans (with bonds β(2-6)), the levan type neoseries, inulin type neoseries, and mixture of branched fructans (Carvalho and Figueredo 2001; Vijn and Smeekens 1999).

Inulin is a mixture of polysaccharides composed of fructose chains linked by β(2-1) bonds with a terminal unit of glucose and fructose chains linked by β(2-1) without a terminal unit of glucose (Fig. 1; Barclay et al. 2010; Franck and De Leenheer 2002). The general formula is represented by

$$GF_n \text{ or } F_n$$

where

G is the terminal glucose unit.
F represents the residue of fructose.
n characterizes the number of fructose units (2–60).

From the chemical point of view, inulin is described as a polyose resulting from the polymeric condensation of fructofuranose (cyclically fructose) monomers linked through a glycosidic bond. In a simpler way, inulin may be defined as a polymer of fructans consisting of chains of fructosyl groups joined by terminal glycosidic bonds. When the reducing end presents a glucose molecule, the structure is named D-glucopyranosyl; when the end molecule is a terminal fructan, the structure is named β-D-fructopyranosyl. Frequently, the linear chains in inulin have a length in the range of 3–60 units of fructose, with a molecular

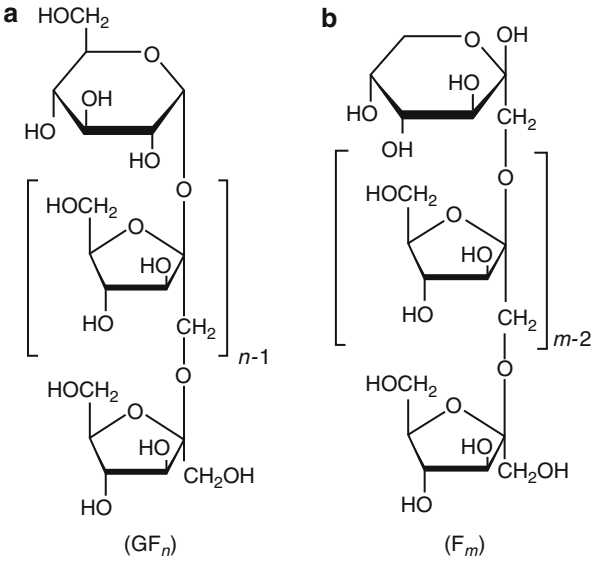

Fig. 1 Structure of inulin

weight in the range of 3,500–5,500 Da (Derycke and Vandamme 1984; Kay and Nottingham 2008).

Inulin is presented in the form of an odorless white powder with neutral flavor. Most of its physical properties are established in the amorphous solid state. This state is reached as the result of several processes such as melting, denaturation, gelatinization, mechanical treatment, solvent removal, and depolymerization of complex structures. Figure 2 depicts a diagram relating the different processes industrially employed to reach the final structure of inulin. The most common processes for reaching the amorphous state are drying, spray drying, freezing, and hot extrusion. In general, a melted material may be converted into an amorphous matrix if the cooling rate is fast enough for avoiding the undesired crystallization (Bhandari and Hartel 2005; Roos 2002). In this sense, Ronkart et al. (2006, 2009) and Zimeri and Kokini (2003) demonstrated that some physical properties such as glass transition temperature, crystallization temperature, and melting temperature are not accurately determined from semicrystalline systems. Table 1 summarizes some physicochemical properties and characteristics observed in inulin.

2.2 Effect of the Degree of Polymerization in Inulin

Microscopically, polymers are comprised of a large number of entangled chains which are conformed of many monomer units chemically bonded by the polymerization process. The length of these chains is not constant through the polymer, which is observed macroscopically as a molecular weight distribution (MWD). MWD may be expressed in different ways depending on the fashion it was determined. The number average molar mass (M_n) and mass average

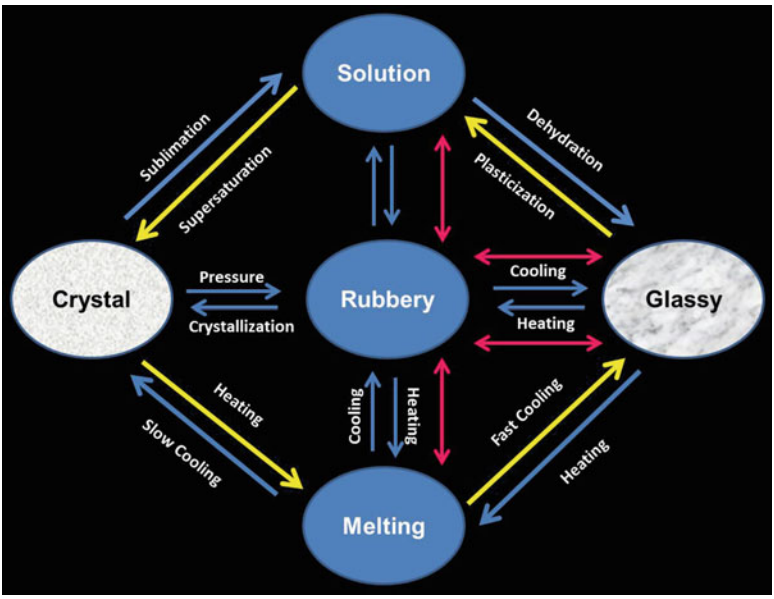

Fig. 2 Mechanism to obtain an amorphous material (Roos and Karel 1991)

Table 1 Physicochemical properties and characteristics of inulin

Properties/characteristic	Value
Appearance	Granular white powder
Flavor	Neutral
Hygroscopicity	High
Range of polymerization degree	2–60 units
Average polymerization degree	12 units
Average molecular weight	5,000 Da
Solubility in water (25 °C)	120 g/L
Viscosity in aqueous solution (5 % w/w and 10 °C)	1.6 mPa.s
Melting temperature (Tm)	165–180 °C
Glass transition temperature (Tg)	125–140°

molar mass (M_w) are the two common average values for describing the molecular mass of a polymer. The former is employed for calculating the degree of polymerization (DP) which is defined as the average number of base units (monomeric units) forming a molecule or chains. Inulin can be characterized according to the molecular size of the chains, i.e., by expressing the DP in terms of the total number of fructose units forming the molecule. The DP strongly influences the final properties of inulin, and it varies according to the type of plant from which the inulin is extracted, the environmental conditions exerted during the farming, and the physiological age of the plant.

In the vegetable origin inulin has been found a maximum DP value of 200 monomers units. Although, inulin from plants was considered only with a linear molecular structure, recently it was found to have a very low percentage of branches (1–2 %) (Franck and De Leenheer 2002; Conceiçáo et al. 2014). Native inulin (NI) is considered as all the powder extracted with an average long chain associated to a DP from 3 to 60 monomer units. Short chain inulin molecules presenting a DP from 1 to 10 monomer units are known as oligofructans (OS). OS are more soluble and sweet than NI and long chain inulin (LCI) and may contribute to increase the mouthfeel because some properties are very similar to those from other sugars. In this sense, OS can be used for replacing sucrose because they possess a similar sweetness profile, but with lower caloric content (1–2 kcal/g) and sweeting powder (30–25 %). In the other hand, LCI is less soluble and more viscous and thermostable than NI. These properties may be used in a beneficial way to improve the sensory properties of dairy products when LCI is used as fat substitute. LCI acts as filler, breaking the structure in the same way with that of fat globules. When mixed and sheared with water or milk, crystals are formed, providing a smooth texture and the creamy sensation of fat in the mouth. Other properties influenced by DP include melting, glass transition temperature, gel formation, and strength (Sobral et al. 2001).

Because inulin is a mixture of polysaccharides, the potential application depends on the distribution size of the fructose units; for example, inulin with a high DP presents a lower solubility in water and higher resistance in the gel form (Mancilla and López 2006). In this regard, only few works have been reported in the literature: Vereyken et al. (2003) found that the presence of fructooligosaccharides (FOS) in plants favors the decrease in the transition temperature from liquid to gel phase. Meyer et al. (2011) evaluated the effect of inulin in nonfat yogurt, finding that after 4 weeks the yogurt with inulin and higher DP FOS presented less syneresis, more water retention, and greater firmness than the control. Nguyen et al. (2005) observed that skim milk supplemented with 1 % of fructan can generate a product with similar sensory attributes with that of a yogurt made of whole milk. Castellanos et al. (2012) indicated that the physical properties of inulin make it unique for a high water retention capacity, synergism in rubbers, and the ability to stabilize foams and emulsions. In addition, the use of inulin as fiber in foods improves the properties of the final product without modifying the production process.

2.3 The Amorphous State of Inulin

Overall, the term amorphous or glassy state is employed for describing those materials showing low molecular mobility, brittleness, high resistance, and transparency. In the fields of polymers physics, the term is referred to a metastable state where molecules have a disordered arrangement, occupying a larger volume than the crystalline state (Roos 1995; Slade and Levine 1988). The structure of the glassy or amorphous solid is described taking as a reference the crystalline structure,

where molecules are well arranged (Liu et al. 2006; Rhaman 2010; Schenz 1995; Yu 2001). Although the general structural arrangement of an amorphous solid is disordered, it may present some short-range ordered regions similar to those found in the crystalline state. Certainly, this mixture of structures may develop different properties from those of the single structures. Because of its high viscosity, amorphous solids show a reduced molecular mobility with limited diffusion phenomena (Rhaman 2010; Ribeiro Santivarangkna et al. 2011; Zimeri and Kokini 2003), where the molecular movements are restricted only to vibrational and short-range rotational displacements (Labuza and Hyman 1998). Then, the alterations occurring in the amorphous state are extremely slow and commonly are observed as the physical aging and changes in the properties as: (i) mechanical and flow properties as elastic modulus and viscosity, both of interest in the texture of food products, and (ii) diffusional properties as diffusivity, on which depend the adsorbed moisture and the crystallization temperature (Roos 1995).

The most important parameter for characterizing an amorphous material is the glass transition temperature (Tg), which is a time-dependent second-order transition. This parameter is the temperature at which the system undergoes in either direction, the transition from the glassy state into the rubbery state. However, glass transition temperature is understood as a state transition rather than a phase transition, and this is because in the metastable nature of the amorphous system, the vitrification process can occur in a wide range of temperatures (Kasapis 2006; Liu et al. 2006; Rhaman 2010). The Tg contributes for the identification, prediction, and control of the stability of food products during the processing and storage. The value has been employed in lipids with different polymorphic forms to differentiate the type of lipid; in proteins it is related to the thermal stability (Bell and Hageman 1996), while in sugars it is the technological base for the processing of amorphous sugars in dairy products (Haque and Roos 2006). In the field of food products, glass transition temperature may be employed for predicting the stability of the products during the processing and storage. In carbohydrate polymers as inulin, glass transition temperature also depends on the concentration of the adsorbed water (Rhaman 2010; Sablani et al. 2010; Sablani et al. 2007; Santivarangkna et al. 2011). In the literature the reported Tg values for amorphous inulin are in the range of 125–140 °C (Kawai et al. 2011; Ronkart et al. 2006, 2009; Saavedra-Leos et al. 2014; Zimeri and Kokini 2003). In these investigations, the first reported results showed X-ray analysis to determine the overall state of inulin at different moisture conditions. Kawai et al. (2011) reported the effect of water content, molecular weight, and crystallinity on the Tg of inulin, emphasizing the importance of knowing the initial state of inulin. In this sense, Saavedra-Leos et al. (2014) reported Tg values for inulin stored at different water contents, finding that for the proper determination of Tg it is necessary to start from the fully amorphous state. The procedure for achieving the desired final state is shown schematically in Fig. 3 and is described as follows: (i) determine the initial state of inulin. If inulin is in the semicrystalline or crystalline state, then the amorphous state must be induced. (ii) Determine the thermal degradation temperature in order to find the maximum temperature supported by the material. (iii) Finally, determine the melting

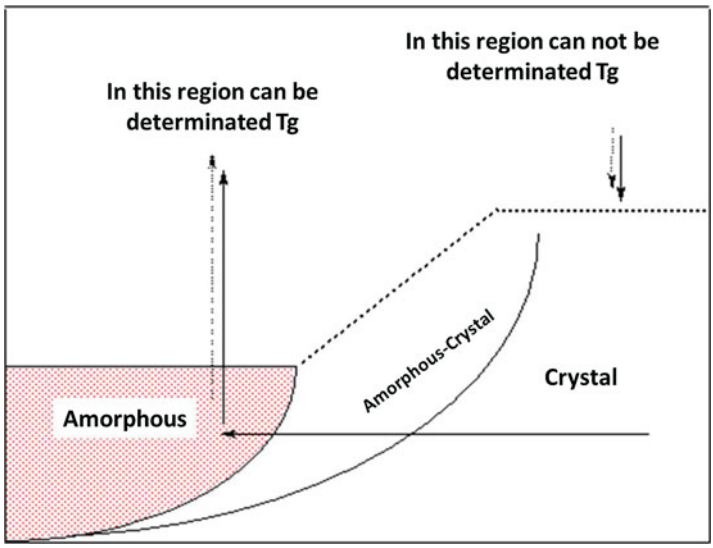

Fig. 3 Diagram of procedure for determining Tg

temperature and glass transition temperature. Following this procedure allows obtaining amorphous inulin powder. Jiang et al. (2008) proposed a method based on calorimetric experiments for determining the Tg of amorphous structures from the melting of crystalline sugars. The method was improved by Saavedra et al. (2011) who employed sugar-rich systems with an initial crystalline structure. As mentioned earlier, inulin is a white powder which apparently looks amorphous. However, X-ray diffraction (XRD) results obtained from Saavedra-Leos et al. (2014) showed that the structure of inulin powder depends on the adsorbed water content and can be either semicrystalline or crystalline. Therefore, for determination of the Tg, the amorphous state in inulin powder must be first induced.

Differential scanning calorimetry (DSC) is a technique widely used for determining phase transitions in organic compounds and food products. The bases of the technique are reported elsewhere. However, if the structure is semicrystalline, Tg may not be determined correctly by this technique. Thus, for the proper determination of Tg, it is necessary to induce the amorphous state according to the procedure shown in Fig. 3. In some reports, DSC has been employed together with a standardized thermal treatment for reaching the amorphous state (Badrinarayanan et al. 2007; Plazek and Bero 2003). Even with the efforts made in this direction, different Tg values have been reported for the same carbohydrate, i.e., for sucrose, a Tg in the range of 60–79 °C is reported. These results are explained in terms of physical or chemical changes exerted on the carbohydrate, which may occur during the thermal treatments (Kalichevsky et al. 1993; Roos 1995). Vanhal and Blond (1999) found that for sucrose, the melting is followed by a decomposition process that starts with the breaking of the glycosidic bond and the further production of glucose and fructose. Unfortunately, the degradation process

is carried out simultaneously during the melting; therefore, the chemical composition of the carbohydrate is varying along the thermal treatment. In this regard, Saavedra et al. (2012) developed a methodology for accurately determining the Tg from mixtures of sugars such as glucose, fructose, and sucrose in the crystalline state. The method consists in preparing an amorphous sugar powder without modifying the chemical composition: (i) the crystalline sugar is heated to the melting temperature (T_1) and kept at this temperature for 2 min. (ii) The sample is rapidly quenched until a temperature of −15 °C (T_2) and held there for 2 min. (iii) The sample is heated again to the temperature (T_3) and the Tg is determined from this heating curve.

For the accurate determination of the Tg, besides the induction of the amorphous state in the sample, it is recommendable to employ the modulated differential scanning calorimetry (MDSC). This technique is similar to DSC but differs in the way the sample is heated. In MDSC the heating is controlled by modulating the temperature amplitude and the period. The advantage from MDSC is the possibility to separate the signals forming the heat flow curve into reversible and nonreversible transitions. This allows to accurately differentiate the events taking place during the heating. For example, in the reversible heat flow curve, the glass transition temperature is observed as the slope of the curve changes, while melting is presented as an inflection point. In the nonreversible heat flow curve, the nonreversible events such as thermal degradation are observed. Recently, Saavedra et al. (2014) employed MDSC and reported accurate values of Tg for inulin and inulin-complex systems stored at different water activities. Among their findings is the observation in the evolution of Tg as water by the systems was adsorbed.

2.4 Undesired Characteristic: Stickiness, Agglomeration, and Crystallization

Stickiness, agglomeration, and caking are three undesired characteristics presented during the processing, handling, and storing of sugar-rich food products. These characteristics are related to the collapsing of the microstructure carried out when the matrix cannot support its own weight, producing radical changes in the macroscopic structure as the decrease of the volume, the stuck of the powder particles, color change, etc. During the collapse, water is released, and as a consequence, the viscosity of the system decreases up to values of 10^{12} (Pa s). Stickiness is defined as the capacity of a material for adhering on a solid surface under the effect of a slight pressure. Again, at the microscopic level there is a competition between the cohesive and attractive forces acting on the sticky material and the solid surface. Cohesion is defined as the attractive forces acting between adjacent particles or molecules in the same matrix; adhesion is the interaction among the surfaces of different bodies. Cohesion is the force responsible for caking and agglomeration of foodstuff powders, while adhesion is responsible for the stickiness presented on the walls of a dryer during the drying process of the powder. Foster et al. (2006) studied the effect of storing time on the cohesion of sucrose, maltose, glucose, galactose,

and fructose powders under environmental controlled conditions of humidity and temperature. They reported that the cohesion of the powders is directly related to the amount of adsorbed water and the glass transition temperature. Likewise, at temperatures above the Tg of the system, agglomeration is promoted because of the formation of liquid bonds inside the powder matrix. Moreover, adhesion which takes place among the drops of liquid and the walls of the drier during the spray drying process was studied by Truong et al. (2005). They reported that some sugar-rich systems behave as hygroscopic thermoplastics which lead to problems of sticking of powder particles on the walls of the dryer. In the case of inulin, Saavedra et al. (2014) showed that the problems of agglomeration and stickiness are presented only when powders were stored at temperature above 60 °C and water activity (a_w) of 0.5. Similarly, they reported the storage conditions for the inulin-orange juice system. The complex system presented agglomeration when stored at temperatures above 45 °C and a_w of 0.2.

The interaction mechanisms between the two surfaces are divided into four groups: intermolecular forces, electrostatic forces, liquid bridges, and solid bridges; all of them are closely dependent on the particle size. If the particle size is smaller than 1 μm, the overall surface area is increased. Then, the attractive forces between the particles are much bigger, observing larger effects of agglomeration and caking in the powder product. The interaction mechanism in stickiness, which is presented during the drying process, corresponds to the liquid bridge. The liquid bridges may be pendulum, funicular, or capillary type. Pendulum type is presented when the liquid is distributed on the solid in a discrete way, forming a mixture of liquid drops and air bubbles. Funicular bonding is similar to pendulum interactions but with air bubbles trapped inside the liquid. In capillary type the liquid is homogeneously distributed on the surface of the solid, creating a continuous liquid phase. Andhikari et al. (2003) studied the stickiness for solutions of fructose and maltodextrin during the drying process. They related the molecular weight with the force required to separate the solution of fructose/maltodextrin from the contact surface. The required force was larger for the fructose than for maltodextrin, because in the former the adhesive interacting forces are larger than the cohesive forces; the opposite was for the maltodextrin. In this sense, inulin is a carbohydrate similar to fructose and maltodextrin with long chain molecules and molecular weight higher than 52 kD. Obón et al. (2009) prepared a powder of red-purple colorant employed as functional food from the juice of *Opuntia stricta*. This powder presented stickiness problems associated to the phenomena of cohesion and adhesion. The solution proposed for this problem was to employ mixtures of inulin or maltodextrin for encapsulating the powder particles while keeping the amorphous state in the fruit juice.

2.5 Phase Stability

Phase stability is related with the structural changes carried out during the processing and storage of food products. For inulin, the study of phase stability is

a very interesting topic because of the potential improving properties on the food product during storage. In this sense, the performance of inulin has been studied as a functional agent in the pharmaceutical and food industries. As functional agent, inulin has been employed as excipient, additive, thickener, emulsifier, gelling agent, sugar and fat substitute, etc. From the point of view of phase stability, the most common problems presented during the handling of amorphous powders are stickiness, agglomeration, crystallization, loss of volatile compounds, and crush (Andhikari et al. 2001; Kay and Nottingham 2008; Khalloufi et al. 2000). Recently, the stability of inulin during the storage has been evaluated and related to the concepts of a_w and Tg, where a_w is expressed as the amount of water adsorbed per 100 g of dried sample. In other works, inulin was analyzed as a model system: Blecker et al. (2003) discussed about the structure, stability, and texture of inulin as a fiber, highlighting the importance of this ingredient in the dietary fiber, fat substitute, and in other applications in the food industry; Kawai et al. (2011) investigated the effect of water content, molecular weight, and crystallinity on the Tg; Zimeri and Kokini (2002) determined the Tg for inulin and inulin-water mixtures at different water contents, finding that Tg increases at low water contents, when inulin is semicrystalline and at high molecular weights; Ronkart et al., (2006, 2009) studied the Tg for inulin with different polymerization degrees, finding that Tg increases with the degree of polymerization. Saavedra et al. (2014) found that, up to a water content of 10 % and water activity of 0.6, the structure of inulin remains stable and without agglomeration; however, at levels higher than 15 % and 0.8, agglomerations were observed. They concluded that inulin was more stable when stored at temperatures lower than its Tg and water content below the monolayer level. Therefore, undesired characteristics such as stickiness, agglomeration, and crystallization, which largely depend on the processing and storage conditions, may be overcome if inulin is kept under those conditions of temperature and humidity.

2.6 State Diagram for Stability of Inulin

In food products, the amount of water adsorbed depends on different characteristics from sample and environmental conditions such as chemical composition, structure, temperature, and relative humidity (Jaya and Das 2009; Roos 1995; Sablani et al. 2010). All of these characteristics and others can be displayed together in a single plot known as state diagram. A state diagram is a graphical representation of the different states observed in a material or mixture of materials at different conditions of temperature, pressure, composition, etc. Thus, in this type of diagram, the final state, i.e., if the structure of the material is amorphous or crystalline, may be predicted for given conditions of temperature and composition. Several authors have reported state diagrams based on Tg and a_w measurements, for systems rich in sugars such as dehydrated fruits and fruit powders (raspberry, blueberry, strawberry, kiwi, grape, tomato, mango, pineapple, apple, etc.): Sá and Sereno 1994; Welti-Chanes et al. 1999; Khalloufi et al. 2000; Bai et al. 2001; Sobral et al. 2001;

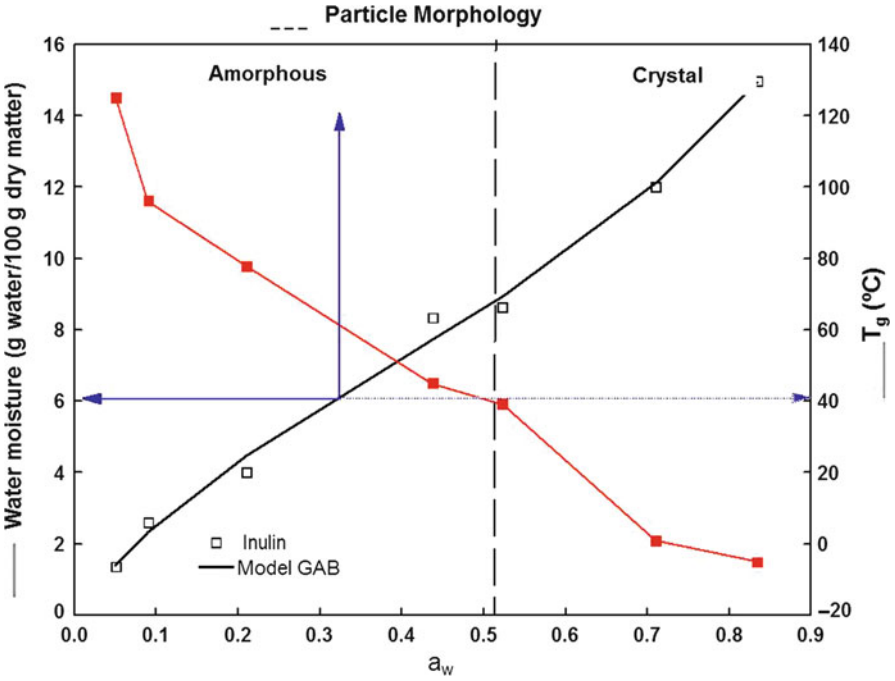

Fig. 4 Diagram for predicting the stability of inulin during storage at different a_w

Telis and Sobral 2001, 2002; Moraga et al. 2004; 2006; Silva et al. 2006; Goula et al. 2008; Wang et al. 2008; Jaya and Das 2009; Fabra et al. 2009; and Tonon et al. 2009. However, for inulin Saavedra-Leos et al. (2014) reported a state diagram including other aspects such as water content, microstructure, and morphology. The data plotted in that diagram provides information for predicting the stability of inulin during storage. Figure 4 presents a state diagram for inulin including the aspects aforementioned. For example, at a (a_w) of 0.31, inulin adsorbed a water content of 6 g of water/100 g of dried material; Tg at these conditions is about 60 °C. This means that inulin will remain in the amorphous state when stored at these conditions of humidity and temperature. On the other hand, if inulin contains an amount of water higher than 8 g of water/100 g of dried material (a_w of 0.523), the powder must be stored below 40 °C in order to avoid crystallization and agglomeration. These results were confirmed by scanning electron microscopy (SEM) and optical microscopy (OM) observations. In Fig. 5 SEM and OM images for inulin at three levels of water activity (a_w = 0.05, 0.523, and 0.71) are presented. In the images the microstructure developed by inulin at the given a_w at room temperature is observed. At a_w of 0.05 and 0.523, the amorphous state is observed in the form of spherical particles of inulin with sizes of about 20 μm in diameter. At a_w of 0.71, the spherical morphology disappeared and inulin is observed agglomerated. In OM images the characteristics of inulin in bulk are observed: powder is observed in white color and the color remains unchanged as

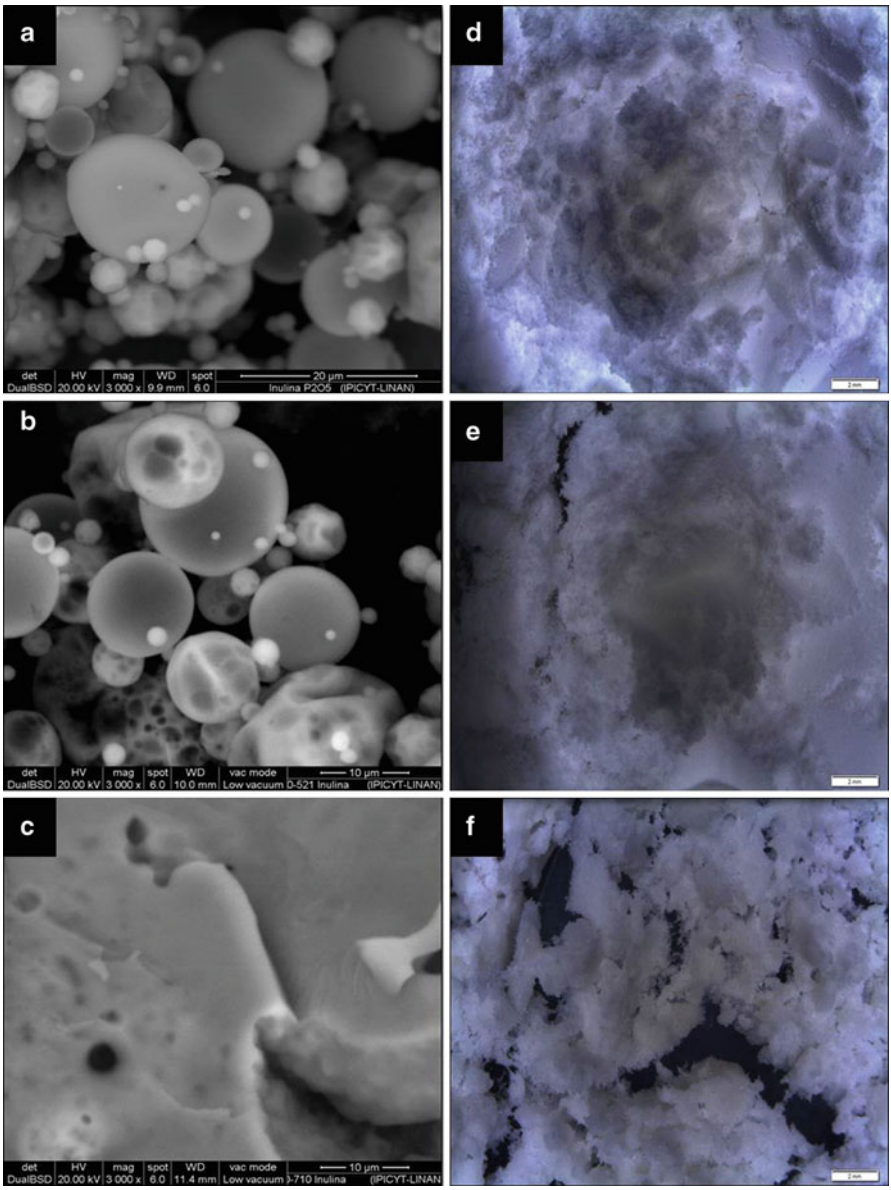

Fig. 5 SEM and OM images from powdered samples of inulin at three water activities corresponding to aw = 0.050, aw = 0.523, and aw = 0.710 and arranged in the figure from *top* to *bottom*, respectively. Images on the *left* column correspond to SEM images (**a**, **c**, and **d**), while those on the *right* side correspond to OM images (**b**, **d**, and **f**)

water adsorbed was increased. However, at this magnification, the only noticeable feature is the morphology of the particles. At low and medium water activities, the particles are randomly accommodated, while at high water activity, particles developed a needlelike morphology. Clearly, state diagrams can be very complex containing much more information measured in the laboratory and are not restricted only to temperature and compositional relationships. As more information is included in the diagram, this will be more useful and thus the physical properties of the material will be easier to predict.

3 Technological Applications of Inulin

From the nutritional and technological points of view, inulin may be considered as an outstanding biopolymer because of its wide range of applications in the pharmaceutical, chemical, and food industries (Franck and De Leenheer 2002). In the pharmaceutical industry, inulin offers a great potential as an excipient material in pills, adjuvant in vaccines, and encapsulating material for a wide variety of bioactive compounds (Rodriguéz et al. 2012). In food industry, inulin is largely employed as an ingredient in healthy food development; this application has been growing because of the benefits offered as natural prebiotic and dietary fiber. However, the interest in this polysaccharide is associated with food technology functions, where it has been employed as stabilizer, fat substitute, and improvement on the texture of food and other applications. In Table 2 some applications where inulin is employed and its functionality in the final food product are summarized.

Inulin represents a renewable resource that can be modified to form novel products, many of which have superior attributes to similar products derived from glucose polysaccharides. Modifications of inulin may expand the amount and type of technological applications because most of these compounds are readily biodegradable. For example, in the chemical industry, modified inulin by different chemistry processes is used as chelating agent and antifouling in pipes, containers, reaction chambers, and separation equipment, among others, or converted into plastic films as a proposed alternative for the substitution of petroleum polymers (Grandther et al. 2005; Kasapis 2006; Verraest et al. 1996).

3.1 Pharmaceutical Applications

The aim of a drug delivery system is to maximize the exposure of a drug into an organ, tissue, or cells requiring treatment. Also it may be used to stabilize labile drugs such as proteins and peptides. This helps to minimize dosage, reducing side effects and cost, while maximizing efficacy and allowing the patients to choose the preferred route of administration, such as oral rather than injected routes. Inulin is generally biochemically inert and nontoxic and can form hydrogels.

Inulin is an ideal vehicle for drug delivery into the colon since the β(2-1) glycosidic linkages are stable to the range of pH and ionic strength observed in

Table 2 Application of inulin in food products

Functionality	Product
Prebiotic	Baked goods and bread
	Dairy products
	Dietetic products
	Frozen desserts
Fiber	Baked goods and bread
	Dietetic products
	Frozen desserts
Fat substitute	Baked goods and bread
	Chocolate
	Dairy products
	Meat products
Sugar substitute	Baked goods and bread
	Chocolate
	Frozen dessert
Texture modifier	Baked goods and bread
	Dairy products
	Meat products
	Salad dressings
Wall material	Bioactive compound encapsulation
Emulsifier	Baked goods and bread
	Salad dressings
	Dairy products
	Meat products

the human gastrointestinal tract, thus avoiding the endogenous enzymatic action of the human digestive system and reaching the colon without reacting. Once there, the glycosidic linkages are broken by the colonic bacteria, releasing the loaded drug directly in the organ. Therefore, while inulin hydrogels might be ideal for drug transport into the colon, the gel swelling characteristics must remain unchanged during the journey inside the digestive system. Hydrogel stability can be increased by modifying the crosslinking conditions, and for inulin polymers this has been achieved through different chemical modifications (Barclay et al. 2010; Kasapis 2006). For example, inulin, inulin acetate, and modified inulin acetate microspheres have been used for the transport of water-soluble model drugs with the modified inulin acetate microspheres being supramolecularly associated with 1,2-dodecanedicarboxylic acid. Encapsulation was similar for all three species, being maximized at 65 %. Drug release was initially quick as the outside of the microspheres. For inulin and inulin acetate, 58–62 % of the drug was released within the first 5 min, whereas for modified inulin acetate, only 32 % was released within the first 15 min. After this time the drug was released slowly by diffusion for approximately 1 day, after which the erosion of the microspheres led to quicker release until complete after 3.5–4 days.

Another application of the inulin in the pharmaceutical area is as a cryoprotectant . In this aspect inulin can be employed in the lyophilization of proteins with for

protecting it from conformational changes and avoiding the loss in its biological activity. In this regard, previous studies have demonstrated that inulin exerts two protective effects: (i) replaces the water that hydrates the proteins and (ii), once the protein is encapsulated within a vitreous structure, prevents its deployment while preserving its morphology and crystallization, inhibiting the kinetics of degradation reactions during storage (Barclay et al. 2010).

3.2 Food Applications

Inulin was first studied as stabilizer by Staffolo et al. (2004), using 1.3 % of inulin fibers for the stabilize bamboo, wheat, and apple in diary medium. They reported a larger stability for the yogurt with inulin fibers than for the control test. The success of this property was observed as the high level of acceptation of yogurts with fiber. The sensory responses were carried out according to a hedonic response, where the texture property was widely accepted in more than 50 % of the individuals surveyed. As fat substitute, inulin has been largely employed in the development of dietary products in the food industry of countries with developed economies, because of the similar features as in fat but without the caloric contribution. The use of inulin as a fat substitute is related with its solubility and degree of polymerization. The solubility is a property utilized in the formation of gels of inulin in dairy products, spreads, dressings, sauces, and meat products, in which functional properties provided by fats are essential to achieve the desired sensory effects by consumers. Inulin gels can be formed by mechanical or thermal effect; those obtained by heat treatments present better texture and firmness. The characteristics of the gel are dependent on the temperature, stirring speed, degree of polymerization, and concentration of inulin. The gel is a three-dimensional network of insoluble particles with sub-micrometric size. Liquid water is immobilized within these particles, which ensures physical stability. When the concentration of inulin in the solution exceeds 15 %, a gel may be formed providing a creamy effect to the product; below this concentration, inulin solutions behave as low viscosity liquids. Since the firmness of the gel is increased with the concentration of inulin, a maximum level of firmness can be achieved by the combination of shear treatments and the addition of seed crystals during the cooling (Kim et al. 2001). Results showed that only the inulin molecules with DP larger than 10 may contribute to the gel, while shorter molecules remain dissolved in the liquid (Staffolo et al. 2004). Chiavaro et al. (2007) studied the ability of commercial LCI for forming gels at 25 °C and 50 °C. They found that inulin composed of oligosaccharides (ICO) gelatinized at concentrations of 30–60 % (w/w), while that with long chains gelatinized at a lower concentration range of 20–40 %. Textural and thermal properties were evaluated during storage at 4 °C. LCI gels showed better behavior, presenting more firmness, adhesion, and less cohesion, than those gels prepared with inulin of heterogeneous composition at a concentration of 40 %. Glibowski and Pikus (2011) evaluated the effect of filtration and the addition of seed crystals on the formation of gels of inulin. They found a larger gel stability for solutions of

inulin passed through large pore filter (>1 mm). Heating the inulin solution at 100 °C by 5 min produces the collapsing of the gel structure or the formation of inulin sediments. However, the addition of seed crystals after the heating treatment promotes the formation of stable gels again. Tseng et al. (2008) studied the influence of LCI and ICO solutions on the thermal and mechanical properties of soy protein gels. The gels containing 8 % (v/v) of LCI showed an improvement larger than 40 % in both the elastic and loss moduli (G′ and G″) with respect to the control test. According to the reviewed literature, the optimal conditions for the formation of stable inulin gels are a concentration of 20–30 % (w/w), a heating temperature of 80–90 °C during 3–5 min, and pH levels between 6 and 8. When inulin is subjected to temperatures higher than 90 °C or to pH levels below 3, inulin chains are hydrolyzed in shorter length chains, avoiding the formation of the gel. At inulin concentrations below 10 %, there is no gel formation either, because the inulin-water system lacks enough inulin particles for forming the three-dimensional network characteristic of the gel.

Regarding the texture of food products, it is important to note that inulin gels present a texture similar to that from fat while giving a desired taste without the caloric intake. Unlike other insoluble fibers that have been used as fat substitute, inulin may be considered as a better option in this application. Besides its neutral flavor, the outstanding property of inulin in this field depends on the immobilization of water molecules during the formation of the gel. The sensory effect produced by the inulin gel during the tasting of the food product causes the sensation of consuming a fatty product. These characteristics allow inulin to be employed in many meat, dairy, and bakery products. In this field, one of the pioneers was Meyer et al. (2011) who employed inulin for modifying the texture of dairy products. They found that the texture of the dairy products tested (fermented milk, kefir, milk drinks, yogurts, desserts, cheese, and ice cream) depends on both the concentration of inulin and the degree of polymerization. Likewise, the addition of LCI in dairy products may affect not only the texture of the product but the rheology. This depends on the interaction of inulin with the other ingredients and solvents presented in the product, especially other carbohydrates and water. The chemical structure of the product and the presence of ingredients such as hydrocolloids may largely affect the rate and degree of crystallization of inulin, which influence its performance as fat substitute. At concentrations higher than 20 % of LCI, in the solution there is a competition between the starch and inulin molecules for the available water: starch can completely gelatinize or inulin can be either fully dissolved or form a gel. The capability of inulin as fat substitute is not only related to the modification on the rheological behavior of the food product but also on other characteristics such as creaminess and smoothness. In general, in products where these two features are desired, it is necessary to employ high concentrations of inulin to achieve the rheological and textural properties showed by the product elaborated with fat. Zahn et al. (2010) evaluated the effect of the full and partial substitutions of fat in the formulation of bakery products (muffins). The results showed that it is possible to replace up to 50 % of the fat required to bake these products.

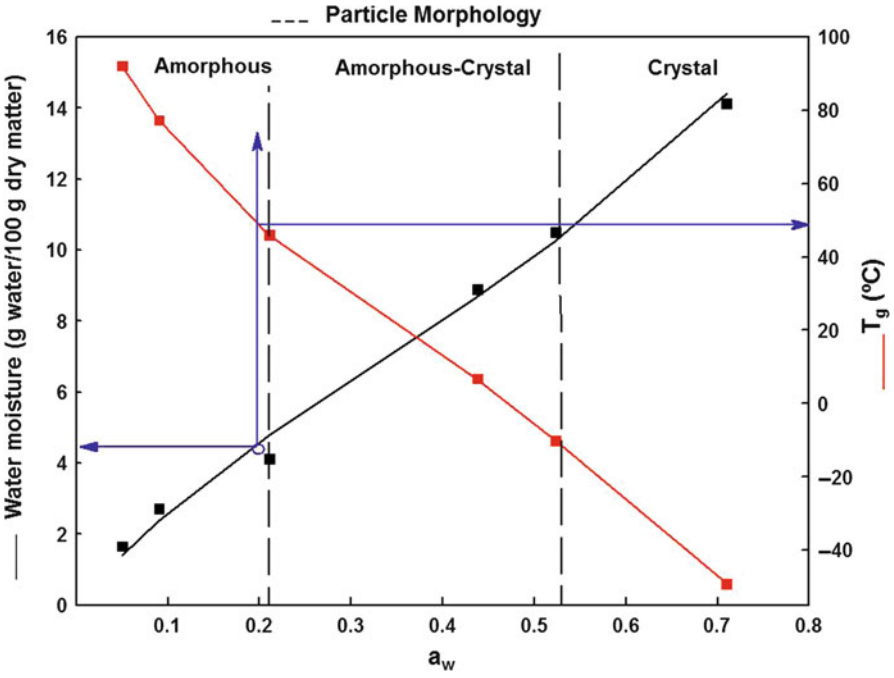

Fig. 6 Diagram for predicting the stability of thr system inulin-orange juice

This fat substitution is equivalent to 45 % of the total fat of the product and reduces the caloric contribution from 13 % to 16 %.

Another application based on the concepts of science and technology was recently reported in literature by Saavedra et al. (2014) who employed inulin for developing a functional food product. The product is a mixture of inulin and orange juice, obtained in the form of a dried powder with an amorphous structure and without sticking problems. The evolution of the properties with the content of water was used as the indicator to set the conservation of the system. They reported a state diagram for the system inulin-orange juice, as that shown in Fig. 6. From the state diagram the optimal storage conditions for the conservation of the amorphous structure were possible to establish: maximum storage temperature of 47 °C, water activity (a_w) below 0.21, and water content below 4.2 g of water per 100 g of dried material. The very first feature observed in this state diagram is the presence of three structures developed by the system: amorphous, semicrystalline, and crystalline. The intermediate structure is a region where both amorphous and crystalline moieties coexist; in the intermediate region, phase transitions have begun producing a physical change in the food product. These zones were established by XRD analysis, SEM, and OM observations at room temperature. The second noticeable feature is the relative position of the curves when compared to the inulin state diagram (shown in Fig. 4). The vertical line separating the

amorphous state from the semicrystalline state has shifted towards the left, thus to lower water activities. Physically, this shift means that the inulin-orange juice powder must be stored under a temperature of 47 °C and with a maximum water content of 4.2 g of water per 100 g of dried material in order to avoid undesired phase transition. The main difference is the amount of water withheld by the systems. While inulin may support approximately 8.8 g of water per 100 g of dried material, inulin-orange juice may withhold only 4.2 g of water. The orange juice is a chemical composition complex system characterized by a high content of low molecular weight sugars (fructose, glucose, and citric acid) which have a wide range of glass transition temperature, from 12 °C to 62 °C (Slade and Levine 1994). The chemical composition of these sugars produces a greater availability of active sites generating more chemical interaction types: van der Waals and hydrogen bonding. Then, water is preferentially adsorbed by the inulin-orange juice system. At the microscopic level, water molecules act as a plasticizer into the polymeric chains of the system intensifying the intermolecular distance and the molecular mobility, which is observed as structural disorder. Macroscopically, this disorder is observed as a decrease in the viscosity of the system and the phase transformation from an amorphous into a crystalline structure.

Another characteristic observed in the state diagram is a small difference in the Tg of both systems, 40 °C and 47 °C for inulin and inulin-orange juice, respectively. Although this small difference in temperatures may be considered as a positive feature for the complex system, it is actually a disadvantage. This is because the slope of the curves is much pronounced for the orange juice complex system than for the inulin. This indicates that small changes in the adsorption of water will require lower storage temperatures. In example, while the inulin system can be stored at a temperature of 40 °C and absorbs water from the environment up to a value of 8.8 g per 100 g of dried material, if the inulin-orange juice powder is stored at 47 °C and water is adsorbed from environment reaching a maximum value of 10.5 g of water per 100 g of dried material, the temperature of the room must be lowered to −9.9 °C in order to avoid crystallization of the powder and product deterioration. Clearly, from the economic point of view, this is a disadvantage for the complex system since it requires more care during handling and storage of the food product. Moreover, higher Tg values means that powders must be stored in warehouses properly conditioned for supporting and maintaining those temperatures. Sugar-rich systems require keeping high room temperatures in order to maintain the product in good conditions. Obviously, this is a dangerous situation for any person handling the powder in a room set at temperatures higher than 35 °C.

As mentioned before, the results were confirmed from SEM and OM observations; these two characterization techniques have the advantages of availability and relative low cost, in industries such as food product companies. Furthermore, the results obtained from these techniques are easy to understand without further interpretation. Figure 7 shows SEM and OM micrographs from the inulin-orange

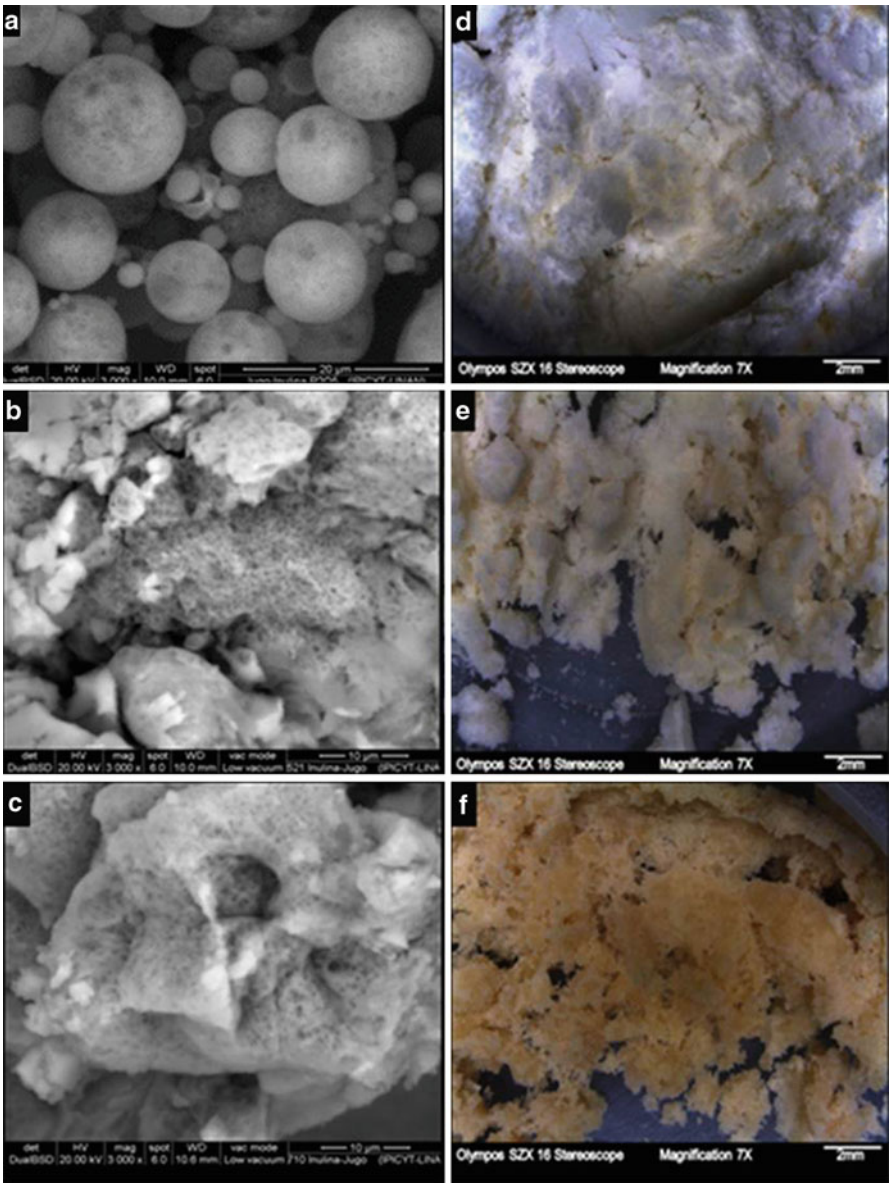

Fig. 7 SEM and OM images from powdered samples of the system inulin-orange juice at three water activities corresponding to aw = 0.050, aw = 0.523, and aw = 0.710 and arranged in the figure from *top* to *bottom*, respectively. Images on the *left* column correspond to SEM images (**a**, **c**, and **d**), while those on the *right* side correspond to OM images (**b**, **d**, and **f**)

juice system. From the SEM images, it is observed that the microstructure of inulin rapidly changes even at low water activities. The well-defined spherical morphology is lost and the material is observed as agglomerated. This effect is magnified at higher water activity. In the OM images another feature is observed: the color of the powder. The inulin-orange juice powder is observed in white color at low water activity. As water adsorption was increased, the color changed to yellowish and later to orange at the major water content. Evidently, these changes are produced by the crystallization of the low molecular weight species content in the orange juice. The overall morphology of the powder also presented a change at these water activities. Morphology was first observed as particles randomly accommodate, i.e., without any specific shape. As orange juice components crystallized in inulin, the morphology changed to needle-shaped particles. These modifications in the microstructure produce an overall appearance that can be easily related to the effect of the orange juice in the system and may help to visually distinguish a crystallized system from an amorphous one.

All the above provides an overview on the applications of inulin; depending on the desired final properties of the food product, inulin may positively affect the performance and in consequence influence the acceptance of the product in the market. Finally, from any point of view, scientific or industrial, this type of information can be very helpful in decision making.

4 Conclusions

Through these pages, inulin a very important natural carbohydrate polymer was reviewed. This polysaccharide is naturally found in a wide variety of plants and is industrially extracted mainly from the chicory root in the form of white, neutral flavor, odorless powder. In the recent decades, scientists and technologists from food, pharmaceutical, and chemical industries have shown great interest in studying the physicochemical properties of inulin to find alternatives in the development of new products. Inulin has a chemical structure mainly made of linear chains of fructose, which imparts very important physical properties. The degree of polymerization largely influences the final properties of the inulin. The application field of this carbohydrate polymer is wide since it can satisfy the requirements from industry related with the development of new products and the needs from consumers related with healthy products. Products formulated with inulin have been well accepted by consumers because the textural, rheological, and sensorial effects are maintained while lowering the caloric ingest in diary, meat, bakery, and candy products. The aim of the work was to provide an overview of the main physical properties of inulin to demonstrate the applicability of this carbohydrate polymer in many products consumed in daily life.

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Part III

Methods

Microwave-Assisted Extraction of Polysaccharides

34

Ana Cristina Soria, Laura Ruiz-Aceituno, Lourdes Ramos, and Luz Maria Sanz

Contents

Abstract

In this chapter, the use of microwave irradiation has been reviewed and discussed for the extraction of polysaccharides as well as for combined processes involving extraction and hydrolysis of these compounds. Special attention has been paid to polysaccharides with bioactive properties. Fundamentals and instrumentation, together with a detailed discussion on the effect of the most important parameters affecting the microwave-assisted extraction (MAE) process, are presented. Some of the most recent and outstanding applications of MAE for the extraction of polysaccharides, mainly from food matrices or food by-products, are described and classified according to the type of polysaccharide extracted. The comparison in terms of speed, yield, etc. of MAE with other conventional (solid–liquid extraction) or emerging techniques

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(pressurized liquid extraction, ultrasound-assisted extraction) is also shown. The scale-up of MAE technique and the development of hybrid systems (e.g., ultrasonic–microwave-assisted extraction, UMAE) are shown as future trends. To conclude, MAE is shown as a promising emerging technique for extraction of polysaccharides from natural sources.

Keywords
Microwave-assisted extraction (MAE) • Pectins • Galactomannans • Arabinogalactans • Xylans • Sulfated polysaccharides • Optimization

1 Introduction

Different bioactivities (immunostimulatory, prebiotic, antioxidant...) and technological functionalities (emulsifying, gelling...) have been attributed to polysaccharides (Simões et al. 2009; Zeng et al. 2012; Holck et al. 2014), as mentioned in previous chapters.

Extraction of these compounds from plant and food sources has attracted a great deal of interest with a view to provide natural extracts rich in bioactive ingredients of potential application, among others, in the food industry. It is also known that the composition of extracts and their bioactivity are widely dependent on the extraction technique and experimental conditions used. Conventional treatments based on sequential acid or basic extraction processes have been commonly used for polysaccharide extraction. However, these methods are time-consuming, and yields and purity of the final extract are relatively low. Therefore, there is a wide interest in the search for faster and more efficient alternative methods for the extraction of these compounds. In this sense, different emerging techniques such as microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and ultrasound-assisted extraction (UAE) have been described (Meireles 2009).

In this chapter, MAE will be evaluated as it generally provides mild extraction conditions, extracts of high purity with high yields of extraction, short processing times, low production cost and waste generation, etc. (Wang and Weller 2006). In addition to an overview of fundamentals and mechanisms of MAE, the characteristics of the most common instrumentation used for polysaccharide extraction and the effect of the main variables affecting the MAE process will be discussed in detail. Some of the most recent applications regarding the extraction of polysaccharides, mostly from foods and food by-products, will be described. Extractions from other biological matrices will also be mentioned in specific cases. For concluding, advantages and disadvantages of this technique and future perspectives will be discussed.

2 Fundamentals

2.1 Operation Mechanism

Microwave (MW) energy is a nonionizing type of electromagnetic radiation with wavelengths typically in the 1 mm to 1 m range, corresponding to 0.3–300 GHz. Nevertheless, most of the commercialized instruments, both for domestic and industrial application, operate at fixed frequencies (typically, 2.45 GHz).

The application of microwaves to a medium promotes the electrophoretic migration of ions following the electric field produced by the microwave (Nadagouda et al. 2011). The direction of the ions changes as many times as the field changes its sign. The resistance of the medium to the ionic migration generates heat as a result of collision between molecules. At the same time, dipolar molecules attempt to align with the electric field. The promoted phenomenon of alignment, randomization, and realignment of the dipoles leads to collisions between them and the surrounding molecules, which generates energy and increases the medium temperature. In general, ionic conduction and dipole rotation occur simultaneously and change microwave energy into thermal one (Zhang et al. 2011), resulting in an almost immediate heating up of the sample.

The mechanism through which the microwave energy is absorbed is consequently not unique and depends on the nature of the considered substance. Thus, molecules that do not have a dipole moment (or in which a dipole moment cannot be induced) cannot be directly heated by microwaves. Gases cannot be heated by this energy either due to the large distances existing in between molecules. However, when applied to solid or dissolved samples, microwave energy is directly absorbed by the medium in which the molecules are imbibed. This is the reason why this form of energy results in a more efficient source of heating than any other conventional thermal heating procedure, in which the walls of the container are primarily heated and heat is then transferred to the sample. The fact that the microwaves penetrate uniformly throughout the volume of the product (liquid, suspension, or semisolid) being heated is usually referred to as microwave volumetric heating (MVH).

Materials can be classified on the basis of their ability to absorb microwave energy as: conductors, whose surfaces reflect microwaves (e.g., metals); insulators, which are transparent to this energy and can be used to support the materials to be heated (e.g., plastic); and dielectrics, which absorb the microwaves and are easily heated (e.g., polar liquids).

In general, the capability of molecules to absorb microwave energy increases as their dielectric constant (ε') does, which in turn results in a faster solvent heating and extraction at higher temperatures. Nevertheless, solvents with low dielectric constants can also be used for MAE. In this type of approach, microwaves are absorbed by the matrix promoting internal heating, which results in cell structure disruption by expansion. The target compound is then liberated to the cool medium.

This procedure has been proved to be useful, for example, for the extraction of thermolabile nonpolar compounds into transparent solvents (Eskilsson and Björklund 2000; Romanik et al. 2007). However, in most applications, polar or mixtures of nonpolar−polar solvents are used as extractants. Table 1 summarizes relevant physical constants for selected solvents commonly used in MW extraction.

The dielectric constant of a solvent can be considered as an indication of its capacity to be polarized in an electric field. However, the so-called dielectric loss (ε''), a measure of the efficiency of the solvent to dissipate the absorbed microwave energy into heat, should also be considered. The ratio between these two values is the dissipation factor ($\ln \delta = \varepsilon''/\varepsilon'$) and determines the efficiency of the extraction process. As previously indicated, polar solvents and ionic solutions absorb microwave energy strongly due to the presence of permanent dipoles, whereas nonpolar solvents, such as hexane, are not really indicated for use in MAE, unless they are mixed with a polar solvent (Table 1). On the basis of their dielectric constant, water has higher ability to obstruct the microwaves as they pass through than, for instance, methanol and, thereby, it will improve microwave penetration. However, methanol has higher ability than water to dissipate the microwave energy as heat (Eskilsson and Björklund 2000). According to these considerations, heterogeneous samples or mixtures containing species with different dielectric properties dispersed into a homogeneous medium will have different capability to absorb microwaves at different points of the mixture. This can result in selective heating of specific components or sample areas. This phenomenon is called superheating (Baghurst and Mingos 1992) and has been exploited, for instance, in the extraction of nonpolar analytes into transparent solvents (Vetter et al. 1999; Hummert et al. 1996).

Table 1 Physical constants and dissipation factors for some solvents commonly used in MAE

Solvent	Dielectric constant[a], ε'	Dipole moment[b]	Dissipation factor, tan δ ($\times 10^{-4}$)	Boiling point[c] (°C)	Closed-vessel temperature[d] (°C)
Acetone	20.7			56	164
Acetonitrile	37.5			82	194
Ethanol	24.3	1.96	2,500	78	164
Hexane	1.89			69	–[e]
Methanol	32.6	2.87	6,400	65	151
2-Propanol	19.9	1.66	6,700	82	145
Water	78.3	2.3	1,570	100	
Acetone–hexane (1:1, v/v)				52	156

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[a]Determined at 20 °C
[b]Determined at 25 °C
[c]Determined at 101.4 kPa
[d]Determined at 1,207 kPa
[e]Indicates no microwave heating

2.2 Instrumentation

At present, two types of microwave heating systems are commercially available at laboratory scale: open- and closed-vessel systems (Fig. 1). The former, also named as focused microwave-assisted solvent extraction (FMASE) system, uses open extraction vessels under atmospheric conditions. Consequently, their maximum operation temperature is the solvent boiling point at atmospheric pressure and is frequently refluxed to prevent solvent evaporation. In this type of systems, irradiation is continuous, power can be regulated, and up to eight samples can be simultaneously processed in glass or quart vessels (Milestone 2007). These instruments have been mainly used for the extraction of organometallic compounds (Takeuchi et al. 2008).

Closed MAE systems operate with closed extraction vessels, which allow the use of temperatures above the boiling point of the solvent [up to three folds higher under superheating conditions (Takeuchi et al. 2008)] without a significant solvent evaporation. This high operation temperature frequently results in high extraction efficiencies in short times and with minimum solvent consumption. In these instruments, the temperature and the pressure inside the vessel are typically controlled in only one out of the 4–48 cells that can be simultaneously processed. This makes mandatory that all samples are identical in terms of solvent nature and solid–liquid ratio (SLR). Alternatively, systems provided with infrared sensors are also commercially available for monitoring of the temperature in every extraction vessel. All these MAE systems allow setting different powers and modification of irradiation cycles in a multimode cavity equipped with a turntable that rotates for improved irradiation homogeneity. Due to their advantageous features, these systems are the most profusely used in all application fields.

The basic setup for closed-vessel MAE consists of a magnetron tube (although systems with sequential magnetrons to help fine-tune and maximize MW power are also available), an oven where the extraction vessels are placed upon a rotating

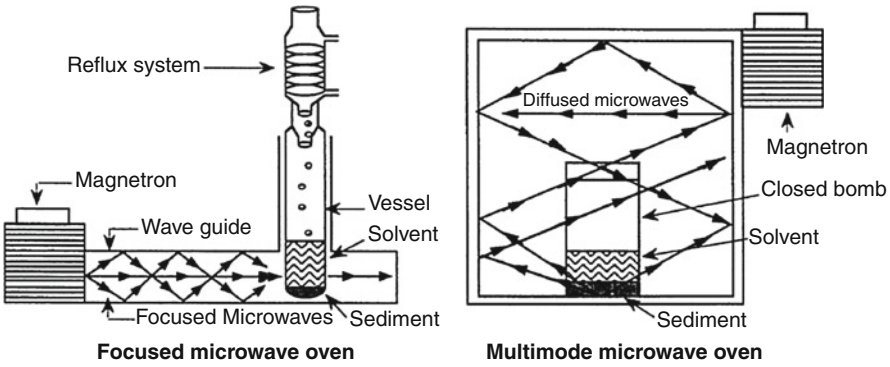

Fig. 1 Schematic view of focused microwave oven and multimode microwave oven (Reprinted from Letellier and Budzinski (1999), with permission of EDP Sciences)

carrousel, and different temperature and pressure sensors. In addition, a number of electronics and safety features can be included, such as a solvent vapor sensor which constantly monitors the cavity to detect solvent leakages, a chamber located in the center of the carrousel and connected to each vessel for vapor collection in the event of solvent leakage, an exhaust fan in the oven, extra oven isolators, and a movable door to release the pressure inside the instrument cavity. Other safety accessories are rupture membranes for the extraction vessels that burst at pressures above 200 psi or resealable vessels that open and close rapidly to prevent over-pressure inside the extraction cells. Vessels are typically made of microwave transparent materials, such as glass and a variety of plastics (polyether imide, tetrafluoromethoxyl, Teflon®, etc.); their selection is usually conditioned by the extractants and the pressure and temperature used during the MAE process. In most models, magnetic stirring is feasible for improved and rapid homogenization of the extraction medium temperature.

A typical MAE experiment consists of loading of the homogenized and, when required, pretreated sample into the extraction vessel, followed by solvent addition and closing of the vessel. Once the vessels are installed in the carrousel, microwave irradiation starts with a short pre-extraction step that allows heating of the solvent at a selected temperature. The duration of this step (typically a few minutes) depends on the set value, the power applied, as well as the type and number of samples. Then, the sample is irradiated for a preselected time under some specific power conditions. Once the MAE step is completed, vessels are allowed to cool down to room temperature before proceeding with solvent and sample separation and, when required, further cleanup and/or concentration of the extracts.

2.3 Parameters Affecting MAE Efficiency

The solvent nature and volume, the extraction temperature and time, and the raw material humidity and polarity have commonly been identified as the most relevant parameters affecting the MAE process. Due to the mutual dependence among some of these parameters, in general, the optimization of the experimental extraction conditions is addressed by using experimental design approaches (Prakash Maran et al. 2013; Rodriguez-Jasso et al. 2011).

This section provides a brief discussion on the effect of some of the most important variables influencing the MAE process in closed-vessel systems, as these setups are the most frequently used for the extraction of polysaccharides.

Solvent nature. The solvent composition has a profound effect on the efficiency of the MAE process. When selecting the extraction solvent for a given application, not only the dielectric properties of the solvent (or mixture of solvents) selected but also the interaction of the extraction solvent with the investigated matrix and the analyte solubility on the solvent should be considered (Eskilsson and Björklund 2000).

Three types of solvents can be used in MAE (Jassie et al. 1997): (i) a solvent (or mixture of solvents) that absorbs microwaves strongly, (ii) a mixture containing

solvents with high and low dielectric losses, and (iii) microwave transparent solvent(s). The former case includes the use of polar organic solvents, water and acidic water mixtures, as well as novel green solvents, such as ionic solvents (Guolin et al. 2012). In all instances, it is the solvent which absorbs the microwave energy so its capability to wet (i.e., penetrate) and/or dissolve the matrix should be taken into consideration to improve the selectivity of the MAE step and to avoid any possible readsorption of the extracted analytes in (previously dissolved) matrix components during the cooling step. The second category of solvents corresponds to mixtures containing variable percentages of polar and nonpolar organic solvents such as acetone–hexane (1:1, v/v) mixture. Alternatively, the use of microwave transparent solvents modified with a relatively small amount of water (typically 10 %) has also been proposed in the literature. As mentioned above, this third possibility takes advance of the so-called superheating phenomenon and performs well with matrices having a high dielectric loss (and with, for instance, a high water content) but results in the destruction of the plant structures. The efficiency of this approach for the MAE of natural products such as essential oils has been illustrated in a number of application studies (Jocelyn Paré et al. 1994).

Finally, in the solvent-free microwave extraction (SFME), the internal heating of the water within the sample, as no additional solvent or water is required, distends the cells and leads to rupture of the glands and oleiferous receptacles of the plant. This process gives rise to the rapid extraction of essential oils from aromatic herbs, spices, and dry seeds (Mircioaga and Calinescu 2011).

Solvent volume. The solvent volume must be sufficient to ensure complete sample immersion. In this sense, special caution should be taken with matrices that swell during the extraction process. Typical solvent volumes used for the extraction are in the 10–30 mL range per gram of analyzed sample (Table 2). Unlike conventional extraction techniques where the higher the volume the higher the recoveries, in MAE, increasing the solvent volume may result in lower recoveries (Prakash Maran et al. 2013). The higher difficulty for homogeneous heat dissipation in the extraction medium as the solvent volume increases has been suggested as a possible explanation for this type of observation (Eskilsson et al. 1999). In such a case, stirring could be considered an appropriate solution to the problem. In this way, according to previous considerations, it looks evident that in MAE the SLR could be a more adequate variable to be considered during method optimization than the solvent volume itself.

Extraction temperature. As in any other extraction technique, the temperature is a key factor affecting the MAE efficiency. In general, an increase of the operation temperature reduces the surface tension and viscosity of the extraction solvent thus increasing its diffusivity into the sample matrix and improving its capacity to dissolve analytes. All together leads to improve the extraction efficiencies and, consequently, to shorten the analytical times. When MAE is performed in closed vessels, temperatures above the atmospheric boiling point of the solvent are frequently applied. Although this can be advantageous from the extraction yield point of view, it can also cause the degradation of thermolabile compounds. In practice, the optimal extraction temperature will depend on the nature of the extraction

Table 2 Some of the most recent applications of MAE of polysaccharides

Polysaccharide	Source	Solvent	Optimal MAE conditions	Yields (%, w/w)	Reference
Pectin	Grapefruit peel	Acidulated water	900 W; SLR[a]: 1:50; pH 1.5; 6 min	26.27	Bagherian et al. 2011
	Lemon peel	Ionic liquids in water	88.4 °C; SLR: 1:15; 9.63 min	25.10	Guolin et al. 2012
	Orange peel	Acidulated water	422 W; SLR: 1:16.9; pH 1.4; 2.81 min	19.24	Prakash Maran et al. 2013
	Sugar beet pulp	Acidulated water	152.63 W; SLR: 1:18.92; pH 1.57; 3.53 min	32.4	Li et al. 2012
	Berries	Water	50–700 W; SLR: 1:10; pH 3.2–4.5; 30 min	59.4–83.7	Bélafi-Bakó et al. 2012
	Citrullus lanatus	Acidulated water	477 W; SLR: 1:20.3; pH 1.52; 2.13 min	25.79	Prakash Maran et al. 2014
	Lime flavedo, albedo, and pulp	Acidulated water	630 W; SLR: 1:25; pH 2; 3 min	14.6	Fishman et al. 2006
	Apple pomace	Acidulated water	499.4 W; SLR: 1:14.49; pH 1.01; 20.8 min	15.75	Wang et al. 2007
	Pumpkin flesh	Acidulated water	60/80 °C; 20/10 min; pH 1.0	11.3	Yoo et al. 2012
	Pericarpium granati	30 % polyethylene glycol with MW 400 Da	Ultrasonic–microwave-assisted extraction (UMAE): US (240 W); MW (365 W); 90 °C; 10 min; SLR: 1:20	7.94	Zhou et al. 2014
Galactomannans and arabinogalactans	Spent coffee grounds	Water	900 W; 200 °C; 5 min; 16 bar; SLR: 1:10; 2 MAE cycles	42 (mannose: 66 %)	Passos and Coimbra 2013
	Spent coffee grounds	Water	900 W; 200 °C; 5 min; 16 bar; SLR: 1:30; 5 MAE cycles	82 (mannose: 69 %)	Passos et al. 2014

Arabinoxylans	Brewers' spent grains (barley husks)	Water/ water/ 0.1 M KOH	3 MAE cycles; 140 °C; 2 min; 180 °C; 2 min; 180 °C; 2 min; SLR: 1:6	62	Coelho et al. 2014
	Wheat bran	4 % (wt) NaOH	110 W; 10 min; SLR: 1:10	60	Panthapulakkal et al. 2013
Arabinoglucuronoxylans	Corn fiber	Water	180 °C; 7 min; SLR: 1:20	20	Benkő et al. 2007
Inulin	*Cynara scolymus* bracts	Water	120 °C; 3 min; SLR: 1:33	11.4	Ruiz-Aceituno et al. (2014)
Fucoidan	*Fucus vesiculosus*	Water	120 psi; 1 min; SLR: 1:25	18.22	Rodriguez-Jasso et al. 2011
Carrageenan	*Hypnea musciformis*	Water	85 °C; 10 min; SLR: 1:50	22.7	Vázquez-Delfín et al. 2014

[a]*SLR* solid–liquid ratio (g mL^{-1})

solvent, the raw sample properties (e.g., polarity, water content, and swelling capability), and the thermal stability of the target compounds.

Extraction pressure. Pressure is a relevant parameter to be considered when MAE is performed in closed vessels. In these systems, elevated pressures are typically applied to ensure that the extraction solvent is kept as a liquid at the high extraction temperatures reached during the extraction step. High pressures also improve solvent penetration into the sample, which facilitates the desorption of the analyte from the active sites of the matrix. Due to the direct relationship existing between these two parameters in MAE, temperature is the variable most commonly considered during method optimization. However, in instruments without temperature control, pressure in the vessel can be optimized instead (Eskilsson and Björklund 2000).

Extraction time. As compared to conventional heating extraction techniques, MAE involves much shorter extraction times (typically in the 3–30 min range for food matrices). In instruments operating at a fixed irradiation power, the time required for quantitative analyte extraction depends on the nature of both the extraction solvent and the sample nature, as well as on the type of targeted compounds. In MAE systems with separate irradiation power control, the influence of this parameter on the extraction yield and its relationship with the extraction time should be considered during method optimization. In any case, for thermolabile compounds, one should consider that long extraction times (as well as high irradiation powers) can result in analyte degradation.

Irradiation power. The selection of the irradiation power is governed by the number of extraction vessels simultaneously processed. Power should be chosen to minimize the extraction time while avoiding solvent projections and degradation of thermolabile analytes. The possibility of system leaks at relatively high irradiation powers (Eskilsson and Björklund 2000) should also be considered when optimizing this experimental parameter.

3 Applications to Polysaccharide Extraction

Extraction temperature and time and SLR are the most common parameters considered for the optimization of MAE of polysaccharides (Prakash Maran et al. 2013). Depending on the nature of the considered polysaccharide, solvent pH is also occasionally selected as a variable in experimental designs aimed to the optimization of experimental conditions. In addition to the effect that the operating conditions play on extraction yield, they also affect the possibility and extent of other phenomena such as depolymerization of polysaccharides that can also take place along the extraction process (Holck et al. 2014). Because bioactivity of polysaccharides usually depends on their molecular weight and their monomeric composition, a trade-off between the extracted amount and the structural composition is usually aimed during the extraction processes.

Before MAE of polysaccharides, samples are usually dried either in convection ovens (45–60 °C; (Bagherian et al. 2011; Li et al. 2012; Prakash Maran et al. 2013;

Panthapulakkal et al. 2013) until constant weight or freeze-dried, ground, and sieved to obtain powdered samples. In some cases, samples are subjected to different pretreatments to enhance extraction efficiency. For instance, sugar beet pulp was bleached at 95 °C for 5 min (Li et al. 2012), pumpkin flesh powder was treated with 0.1 N HCl (pH 1.0) at 25 °C for 1 h (Yoo et al. 2012), birchwood powder was treated with 0.05 M HCl at 70 °C for 2 h followed by a treatment with 14 M NH_4OH (Panthapulakkal et al. 2013), etc.

Although MAE has been applied for the extraction of polysaccharides with a variety of structures, most works have focused on the extraction of pectins and xylans from fruits and cereals, respectively. Table 2 summarizes some of the most recent applications of this technique for extraction of polysaccharides from different matrices. The approaches followed will be discussed in the subsequent subsections according to the type of polysaccharide extracted.

3.1 Pectins

Polysaccharides from fruits and vegetables are mainly pectins consisting of homogalacturonan and rhamnogalacturonan I (RGI) that forms the backbone with arabinan, galactan, and arabinogalactan neutral sugar side chains attached to rhamnose in RGI regions (Holck et al. 2014). Although commercial pectins are extracted from apple pomace and citrus peel, new potential sources such as sugar beet pulp, berries, etc. have been recently investigated for extraction of these polysaccharides (Li et al. 2012; Bélafi-Bakó et al. 2012).

MAE can be applied for fast and low exposure to harsh extraction conditions of pectins (Holck et al. 2014). In general, pectin extraction efficiency is improved by raising microwave power. Microwave radiation loosens the cell wall of plant material and the skin tissues are opened (Kratchanova et al. 2004; Prakash Maran et al. 2014). Therefore, the extracting agent can easily interact with the matrix to extract the pectins.

Irradiation time should be also carefully selected, as an increase of this parameter can help the extraction but an excessive time exposure can give rise to the degradation of pectins (Prakash Maran et al. 2014). These effects are shown in Fig. 2a where maximum yield of pectins from orange peel was achieved at the highest microwave power assayed (480 W) for 125 s; above this time yield decreased (Prakash Maran et al. 2013).

As previously mentioned, both the SLR and the nature of the solvent are important variables to achieve an efficient extraction. Pectin yield increases with solvent volume up to the solution gets saturated, which negatively affects the mass transfer rate into the solution (Prakash Maran et al. 2013).

Some experiments have demonstrated that pectin could not be extracted by MAE under pressure using pure water as solvent (Fishman et al. 2006), and consequently, pectin extraction is usually carried out using acidulated water (pH adjusted to 1.4–1.5 with 0.1 N HCl or H_2SO_4) (Bagherian et al. 2011; Li et al. 2012). Acidic conditions can promote the hydrolysis of insoluble pectin constituents into soluble

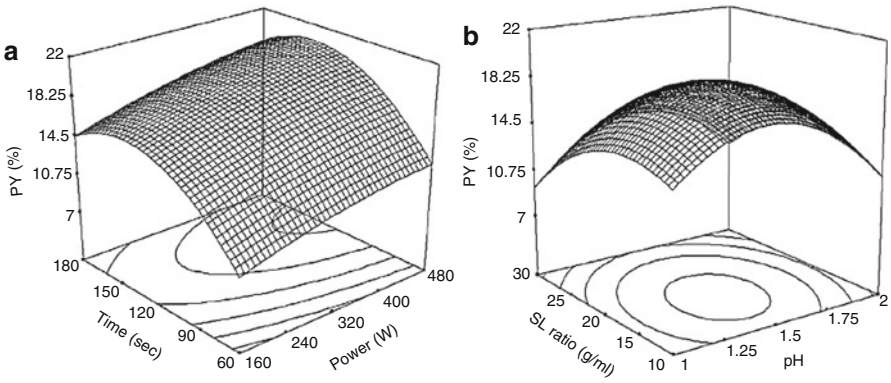

Fig. 2 Effect of irradiation time and microwave power (**a**) and solvent pH and solid–liquid ratio (**b**) on the extraction yields of pectins (PY) from orange peel (Reprinted from Prakash Maran et al. (2013). Copyright (2013), with permission from Elsevier)

pectin, increasing its recovery (El-Nawawi and Shehata 1988; Prakash Maran et al. 2013). However, as shown by Prakash Maran et al. (2013), above pH 1.5 pectin aggregation can take place, delaying its release and decreasing yields (see Fig. 2b as an example). Acidic conditions have been also proposed to obtain good galacturonic acid yields (Buchholt et al. 2004), whereas alkaline conditions have been used in order to achieve high yields of arabinan side chains. These extraction conditions have been combined with chelating agents for preservation of ferulic acid units of pectins (Holck et al. 2014).

The use of ionic liquids (ILs) as extracting agents in MW processes has recently been suggested (Guolin et al. 2012). ILs are low-melting-point salts that exist in liquid phase at relatively low temperatures (generally speaking, below 150 °C) and possess unique and attractive properties such as low volatility, variable viscosity, chemical and thermal stability, and tunable solubility properties, among others (Ruiz-Aceituno et al. 2013). Guolin et al. (2012) assayed different concentrations of IL solutions ([BMIM][Br], [BMIM][Cl], [EMIM][Br], [BMIM][BF_4], and [AMIM][Cl] in distilled water) for pectin extraction. The highest yield (25.1 %) was achieved using 1.0 mol L^{-1} [BMIM][Cl] under optimal conditions (88.36 °C, 9.63 min, SLR: 1:15 w/v). Recently, the use of aqueous polyethylene glycol solutions has also been proposed for the extraction of polysaccharides from *Pericarpium granati* (Zhou et al. 2014). Although yields were not notably high, this solvent could be a good alternative to other more aggressive solvents considering its biodegradability, low flammability, nonvolatility, and stability at high temperature (Bulgariu and Bulgariu 2008).

The effect of microwave irradiation on the pectin structure (Bélafi-Bakó et al. 2012) is also discussed in different manuscripts. As commented before, microwave irradiation could cause a deeper rupture in the cell wall which could result in a more compact pectin structure as compared to conventional extraction.

Heating time and temperature also affect the pectin structure (Holck et al. 2014). As an example, molecules of pectin from lime albedo, flavedo, and pulp became less compact and with lower molar mass and intrinsic viscosity with increasing MAE heating time and temperature (Fishman et al. 2006).

MAE has also been proposed for the sequential isolation of different polysaccharides. As an example, pectin, alkaline-soluble polysaccharides, and cellulose have been successfully isolated from sugar beet pulp by different sequential MW treatments under basic (pH 11.5) and acid (pH 4.0–4.5) conditions (Fishman et al. 2011).

After extraction, samples are usually filtrated. The filtrate (containing pectin) is cooled down and centrifuged. The supernatant is precipitated with absolute alcohol or isopropyl alcohol and left to rest in order to float pectin which is finally dried to constant weight (Li et al. 2012; Bélafi-Bakó et al. 2012).

3.2 Galactomannans and Arabinogalactans

Coffee beans are a rich source of polysaccharides, specifically cellulose, galactomannans (low-branched polysaccharides composed of a backbone of β-(1 → 4)-linked mannose residues, branched at *O*-6 by single α-(1 → 6)-linked galactose and single (1 → 5)-linked arabinose residues) and type II arabinogalactans (high-molecular-weight highly branched polysaccharides composed of a backbone of β-(1 → 3)-linked galactose residues, branched at *O*-6 by β-(1 → 6)-linked galactose and/or α-(1 → 5)-linked arabinose residues) (Passos and Coimbra 2013).

Most of these polysaccharides are not extracted during the beverage preparation and remain present in the spent coffee ground matrix. MAE has been proposed as an efficient technique for the recovery of these polysaccharides. As an example, Passos and Coimbra (2013) evaluated the effect of SLR on the extraction yield of polysaccharides from spent coffee grounds. These authors proposed two consecutive extraction processes using water as solvent, 900 W and 200 °C for 5 min under stirring as extraction conditions. Even at this high temperature, no degradation of polysaccharides was observed. The highest yields (55 %) of galactomannans and arabinogalactans were achieved at intermediate conditions (SLR 1:10) (Fig. 3). These authors also improved this method using five sequential MAE cycles to achieve an extraction yield of 82 % (69 % mannose) (Passos et al. 2014). Fractionation of mannans from cellulose, which remained in the insoluble material (84 %), was also successfully achieved.

3.3 Xylans

Xylans possess a backbone made up of xylose units linked by β-(1 → 4) bonds with different substituents as side chains, namely, acetyl, arabinosyl, glucuronic acid, etc., and frequent esterification by phenolic acids. Heteroxylans occur in

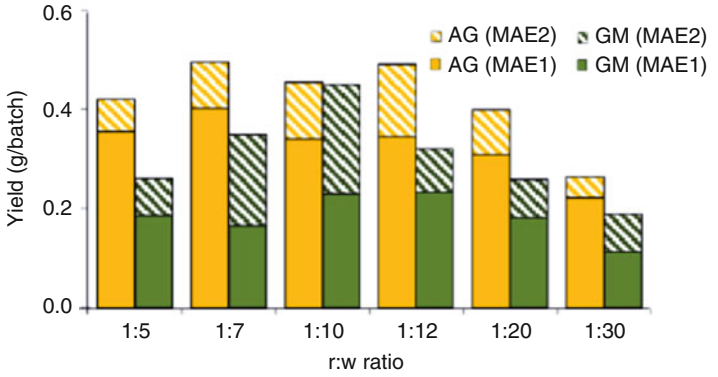

Fig. 3 Amount of arabinogalactans (*AG*) and galactomannans (*GM*) recovered during two cycles of MAE extraction (Reprinted from Passos and Coimbra (2013). Copyright (2013), with permission from Elsevier)

hardwoods, agricultural products of lignocellulosic nature (straw, herbs, and the like), and cereals (Gullón et al. 2014).

In general, low power levels are used for the extraction of xylans, considering that a degradation of these polysaccharides can occur as a result of an increase of the temperature in a short time (Panthapulakkal et al. 2013). Coelho et al. (2014) found that temperatures higher than 180 °C, similar to those proposed by Passos and Coimbra (2013) for the extraction of galactomannans, produced depolymerization, debranching, and de-esterification of arabinoxylans, with the formation of brown products. Similarly, Benkő et al. (2007) observed a decrease in the molecular weight of arabinoglucuronoxylans isolated from corn fiber when temperature was increased from 160 °C to 210 °C. However, recovery of this polysaccharide increased at higher temperatures. These results confirm the relevance of optimizing each extraction method depending on the nature of the polysaccharide and the purpose of the extraction.

The effect of solvent pH and extraction time in the recovery and molecular weight distribution of xylans during MAE is also crucial. In general, lower extraction yields and shorter polymer chains are obtained when low pHs are used (Benkő et al. 2007; Roos et al. 2009). Xylan extraction yields increased with time of extraction, whereas changes in the molecular structure were observed (high-molecular-weight fraction decreased (from 36 % to 25 %) and low-molecular-weight fraction increased (from 21 % to 30 %)) (Panthapulakkal et al. 2013).

Sequential MAE treatments using different solvents and/or conditions have been proposed to improve the recovery of xylans. As an example, Coelho et al. (2014) obtained high yields of arabinoxylans (62 % of poly- and oligosaccharides) from brewers' spent grains (mainly composed of arabinoxylans (22–23 %), followed by cellulose, starch and β-glucans) using firstly water at 140 °C to remove starch and β-glucans and two consecutive treatments at 180 °C with water and 0.1 M KOH to recover the target carbohydrates.

3.4 Inulins

Inulin is composed of a series of oligomers and polymers of several fructosyl moieties and a terminal glucose unit which are linked by β-(2 → 1) bonds. It is naturally occurring in land plants and in some bacteria and it is a well-known prebiotic carbohydrate. Studies on inulin extraction by MAE are very scarce. Recently, a Box-Behnken experimental design has been applied to optimize the extraction of inulin and inositols (cyclic polyalcohols with recognized bioactive properties) from *Cynara scolymus* bracts (Ruiz-Aceituno et al. 2014). The highest yield of inulin was obtained at high temperature (120 °C) and at a short time (3 min). However, 60 °C, 3 min, and SLR 1:33 were selected as optimal conditions when a multiple response analysis was carried out to simultaneously maximize the extraction yield of both bioactive carbohydrates.

3.5 Sulfated Polysaccharides

Sulfated polysaccharides include a wide range of anionic polysaccharides with different bioactive properties (anticoagulant, antioxidant, anti-inflammatory, anti-adhesive, etc.). Among them, fucoidan is a sulfated polysaccharide mainly consisting of fucose units esterified with sulfate groups and found in various species of brown seaweeds. Before extraction of fucoidan from seaweeds, a pretreatment, usually carried out with methanol/chloroform/water (4/2/1, v/v/v), to avoid coextraction of other algal compounds, is required. Rodriguez-Jasso et al. (2011) extracted high yields of fucoidan (18.22 %) from *Fucus vesiculosus*, a brown seaweed specie from North Portugal, by MAE at 120 psi, 1 min, using 1 g algae/25 mL water.

Carrageenans are sulfated polysaccharides of alternating 3-linked β-galactose and 4-linked α-galactose units or 3,6-anhydrogalactose present in edible red seaweeds. These polysaccharides are of great interest for the food industry mainly due to their gelling, thickening, and stabilizing properties. MAE has been reported for the extraction of carrageenan from *Hypnea musciformis* (Vázquez-Delfín et al. 2014). Both aqueous and alkaline (3 % KOH) treatments have been assayed; however, carrageenan yields were higher under aqueous conditions (85 °C, 10 min), as a consequence of the degradation experimented by this polysaccharide under basic conditions.

4 Comparison with Other Extraction Techniques

As previously mentioned, the MVH phenomenon makes of MAE a promising extraction technique. In contrast to conventional thermal methods, which rely on conduction and convection from hot surfaces to deliver energy, MAE provides shorter extraction times and higher extraction yields, due to the effect of microwaves on both the solvent (volumetric heating) and the sample (increased release of target compounds from the matrix into the solvent) (Wang and Weller 2006; Ying et al. 2011).

As for polysaccharides, in a study by Zhang et al. (2012) on the extraction of these compounds from *Artemisia sphaerocephala* seed de-oiling dregs, MAE showed a two times higher extraction yield and a 20–30 times lower extraction time (depending on experimental conditions) over conventional hot-water extraction. Similar advantages in terms of speed and yield have also been reported in the MAE of pectins from pumpkin flesh [10–20 min vs. 2 h; 11.3 vs. 4.2 % (w/w) yield (Yoo et al. 2012)], in the extraction of xylan from birchwood [10 min vs. 1.5 h; 60 vs. 56 % (w/w) yield (Panthapulakkal et al. 2013)], etc.

MVH also minimizes damage to thermolabile compounds, as samples do not come into contact with hot surfaces. This justifies the current acceptance of the MAE technique in many applications related to the extraction of bioactive compounds of interest for the food and pharmaceutical industries (Proestos and Komaitis 2008; Routray and Orsat 2012; Karabegović et al. 2013). Additional advantages of MAE include lower solvent consumption and high versatility, as selectivity and efficiency of extraction can be finely modulated as a function of experimental conditions selected. Moreover, as in the case of polysaccharides subjected to selected operating conditions, combined processes such as extraction and degradation can also simultaneously take place (Prakash Maran et al. 2013).

When compared to conventional heating techniques, MAE at industrial scale usually requires a higher initial investment. However, the low maintenance costs, the energy savings, the reduced equipment size, and the ease of operation are increasingly promoting the use of MAE for extractions at larger scale of different bioactives from plant and other natural sources (Terigar et al. 2011; Ibañez et al. 2012; Li et al. 2013). Despite the above-referred advantages, as far as we know, no reference has yet addressed the extraction of polysaccharides either at pilot or industrial scale.

In MAE methods, it can be difficult to predict the effect of the interaction of the electromagnetic field with the sample to be extracted, as variations in the sample volume, shape, moisture content, and chemical structure, as well as in the microwave system (extractor geometry, vessels, etc.) and operating conditions, might greatly affect the results. This unpredictability is easily overcome by experimentation and, therefore, the optimization of methods for every new application is always mandatory to achieve optimal extractions. In this regard, and despite the large number of applications already available in the literature for extraction of polysaccharides from different sources (see Table 2), extrapolation of methods and immediate implementation is not usually possible.

Regarding the comparison of MAE with other emerging techniques such as UAE, PLE, etc., several papers have compared their performance in its application to the extraction of polysaccharides from different sources (Du et al. 2014). In a paper by Buranov and Mazza (2010) on the extraction of high-molecular-weight hemicelluloses (HMH) from flax (*Linum usitatissimum* L.) shives, authors concluded that pressurized aqueous ethanol provided the maximum yield of HMH (15 % of dry flax shives), whereas microwave-assisted ethanol extraction was as efficient as pressurized low-polarity water and much more than microwave-water extraction (Table 3). However, it is worth noting that the maximum yield

Table 3 Experimental conditions and high-molecular-weight hemicelluloses (HMH) yield from 13 g flax shives using different extraction methods at 180 °C, 5.2 MPa, and SLR ratio of 1 g: 27 mL

Exp #	Extraction method[a]	Extraction mode	Flow rate (mL min^{-1})	Extraction time (min)	Solid residue (%)	Extract volume (mL)	HMH yield	
							(g)	(% on DFS [b])
1	PLPW	Flow through	3	117	54.2	351	0.81 ± 0.1	6.2
2	PAE	Flow through	3	117	46.9	351	1.93 ± 0.3	15.0
3		Batch	–	5	84.3	351	0.20 ± 0.01	1.5
4	MW-Water[c]			10	83.5	351	0.27 ± 0.02	2.0
5				20	83.8	351	0.23 ± 0.01	1.8
6		Batch	–	5	76.2	351	0.59 ± 0.03	4.5
7	MW-EtOH[c]			10	75.5	351	0.79 ± 0.05	6.0
8				20	76.1	351	0.56 ± 0.03	4.3

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[a]*PAE* pressurized aqueous ethanol, *MW-EtOH* microwave-assisted ethanol extraction, *PLPW* pressurized low-polarity water, *MW-Water* microwave-assisted water extraction

[b]*DFS* dry flax shives

[c]Pressure for microwave extraction was automatically vented when reached 2.6 MPa

achieved by pressurized aqueous ethanol was obtained at a much longer extraction time as compared to the remaining procedures. Bagherian et al. (2011) also evaluated the performance of MAE and UAE for the extraction of pectin from grapefruit. Both the yield and extraction time were better for MAE (28 % vs. 18 %, 6 min vs. 25 min). Finally, Du et al. (2014) compared the extraction of β-glucans from bran of hull-less barley (*Hordeum vulgare* L. var. nudum Hook), a commodity waste rich in this source of carbohydrates, by using PLE, UAE, and MAE. Although yield of crude β-glucan was similar for both UAE and MAE (0.3 %), extraction times were significantly higher for UAE (1 h vs. 2 min). Results achieved by PLE were the best as yields as high as 8.8 % were obtained in only 18 min (3 cycles of 6 min). However, it is worth noting that results obtained in the application of each of these techniques are dependent on a number of factors such as the operating conditions, the characteristics of the sample, or the composition of the extract and, therefore, no general conclusion can be a priori be drawn.

5 Future Trends

As previously mentioned, the application of microwave technologies has been scaled up in the food and cosmetic industries, among others, because of its economic and environmental advantages (Li et al. 2013). In this regard, the large number of successful laboratory-scale applications previously developed has greatly contributed to identify the factors required for scale-up and has helped to promote the implementation of this technique at industrial scale. Thus, and based on the number of advantages above described for MAE of polysaccharides and the ongoing progress regarding microwave technology, it is expected that research in a near future is aimed to the scale-up of this sort of applications and others involving integrated processes (e.g., extraction and enzymatic hydrolysis) from laboratory to pilot/industrial scale.

On the other hand, and following the trend initiated with the development of microwave-assisted Soxhlet extraction (García-Ayuso et al. 1998), new combined techniques have been recently developed. The simultaneous use of microwaves and ultrasound (ultrasonic–microwave-assisted extraction, UMAE) in single reactors (Fig. 4) has been proposed to merge the advantages in terms of improved matter transportation (breakdown of cell walls, improved solvent penetration, etc.) and enhanced energy provided by each of these techniques (Leonelli and Mason 2010). Although application of this hybrid technology results in better and sometimes more targeted extraction yields of flavorings and nutraceuticals from plants, herbs, and seeds (Cravotto and Cintas 2006; Cravotto et al. 2008), its application to polysaccharide extraction is still limited (Bagherian et al. 2011; Zhou et al. 2014). Furthermore, these combined reactors have only been developed at laboratory scale. Taking into account the advances achieved in the last decade regarding microwave instrumentation for applications other than extraction (Leonelli and Mason 2010), further research would be worth doing to explore the

Fig. 4 Apparatus for simultaneous US/MAE irradiation (Reprinted from Cravotto et al. (2008). Copyright (2008), with permission from Elsevier)

potential of UMAE as a large-scale technique for green and efficient extraction of polysaccharides.

Finally, and despite a great progress on safety issues related to MAE systems (pressure and solvent sensors, pressure-relieving doors, high-capacity exhaust devices, multilayered coated cavities, etc.) has been achieved during the last years, conventional quality control methods have been shown to be insufficient to evaluate and control potential hazards for product safety and risks for operators. Hazard analysis and critical control points (HACCP) and hazard and operability (HAZOP) studies have, however, been described as effective for these objectives and must be considered in the design of large-scale MAE applications (Li et al. 2013).

6 Conclusions

MAE has greatly evolved in the last decade and nowadays it can be considered as a mature and efficient technique for extraction of polysaccharides. Selectivity and yield of extraction depend on both dielectric characteristics of the sample and operating conditions which can be easily optimized by using response surface approaches. Despite their undeniable advantages (short extraction times, high extraction yields, reduced solvent consumption, etc.) over conventional extraction methods traditionally used in industry and their ease of operation and reduced costs as compared to other emerging technologies such as SFE and PLE, the full implementation of this technique for polysaccharide extraction at industrial scale still requires further research. Similarly, there is also a need to evaluate the potential of hybrid technologies such as UMAE in its application to the industrial extraction of these compounds.

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Preparation and Characterization of Polysaccharides from Mushrooms

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Contents

Abstract

Polysaccharides are cellular structural components found in the three domains of life. Polysaccharides possess immune enhancement, anticancer, antioxidant, and antiviral activities. Mushrooms are the fungi that have been used as food and medicines for long time in the Oriental countries. One of main categories of active components from mushrooms is recently identified as polysaccharides. The present work summarizes the preparation, extraction, purification, and characterization along with quantitative analysis of polysaccharides from mushrooms. It reflects on current status of this research area with a view for future direction. This will certainly promote the nutritional, medicinal, and nutraceutical use for mushroom polysaccharides.

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Polysaccharides • Mushroom • Extraction • Isolation • Purification • Characterization

Abbreviations

BRM	Biological response modifiers
EAE	Enzyme-assisted extraction
FT-IR	Fourier transform infrared spectrometry
GC-MS	Gas chromatography-mass spectrometry
HIPEF	High intensity pulsed electric fields
HPE	High pressure extraction
HPSEC	High performance size exclusion chromatography
MAE	Microwave-assisted extraction
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
NMR	Nuclear magnetic resonance
SWE	Subcritical water extraction
UAE	Ultrasonic-assisted extraction

1 Introduction

Mushrooms have been cultivated and used for hundreds of years and are being evaluated as edible and medicinal resources in Asian countries. Moreover, numerous species of wild growing mushrooms are widely consumed as a delicacy in Central and Eastern European countries (Kalac 2009). Mushrooms are macro fungi with distinctive basidiomata or ascomata which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked up by hand (Soares et al. 2013). Mushrooms have been traditionally appreciated due to their excellent sensory characteristics, including the pleasant flavor and texture. They are poor in calories and rich in proteins, fibers, carbohydrates, and important vitamins such as thiamin, riboflavin, and ascorbic acid and minerals (Manzi et al. 1999; Kues and Liu 2000; Mattila et al. 2002; Firenzuoli et al. 2008; Mishra and Singh 2010). As a matter of fact, the particular characteristics of growth and development of mushrooms in nature result in the accumulation of essential cell wall components such as polysaccharides (includes β-glucans) and a variety of secondary metabolites such as phenolic compounds, terpenes, and steroids. Polysaccharides from mushrooms have provoked the increasing interest due to the healthy benefits, such as immunomodulation (Wasser 2002), anticancer activities (Ren et al. 2012), prevention and treatment of cardiovascular diseases (Wasser and Weis 1999), and antiviral and antimicrobial effects (Beattie et al. 2010).

In order to reveal the polysaccharides in mushrooms, most researchers employed a routine procedure to fractionate Basidiomycete mushroom: from crude extraction by boiling water to fraction identification, then to further purification of

polysaccharides using a combination of such techniques as ethanol precipitation, fractional concentration, acidic precipitation, ion-exchange chromatography, and gel filtration chromatography (Zhu et al. 2008; Kim et al. 2003; Park et al. 2003a). The aim of this work is to review the main methods for extraction, purification, characterization, as well as the most important quantitative analysis of the polysaccharides from mushrooms.

2 Sample Preparation of Polysaccharides from Mushroom

Generally, the extract solution of mushroom polysaccharide is precipitated by alcohol, methanol, or acetone to obtain the crude polysaccharides. It can be demonstrated with the preparation of crude polysaccharides from *Ganoderma lucidum* and *Ganoderma sinense* as an example. The process was as follows: the fruit bodies of two species of *Ganoderma* were carefully cleaned and cut into slices and then dried at 40 °C for 12 h. The dried mushroom slices were pulverized and then passed through a 0.8 mm mesh. Sample materials were immersed in water and refluxed in a Syncore parallel reactor for 1 h at 100 °C with stirring at 200 rpm. Then the extract solution was centrifuged at 4,500 × *g* for 10 min. An aliquot of supernatant was evaporated to dryness under vacuum. The residue was dissolved in water; ethanol was added to a final concentration of 80 % (v/v) for precipitation of crude polysaccharides (Xie et al. 2012). Another example, the ground stipe powder of mushroom *Coprinus comatus*, was extracted three times with ethanol under reflux to remove lipid, and the residue was further extracted with hot water (Li et al. 2013a). Moreover, the *Hericium erinaceus* polysaccharides were prepared with deionized water by refluxing. The extract was concentrated to a small volume and then mixed with ethanol to yield a 70 % ethanolic solution. The precipitate thus obtained was lyophilized and ground to obtain the coarse powder of polysaccharides (Han et al. 2013).

3 Extraction of Polysaccharide from Mushroom

Extraction always involves a chemical mass transfer from one phase to another phase. Mushroom polysaccharides exist as a structural component of fungal cell wall (Zhang 2007). Selection of an extraction method depends not only on the cell wall structure but also on different physiochemical parameters, for instance, temperature, pressure, pH and ionic strength of the solvent, and fineness of solid particle ground (Villares et al. 2012). Generally speaking, the extraction of polysaccharides can be classified into three main types: One is a conventional solvent extraction method, using hot water, alkali solution, and polyethylene glycol; second uses microwave, ultrasonic or ultrahigh pressure, etc. to extract the polysaccharides; and third is enzyme-assisted extraction method. The extraction methods with detailed procedure are described below and summarized in Table 1.

Table 1 Extraction, purification, and structural features of polysaccharides from different mushroom sources

Source	Part of mushroom	Extraction process	Purification	Structural features	References
Coprinus comatus	Fruit bodies	Water extraction (80 °C)	DEAE-Sepharose CL-6B column (3.5 cm × 30 cm); Sepharose CL-6B column (1.6 cm × 100 cm); Bio-Gel P-2 column (1.1 cm × 48 cm); Sepharose CL-4B column (2.8 cm × 50 cm)	The disaccharide α, α-trehalose [α-*d*-Glc*p*-(1↔1)-α-*d*-Glc*p*]; high-molecular-mass α-*d*-glucans; lower-molecular-mass linear β-*d*-glucans	Li et al. (2013a)
Ganoderma capense	Mycelia	Boiling water	DEAE-Sepharose CL-6B column (2.6 × 40 cm); Sephadex G-75 column (1.6 × 150 cm)	An α-*d*-glucan with the main backbone chain of (1 → 4)- α-*d*-glucopyranosyl residue interspersed with (1 → 4, 6)-α-*d*-glucopyranosyl residue and the side chain attached to the *o*-6 position with (1→)-α-*d*-glucopyranosyl residue	Li et al. (2013b)
Agaricus brasiliensis	Fruiting bodies	Water extraction (100 °C)	DEAE-cellulose column chromatography; Toyopearl HW-65F column; Con A-Sepharose 4B column	A greater proportion of (1 → 6)-β-side branches on the (1 → 3)-β-backbone	Camelini et al. (2005)
Ganoderma lucidum	Spores	Water extraction (100 °C) and graded ethanol precipitation	DEAE-cellulose column and Sephacryl S-300 column	A 1,6-linked β-*d*-glucopyranosyl backbone with different length of branches consisting of terminal and 1,4-linked glucopyranosyl residues, attached to *o*-4 of alternative glucose residues in the backbone	Dong et al. (2012)

Grifola frondosa	Fruit bodies	Alkaline (5 % NaOH) extraction	DEAE-cellulose column	A *β*-*d*-(1–3)-linked glucan backbone with a single *β*-*d*-(1–6)-linked glucopyranosyl residue branched at C-6 on every third residue	Fang et al. (2012)
Coriolus versicolor	Fruit bodies	Hot water extraction	DEAE-cellulose anion-exchange chromatography with 0.7 M NaCl	–	Kang et al. (2013)
Flammulina velutipes	Fruit bodies	Aqueous and alkaline extraction	–	A main chain of (1 → 3)-linked glucopyranosyl residues, substituted at *o*-6 by single-unit *β*- glucopyranosyl side chains	Smiderle et al. (2006)
Auricularia auricula	Fruit bodies	0.15 M Aqueous NaCl extraction (80–100 °C)	–	A *β*-(1 → 3)-*d*-glucan with two *β*-(1 → 6)-*d*-glucosyl residues for every three main chain glucose residues	Xu et al. (2012)
Dictyophora indusiata	Fruit bodies	Water extraction (90 °C)	Sephadex G-200 gel chromatography	A backbone of *β*-conformation, mainly consist of glucose (98.58 %)	Deng et al. (2012)
Geastrum saccatum	Fruit bodies	Water extraction (100 °C)	–	High amount of glucose and traces of galactose. The signal appearing at 103.5 ppm was assigned to C1 of *β*-glucose	Guerra Dore et al. (2007)
Hybrid mushroom of *Pleurotus florida* and *Lentinula edodes*	Fruit bodies	Hot aqueous extraction	Sepharose 6B gel permeation column	→6)-*α*-*d*-Galp-(1 → 6) -*α*-*d*-Galp-(1→	Maity et al. (2013)
Coprinus comatus	Fruit bodies	Distilled water extraction at 100 °C	DEAE-Sepharose anion chromatography	Was composed of L-Fuc and *d*-Gal in the ratio of 1:3.84 (molecular weight: 1.03×10^4 g mol^{-1})	Zhou et al. (2013)

(*continued*)

Table 1 (continued)

Source	Part of mushroom	Extraction process	Purification	Structural features	References
Coprinus comatus	Fruit bodies	Distilled water extraction at 100 °C	High performance size exclusion chromatography (HPSEC)	Was *α*-*d*-(1 → 4)-glucan with branches at C-6 consisting of non-reducing terminal approximately every fourteen residues (molecular weight: 2.44×10^7 g mol^{-1})	Liu et al. (2013)
Ramaria botrytis	Fruit bodies	Hot water extraction followed by treating NaOH	Dialyzed through cellulose tubing membrane	→3)-*α*-*d*-Glcp-(1→	Bhanja et al. (2014)
Phellinus linteus	Submerged culture	–	Sepharose CL-4B column (2.4 cm × 100 cm)	Spherical form; a random coil in an aqueous solution	Hwang et al. (2003)
Phellinus linteus	Fruit bodies	Hot water extraction	Sepharose CL-6B column (150 × 1.5 cm)	A core *β* (1–3) linked glucan heavily substituted via (1–6) links with *β* (1–3) linked mannose chains	Baker et al. (2008)

3.1 Conventional Solvent Extraction

Hot water extraction has been a popular approach for polysaccharide extraction from mushroom because of its safety and environment-friendly compatibility (Nie et al. 2013). In order to obtain high yields, long soaking time, high water temperature, high liquid-to-solid ratio, and multiple extraction steps are usually necessary (Xing et al. 2013). It was reported that the fruit bodies of *C. comatus* were extracted three times with 2 L distilled water for 2 h at 80 °C, followed by intermediate centrifugation (2,000 × *g*, 15 min). After concentrating the collected aqueous supernatants to 400 mL under vacuum (reduced pressure at 40 °C), a precipitation was performed with three volumes of 95 % ethanol. The precipitate was washed with ethanol and acetone and then dried at 40 °C, and the crude polysaccharide material was finally yielded (Li et al. 2013a). Furthermore, a polysaccharide from *Ganoderma capense* was extracted using boiling water (Li et al. 2013b). Before polysaccharide extraction, the lipids were removed from dried *G. capense* mycelia powder using petroleum ether. Kumari et al. (2008) determined optimal operating conditions using one factor at a time method and response surface method to maximize production of schizophyllan by *Schizophyllum commune* from an initial value of 1.06–8.06 g L^{-1}. Moreover, Kim et al. (2005) used distilled water (100 °C) at a ratio of 1:10 (w/v) to extract β-glucan from *Agaricus blazei* with extraction time of 3 h. Similar extraction procedures were conducted for polysaccharide extraction from *Phellinus linteus*, which was extracted three times with boiling water for 2 h, concentrated and precipitated with three volumes of ethanol (Li et al. 2011).

3.2 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) is widely used in the extraction of bioactive compounds. MAE provides a rapid sample preparation with reduced amounts of solvent. For instance, Ookushi et al. (2006) applied microwave irradiation to extract polysaccharides from the fruiting body of mushroom *Hericium erinaceus*. The results showed that microwave irradiation had an advantage for extraction of polysaccharides in terms of saving extraction time. Moreover, MAE technique was employed for the extraction of himematsutake (*Agaricus blazei* Murill) polysaccharide and optimized by Box-Behnken design. The optimum conditions were extraction time 29.37 min, microwave power 400 W, extraction temperature 74.64 °C, and ratio of water to material 32.7:1 with an enhanced yield of 12.35 % (Zhang et al. 2011b). Huang et al. (2007) studied the MAE of polysaccharides from spores of *Ganoderma atrum* with response surface analysis. The experimental results confirmed that MAE had great efficiency compared with traditional hot water extraction.

3.3 Ultrasound-Assisted Extraction

Ultrasound is primarily associated with cell disruption or disintegration. When sonicating liquids at high intensities, the sound waves that propagate into the liquid

medium result in alternating high pressure and low pressure cycles, with rates depending on the frequency. Ultrasonic-assisted extraction (UAE) is an expeditious, inexpensive, and efficient alternative to traditional solvent extraction techniques. In one study, Pan et al. (2010) determined the optimum UAE conditions for polysaccharides from mushroom *Trametes versicolor*. The ultrasonic power and the ratio of material to solvent were the most significant parameters on polysaccharide extraction. In addition, UAE of polysaccharides from white button mushroom (*Agaricus bisporus*) was studied; the optimal extraction conditions were ultrasonic power 230 W, extraction temperature 70 °C, extraction time 62 min, and water/material ratio 30 mL g^{-1} (Tian et al. 2012). On the other hand, the optimization of polysaccharide extraction from *Pleurotus eryngii* using UAE was investigated and the optimal conditions were obtained using single parameter and orthogonal tests when fruit bodies of *P. eryngii* were suspended in water at a ratio of 1:40 and exposed to 70 W of ultrasonic power for 40 min (Zhang et al. 2010).

3.4 High Pressure Extraction

High pressure extraction (HPE) as a novel technique is used for extraction of active ingredients from plant materials (Prasad et al. 2009). High pressure ranging from 100 to 800 MPa or even more up to 1,000 MPa is considered as an alternative extraction method, which is proven to be fast and more effective (Zhang et al. 2004). For example, Tang et al. (2012) extracted polysaccharide from shiitake mushroom particular ly in a HPE method. Being a cold extraction technique, the super high pressure extraction method generates no high temperature during the whole extraction process, shortens the extraction time to a few minutes, conserves the effective ingredients, and has significant economic benefits and broad prospect of market development. Additionally, Xi et al. (2010a) used ultra HPE to extract lentinan from mushroom. The extraction conditions were optimized with the orthogonal test as follows: extraction time 4 min, pressure 350 MPa, solid to liquid ratio 1:35, and temperature 55 °C. The ultra HPE method showed high extraction efficiency, low energy consumption, and short extraction time and provided a new route for the extraction of lentinan.

3.5 Pulsed Electric Fields Extraction

Pulsed electric fields extraction keeps original properties of nutrients unchanged plus can simultaneously sterilize it. When a plant is treated with high pulsed electric field, the cell membranes are ruptured leading to an increase in permeability of the cell walls (Eshtiaghi and Knorr 2002). In our latest study, three extraction techniques, namely, high intensity pulsed electric fields (HIPEF), MAE method, as well as UAE method, were applied to optimize the extraction conditions of polysaccharide from Jew's ear (*Auricularia auricula*). The results suggested that the HIPEF technique will be an effective method in the manufacture of bioactive natural

polysaccharide (Li et al. 2013c). In another study, Yin et al. (2008) demonstrated the extraction of polysaccharide from mushroom *Inonotus obliquus* using HIPEF. The results showed that HIPEF technique is an effective method to extract polysaccharide from mushroom, with higher extraction yield, shorter time, and fewer impurities.

3.6 Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) methods are gaining more attention because of the need for eco-friendly extraction (Puri et al. 2012). EAE methods have been shown to achieve high extraction yields for compounds including polysaccharides, oils, natural pigments, and medicinal compounds (Barzana et al. 2002; Wu et al. 2005; Passos et al. 2009; Yang et al. 2010). For instance, EAE technology was performed for the polysaccharide extraction from *Ganoderma lucidum* in order to increase the amount of extract and polysaccharide extraction yield (Xu et al. 2014). Complex enzyme amount also presents a positive effect on the polysaccharide yield and amount of extract. Chen et al. (2013) conducted three extraction methods (as hot water, microwave assisted, and enzyme hydrolysis assist) to extract polysaccharides from *Clitocybe maxima* stipe. Results indicated that the complex-enzyme-hydrolysis-assist extraction was the optimal approach used for extracting polysaccharide from *C. maxima* stipe. It showed with the advantages in high efficiency, energy, and running time saving.

3.7 Subcritical Water Extraction

Subcritical water extraction (SWE) is a new and powerful technique at temperatures between 100 °C and 374 °C and pressure high enough to maintain the liquid state. The extraction parameter conditions of SWE of polysaccharides from *Grifola frondosa* were optimized using response surface methodology. The results showed that the SWE technology could be a time-saving, high-yield, and bioactive technique for production of polysaccharides (Yang et al. 2013). Furthermore, Lou et al. (2010) reported the extraction of lentinan from mushroom using SWE. The experimental results indicated that the SWE could shorten the extraction time and improve the yield of lentinan compared with the water extraction method.

Recently, complex extraction method has received much attention in natural product research field. For example, Parniakov et al. (2014) compared the efficiency of extraction and stability of extracts from mushroom *Agaricus bisporus* for different extraction methods: pressure extraction, pressure extraction assisted by pulsed electric field, hot water extraction, and ethanol extraction. Pressure extraction was done at room temperature and 5 bar pressure. Pulsed electric field treatment was done using bipolar near-rectangular pulse protocol. The results showed that pressure extraction assisted by pulsed electric field allowed production of mushroom extracts with high contents of fresh-like proteins and polysaccharides.

4 Purification of Polysaccharide from Mushroom

In the recent years, although polysaccharide has been known for its potent anticancer and antiviral activities, the difficulties in its separation and purification have remained an obstacle for its development. Isolation and purification of polysaccharides commonly involve several steps, and different techniques are actually available in order to increase extraction yield and purity (Villares et al. 2012). Polysaccharides extracted from the source material are generally dissolved in water or aqueous solutions. Other macromolecules (such as proteins) and small molecular substances (such as phenolic compounds, monosaccharides, and amino acids) may be also present in the matrix. Firstly, the samples may be subjected to a previous step consisting of a methanol extraction in order to remove small molecular substances. Secondly, proteins can be removed by precipitation with Sevag method (Whistler 1965) using a mixture of chloroform and *n*-butanol, trifluoroacetic acid (20 %, w/v) or by treating with the enzyme protease at 40 °C for 1 h (pH 7.5). Thirdly, polysaccharides are precipitated from the supernatants by the addition of ethanol in different ratios, for instance, 4:1 (v/v) or 3:1 (v/v). Fourthly, pure carbohydrate fractions obtained from ethanol precipitation can be performed by precipitation with ammonium sulfate. The detailed purification procedures were summarized in the following parts and listed in Table 1.

4.1 Chemical Method

The chemical methods for purification of polysaccharide from mushroom were as follows: distribution precipitation method, quaternary ammonium salt precipitation, salting out method, and metal complexes method (Liu and Xu 2007). Distribution precipitation method, which is the addition of miscible solvents such as ethanol or methanol to a solution, may cause proteins in the solution to precipitate. The common quaternary ammonium salt included hexadecyltrimethylammonium bromide and cetylpyridinium chloride. Salting out is the most common method used to precipitate a target protein. Addition of a neutral salt, such as ammonium sulfate, compresses the solvation layer and increases protein-protein interactions. As the salt concentration of a solution is increased, the charges on the surface of the protein interact with the salt, not the water, thereby exposing hydrophobic patches on the protein surface and causing the protein to fall out of solution.

4.2 Chromatographic Method

Column chromatography is a method used to purify individual chemical compounds from the mixtures of compounds. Different column chromatography methods (ion-exchange chromatography and gel filtration chromatography) can be used in polysaccharide purification. Camelini et al. (2005) extracted β-glucan from mushroom *Agaricus brasiliensis* with boiling water, and then the crude polysaccharide was

passed through a DEAE-cellulose column chromatography for further purification. A similar research was conducted to purify water-soluble polysaccharide from the dried powder of submerged fermentation mycelia of *G. capens*e by successive purification through DEAE-Sepharose CL-6B and Sephadex G-75 column chromatography (Li et al. 2013b). Moreover, crude polysaccharide extracts were further purified through a Sephadex column and eluted with water. A water-soluble polysaccharide was purified from the fruit bodies of *C. comatus* using DEAE-Sepharose CL-6B column, Sepharose CL-6B column, Bio-Gel P-2 column, and Sepharose CL-4B column (Li et al. 2013a). In another study, *β*-glucan from mushroom *Sparassis crispa* was prepared using cold alkali (10 % NaOH/5 % urea), and subsequently the extract was applied to a DEAE Sephadex A25 column for further purification (Shibata et al. 2012). The purification techniques used for extraction of polysaccharides from different sources are summarized in Table 1.

4.3 Membrane Filtration

Because membrane processes are increasingly used for separations of mixtures with high complexity, the focus of fouling studies in ultrafiltration has also been in more complicated and less-defined substances such as polysaccharides (Susanto et al. 2008). Interactions of ultrafiltration membranes with polysaccharides have been investigated during processing of bioproduct (Nataraj et al. 2008). Zhao et al. (2010) chose ultrafiltration methods to purify polysaccharide from mushroom *Tricholoma matsutake* Sing. with two membranes of different molecular weight cut-off, namely, 30 and 10 kD. A low-molecular-weight polysaccharide from mushroom *Ganoderma lucidum* fruit bodies was successfully isolated and purified using membrane ultra filtration, anion exchange, and gel filtration chromatography (Zhu et al. 2013).

5 Chemical Characterization of Polysaccharide from Mushroom

Polysaccharides are polymeric carbohydrate structures, formed of repeating units joined together by glycosidic bonds. These structures are often linear, but may contain various degrees of branching. Polysaccharides consist of polymeric structures composed of at least ten monosaccharides sequentially connected by glycosidic bonds (Bertozzi and Rabuka 2009; Mulloy et al. 2009; Stanley and Cummings 2009). The chemical characterization of polysaccharide is an absolute request for a multitude of scientific and industrial applications that go beyond the simple use of polysaccharides where the physical characterization and the knowledge of usage-dependent behavior by specific tests are sufficient. The structural analyses of *C. comatus* polysaccharides were investigated using (*d*/*l*) monosaccharide/methylation analysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy. The disaccharide *α*, *α*-trehalose [*α*-*d*-Glc*p*-(1↔1)-*α*-*d*-Glc*p*], high-molecular-mass *α*-*d*-glucans, and lower-molecular-mass linear *β*-*d*-glucans were

found to be present (Li et al. 2013a). The structural feature of *G. capense* polysaccharide was investigated by monosaccharide analysis, partial acid hydrolysis, methylation analysis, periodic acid oxidation, gas chromatography-mass spectrometry (GC-MS), Fourier transform infrared spectroscopy (FT-IR), and NMR spectroscopy. The results obtained from the analyses indicated that *G. capense* polysaccharide was an *α-d*-glucan with the main backbone chain of (1 → 4)-*α-d*-glucopyranosyl residue interspersed with (1 → 4, 6)-*α-d*-glucopyranosyl residue and the side chain attached to the *o*-6 position with (1→)-*α-d*-glucopyranosyl residue (Li et al. 2013b). Moreover, Camelini et al. (2005) characterized the *β*-glucans of *Agaricus brasiliensis* fruiting bodies by FT-IR and NMR. The results showed that *β*-glucan had a greater proportion of (1 → 6)-*β*-side branches on the (1 → 3)-*β*-backbone. The structure of β-glucan from a hybrid mushroom, *pfls1h* of *Pleurotus florida and Lentinus squarrosulus* (Mont.) Singer, was characterized using total hydrolysis, methylation analysis, peroxide oxidation, and NMR techniques by Sen et al. (2013). Methylation analysis revealed that purified polysaccharide fraction was composed of (1 → 3, 6), (1 → 3), (1 → 6)-linked and terminal *β-d*-glucopyranosyl residues in a relative proportion of approximately 1:1:1:1. The repeating unit of the glucan consists of a backbone chain of two (1 → 6)-*β-d*-glucopyranosyl residues, one of which is branched at *o*-3 position with (1 → 3)-*β-d*-glucopyranosyl and terminated with a *β-d*-glucopyranosyl residue. In another study, Dong et al. (2012) characterized the chemical structure of *β*-glucan (GLSA50-1B) from mushroom *Ganoderma lucidum* using sugar compositional analysis, methylation analysis, partial acid hydrolysis, acetolysis, and NMR and electrospray ionization mass spectrometry. The results indicated that GLSA50-1B was elucidated to be a novel *β-d*-glucan featured by a 1, 6-linked *β-d*-glucopyranosyl backbone with different length of branches consisting of terminal and 1, 4-linked glucopyranosyl residues, attached to *o*-4 of alternative glucose residues in the backbone. Structural study of a polysaccharide isolated from an edible hybrid mushroom of *Pleurotus florida* and *Lentinula edodes* was investigated based on the results of acid hydrolysis, methylation analysis, peroxidation, and NMR experiments (Maity et al. 2013). Furthermore, the structure of a water-soluble polysaccharide from mushroom *C. comatus* was elucidated by methylation analysis, GC-MS, ^{1}H NMR, and ^{13}C NMR spectroscopy. The polysaccharide moiety was composed of *l*-Fuc and *d*-Gal in the ratio of 1:3.84 (Zhou et al. 2013). Structural characterizations of two water-insoluble glucans from mushroom *Ramaria botrytis* were investigated on the basis of total hydrolysis, methylation analyses, periodate oxidation, Smith degradation, and NMR experiments. The structural features of polysaccharides from different sources are shown in Table 1.

6 Quantitative Analysis of Polysaccharide from Mushroom

An easy quantification method for polysaccharides (like chitin and glucans) from mushrooms, with high precision, is a matter of particular importance (Nitschke et al. 2011). The phenol-sulfuric acid method (Du Bois et al. 1956), which is rapid,

simple, and economic, is a classical colorimetric method for determining total polysaccharides (Xi et al. 2010b). Maity et al. (2013) determined the content of polysaccharides from *C. comatus* based on a phenol-sulfuric acid assay at 490 nm. Kozarski et al. (2011) measured the total polysaccharide content of the extracted polysaccharides from medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum*, and *Phellinus linteus* using the phenol-sulfuric acid method with *d*-glucose as a reference. A number of existing methods that determine the total amount of β-1,3-glucans (Manzi and Pizzoferrato 2000; Mizuno et al. 2001; Park et al. 2003b; Synytsya 2008; Rhee et al. 2008) were reported previously. Nitschke et al. (2011) described a colorimetric method for β-1,3-1,6-glucan quantification based on the dye Congo red and determined the β-1,3-1,6-glucan content in mycelia and fruiting bodies from various mushrooms, such as button mushroom, enokitake, maitake, shiitake, and shimeji mushroom, among others. Congo red was used for characterization of glucan tertiary structures because of its interactions with the triple helix of β-1,3-1,6-glucan (Mao et al. 2007). It is the first mean of analyzing β-1,3-1,6-glucan with high precision, without extensive clean-up. The methods are useful tools to determine β-glucans in selected samples.

The β-glucan contents of three different fractions extracted from the fruiting bodies of *Pleurotus nebrodensis* were quantitatively determined using a β-glucan assay kit (Cha et al. 2012). Moreover, the content of β-glucans in mushroom *Coriolus versicolor* was determined enzymatically using a Megazyme kit (Kang et al. 2013). Minato et al. (1999) and Mizono et al. (1996) reported the development of an enzyme-linked immunosorbent assay to detect levels of lentinan in mushrooms. However, the use of enzymes is expensive and time-consuming and hydrolysis by acids is very unspecific.

At present, Brauer et al. (2002) reported a method to quantitate a fraction that included lentinan, which is labeled as a high-molecular-weight polysaccharide. In this method, aqueous extracts of shiitake mushrooms were fractionated by ethanol precipitation and size exclusion chromatography. Lentinan recovery during these two steps was quantitative. Moreover, Ko and Lin (2004) developed a fluorescence microassay based on aniline blue dye to measure β-glucan content in foods, including cereals, tubers, vegetables, fruits, and mushrooms. This microassay displayed selectivities among various 1,3-β-glucan species. The results indicated that biologically active ones such as pachyman from mushroom *Poria cocos* and yeast glucan possessed much stronger fluorescent signals than others such as barley glucan and laminarin from *Laminaria japonica*.

In addition, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) provides rapid and sensitive analyses of larger biomolecules. Hung et al. (2012) demonstrated an efficient method to give glycanbenzimidazole derivatives for polysaccharide determination in MALDI-TOF MS. Polysaccharides isolated from nutritional supplements of *Ganoderma lucidum* and *Saccharomyces pastorianus* were measured using MALDI-TOF MS with 2,5-dihydroxybenzoic acid as the matrix. These glycans were also derivatized to methylated and benzimidazole-tagged glycans by chemical transformation for

molecular weight analysis. The derivatized polysaccharides showed excellent MALDI-TOF MS signal enhancement in the molecular weight range from 1 to 5 kDa. Further research should be undertaken to improve the sensitivity.

7 Potential Applications of Mushroom Polysaccharide

Mushroom polysaccharides have aroused great interest among biotechnologists because of their wide range of potential applications in such fields as pharmacy, foodstuffs, cosmetics, and the petroleum industry (Llamas et al. 2012). Many edible mushrooms, such as reishi, maitake, shiitake, yamabushitake, etc., can be used as highly functional food materials in dishes, concentrates, extracts, liquor, and powdered mushrooms or mycelia. Polysaccharides from mushrooms have been widely applied in the food and medicine fields as immuno-stimulant and antitumor drugs with no toxicity (Zhang et al. 2011a). In medicines, three kinds of carcinostatic polysaccharide drugs, such as immunopotentiators (BRM, biological response modifiers), have been developed in Japan: (a) "lentinan" from the fruiting bodies of Shiitake, (b) "Krestin" from the cultured mycelia of Kawaratake, and (c) "Schizophyllan" (Sonifilan) from the cultured broth products of Suehirotake (Mizuno et al. 1995). The biological activities of polysaccharides have proven to be invaluable and could be employed in the potential industrial applications as natural functional ingredients to obtain possible health benefits.

8 Conclusions and Future Prospectives

Over the years, polysaccharides with potential pharmacological, nutraceutical, functional food, and cosmeceutical properties have been isolated from mushrooms. These polysaccharides attract a great deal of attention of not only researchers but consumers. In the present review, attempts have been made to discuss the preparation, extraction, purification, and characterization along with quantitative analysis of polysaccharides from mushrooms. It is reasonable to evaluate the medicinal quality of mushroom using the bioactive polysaccharide content as a marker, rather than the total polysaccharide level in the future. A number of polysaccharides from mushrooms are recommended for increasing energy, reducing levels of blood glucose, losing weight, and boosting the immune system. However, reliable data from actual animal experiments and human clinical trials are still relatively lacking. In addition, it is essential to develop effective industrialization-oriented methods for the extraction of mushroom bioactive polysaccharides and exploit nutraceutical products based on the bioactive polysaccharides.

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Isolation, Purification, and Nanotechnological Applications of Chitosan

36

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Contents

Abstract

The advent of nanotechnology has given a new momentum to enhance the inherent characteristics of naturally occurring polysaccharides when these are subjected to nanofabrication. At the nanoscale, the functionality of the polysaccharides is changed drastically. Many properties are concealed in the polysaccharides that are perhaps not yet discovered. The exploration of nanotechnology to engineer polysaccharides may open up a new horizon that can noticeably change every aspect of human life. In this context, chitosan, as one of the important polysaccharides, will only be studied thoroughly not only because of its abundant availability in nature but its versatility in various biomedical and industrial applications as well. Usually, chitosan is found in the exoskeleton of crab, shrimp, prawn, lobster, squid pen, etc. There is a great impact on the properties of chitosan depending on the nature of methods of extraction. That is why the selection of gentle methods for isolation and purification of chitosan

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from various sources plays a pivotal role. This chapter will mainly emphasize on the isolation and purification method of chitosan as well as nanotechnological applications of selective chitosan-based derivatives.

Keywords
Polysaccharide • Chitosan • Isolation • Purification • Nanotechnology

Abbreviations

ACE	Angiotensin-converting enzyme
CDA	Chitin deacetylase
CS-g-PEG	Chitosan-graft-poly(ethylene glycol)
DMAc	Dimethyl acetamide
DMF	Dimethyl formamide
EDAC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetic acid
FA	Folic acid
G. tsugae	*Ganoderma tsugae*
HFIP	Hexafluoroisopropanol
mPEG	Monomethyl poly(ethylene glycol)
$NaBH_3CN$	Sodium cyanoborohydride
$NaBH_4$	Sodium borohydride
NCMC	*N*-carboxymethyl chitosan
NMMNO	*N*-methylmorpholine *N*-oxide
PEI-PEG	Polyethylenimine-poly(ethylene glycol)
SBP	Systolic blood pressure

1 Introduction

Among other polysaccharides, chitosan is only polycationic in nature, and this important characteristic has made it as a suitable candidate for a wide range of biomedical applications. The properties of chitosan get drastically changed with extraction and purification methods adopted. Chitin, a homopolymer of β(1 → 4)-linked *N*-acetyl-D-glucosamine, is found widely in the cell walls of some microorganisms such as fungi, molds, and yeasts and in the cuticular and exoskeletons of invertebrates such as crabs, shrimps, prawn, lobsters, squid pens, and insects (Zheng et al. 2001; Knaul et al. 1999; Dutta et al. 2002; Dutta et al. 2004a, b). Chitosan occurs rarely in nature, and it is usually obtained by extensive deacetylation of chitin with a hot alkali solution. Chitosan is composed essentially of β(1 → 4)-linked glucosamine units together with some proportion of *N*-acetylglucosamine units (Fig. 1; Dutta et al. 2004a, b, 2008; Jiu et al. 2004; Dong et al. 2004). Despite the abundant availability of chitin in nature, it is still recognized as underprivileged resources. Nevertheless, a vast amount of research has already been carried out on chitin and chitosan. In recent studies, especially, chitosan has been a very good biomedical candidate for its potential applications

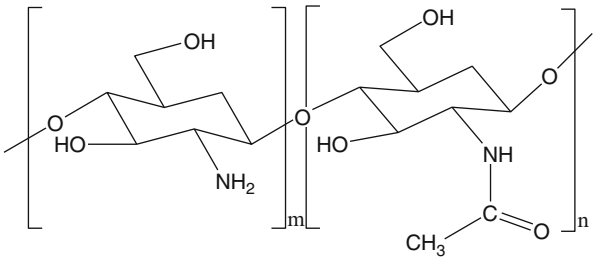

Fig. 1 Chemical structure of chitosan

ranging from drug delivery to cell delivery to gene delivery to wound healing (Khor and Lim 2003; Malafaya et al. 2007; Alves and Mano 2008; Riva et al. 2011; Liu et al. 2011). But the solubility of chitosan has been a major constraint for its versatile applications. Chitosan is not soluble in conventional organic solvents; it is only soluble in acetic acid and a few inorganic acids such as hydrochloric acid (Guibal 2005; Pillai et al. 2009). Therefore, it has been a major challenge to improve the solubility of chitosan. On the other hand, the unmodified forms of chitosan too have confined the practical or commercial uses of it. So it is believed that application of nanotechnology to engineer chitosan may not only boost its solubility properties but also may change its material characteristics that would be beneficial for further processing for its commercialization in various products. This belief has led the chitin and chitosan researchers not only to escalate fundamental research on chitosan but also to work aggressively on applied research to develop chitosan-based nanoproducts by exclusively utilizing its chemical, physical, and biological properties. On the other hand, the variations on the properties of chitosan obtained from various sources are also not yet well documented. This chapter will discuss the different sources of chitosan and its versatile characteristics, isolation and purification method to obtain chitosan, and nanotechnological applications of selective chitosan-based derivatives.

2 History and Origin of Chitin and Chitosan

The word chitin comes from the Greek etymology meaning “envelop.” Prof. Henri Braconnot of France was the first inventor of chitin and he found it in mushrooms in 1811. This was first used by Odier in 1823. Further, in the 1830s, it was isolated from insects and was named chitin. In 1859, Prof. C. Rouget discovered chitosan. Although, in the 1930s and 1940s, the polymers attracted significant attention that is evidenced by about 50 patents but due to the lack of adequate manufacturing facilities and aggressive competition, commercial development of synthetic polymers was restricted. Revived interest in the 1970s encouraged the researchers to better utilize shellfish shells. There are plentiful sources of chitin in the total universe, and chitosan exists naturally only in a few species of fungi (Pochanavanich and Suntornsuk 2002; Bank et al. 2005). Chitin has been found in a wide array of natural sources, namely, crustaceans, fungi, yeasts, insects,

annelids, nematodes, mollusks, coelenterate, marine diatoms, squid pens, etc. (Muzzarelli 1990; Synowiecki and Al-Khateeb 2003; Franco et al. 2005; George et al. 2011). However, chitosan is primarily manufactured from the exoskeleton of crustaceans like crab, shrimp, prawn, lobster, krill, and crayfish, to name a few, because of its abundant availability as a by-product of food processing (Shepherd et al. 1997; Rasmussen and Morrissey 2007).

3 Isolation of Chitin from Plants and Animals

As the principal source of chitin is animal based, this is the reason why it is better to start discussing isolation of chitin from various animals. Over a decade, a small quantity of shell waste was particularly utilized for animal feed or chitin isolation (Synowiecki and Al-Khateeb 2003). Thus, the processing of shellfish was a major concern for environmental pollution. Nowadays, this problem has been overcome to a certain extent because of an increased awareness among the researchers to explore the underprivileged resources (mainly shell wastes) for the isolation of chitin and make it suitable for a variety of biomedical applications. The basic pattern is more or less same for the isolation of chitin from various sources because typically the exoskeleton of crustacean shell consists of minerals (especially calcium carbonate), protein, chitin, pigments, etc. It is quite significant to mention that there are some solvent systems available for direct dissolution of chitin, but no economical and appropriate solvent is, however, known for the dissolution of chitin after isolation of chitin from these exoskeletons (Hudson and Smith 1998; Sannan et al. 2006). The current trend in industry to obtain chitin from their exoskeletons is to make the calcium carbonate and proteins in the exoskeletons soluble in water and then to remove them from the exoskeletons to obtain chitin. Specifically, the exoskeletons of crabs, lobsters, or shrimps are immersed in a dilute aqueous alkaline solution and heated, after which the degraded proteins are washed off with water. Insoluble matter with chitin contained therein is immersed in a dilute aqueous solution of hydrochloric acid to convert calcium carbonate (which is still contained in the insoluble matter) into calcium chloride, which is soluble in water. The insoluble matter is then washed with water to remove the calcium chloride, so that chitin is obtained as insoluble matter. The various sources of chitin differ somewhat in their structure and percent chitin content. Usually, chitin isolation consists of several steps, namely, demineralization, deproteinization, and decoloration. The first two steps can be reversed according to the needs of recovering carotenoids and protein and for further chitin application. Among other crustacean shells, crab and shrimp shells have received a significant attention for the production of chitin and chitosan (Shahidi et al. 1999; Islam et al. 2011). The readers are directed to refer to many reviews, research articles, patents, and books pertaining to traditional isolation of chitin (Okada et al. 1999; Ravi Kumar 2000; Teng et al. 2001; Lertsutthiwong et al. 2002; Wooten and Singer 2003; Sannan et al. 2006; Toan 2009; Das and Ganesh 2010; Kumirska et al. 2011; Limam et al. 2011). Succinctly, demineralization is usually achieved by using diluted

Table 1 Influences on the characteristics of chitin produced by different serial treatments

Treatment 1	Treatment 2
The deproteinization precedes the decalcification	The decalcification precedes the deproteinization
Chitin becomes whiter with increasing concentration of NaOH (1–4 %)	Higher concentration of NaOH (4 %) gave more efficient removal of protein and colored matter
Decalcification with 4 % HCl for only 2 h was sufficient to remove minerals	Decalcification first with 4 % HCl for either 2 or 12 h gave colored matter and the protein still remained bound to the solid matrix
The yield of chitin was 15–20 %. In this case, protein layer gets unprotected as the deproteinization precedes the decalcification. That is why during demineralization more hydrolyses and loss of materials in the solid chitin fraction occur	The yield of chitin was 20–27 % that is attributed to the strong adherence of chitin with protein leading to less hydrolysis of the backbone because the decalcification precedes the deproteinization
Ash content was less than 1 %	Ash content was 1–2 %

hydrochloric acid (1–8 %) at room temperature (Hackman and Goldberg 1965; No et al. 1989). To prevent chitin depolymerization, ethylenediaminetetraacetic acid (EDTA) can be used for the removal of mineral salts (Austin et al. 1981; Roberts 1997). On the other hand, deproteinization involves the usage of aqueous sodium or potassium hydroxide solution, and decoloration is usually carried out by a bleaching treatment with NaOCl or H_2O_2 solutions (Roberts 1992).

The most exploited sources of chitin are shrimp and crab shells. Lertsutthiwong et al. (2002) has studied the effect of chemical treatment on the characteristics of chitosan obtained from fresh local black tiger shrimp shells. In this study, they separately treated the shrimp shells by reversing the conventional order of extraction of chitin from crustacean shells. The differences of the characteristics of chitin produced by different serial treatments are shown in Table 1. Further, they have shown the effect on quality of chitosan obtained from chitin after deacetylation from these different serial treatments. If process 2 is followed by subsequent deacetylation, then there is a possibility of getting chitosan with high viscosity because the protein layer gets protected from more hydrolysis and vice versa in the case of process 1 (See Table 2). Further, to get chitosan with a high viscosity and a high degree of deacetylation at low temperature, the process should be initiated with decalcification and requires multi-deacetylation.

There are many standard operating procedures (SOPs) available for extraction of chitin and chitosan because of their wide range of sources in the earth (Percot et al. 2003; Kim and Rajapakse 2005; Kjartansson et al. 2006; Waldeck et al. 2006; Mahmoud et al. 2007; Rødde et al. 2008; Hayes et al. 2008). Although, lots of works have already been done on extraction of chitin and chitosan from various sources, but a significant research is still required to improve the production process as well as the quality of chitosan, especially from stored material sources. In a study, Toan (2009) has investigated the effect of partial autolysis during storage of shrimp biowaste on the quality of extracted chitin and chitosan. In this study,

Table 2 Influence on various parameter of chitosan during its different serial treatments (Adapted from Lertsutthiwong et al. 2002)

Treatments	Parameter			
	Moisture content (%)	Ash content (%)	DD (%)	Viscosity (cps)
1 % NaOH, 21 h, and 4 % HCl, 2 h	6.71 ± 0.10	0.51 ± 0.04	75.9 ± 0.35	830 ± 30
4 % HCl, 2 h, and 1 % NaOH, 21 h	9.52 ± 0.16	0.89 ± 0.27	74.3 ± 0.07	5,268 ± 146
2 % NaOH, 21 h, and 4 % HCl, 2 h	6.96 ± 0.04	0.52 ± 0.04	75.7 ± 0.71	486 ± 14
4 % HCl, 2 h, and 2 % NaOH, 21 h	8.13 ± 0.09	1.04 ± 0.08	75.5 ± 0.21	6,370 ± 254
4 % NaOH, 21 h, and 4 % HCl, 2 h	8.36 ± 0.06	0.50 ± 0.08	75.7 ± 0.28	435 ± 13
4 % HCl, 2 h, and 4 % NaOH, 21 h	7.70 ± 0.14	1.01 ± 0.001	75.7 ± 0.85	5,238 ± 190
1 % NaOH, 21 h, and 4 % HCl, 12 h	7.44 ± 0.21	0.84 ± 0.08	73.2 ± 2.05	160 ± 9
4 % HCl, 12 h, and 1 % NaOH, 21 h	12.6 ± 0.15	0.87 ± 0.04	74.8 ± 0.57	3,420 ± 174
2 % NaOH, 21 h, and 4 % HCl, 12 h	8.11 ± 0.15	0.71 ± 0.01	73.8 ± 3.0	110 ± 4
4 % HCl, 12 h, and 2 % NaOH, 21 h	9.38 ± 0.04	0.96 ± 0.01	76.3 ± 0.07	2,919 ± 93
4 % NaOH, 21 h, and 4 % HCl, 12 h	7.97 ± 0.11	0.68 ± 0.02	74.1 ± 1.91	106 ± 5
4 % HCl, 12 h, and 4 % NaOH, 21 h	8.33 ± 0.09	0.97 ± 0.01	76.1 ± 0.07	4,470 ± 115

a modified method had been used in which the shrimp shell was allowed to undergo partial autolysis before it was used to produce chitin and chitosan. This revised process for the production of chitin and chitosan is shown in Fig. 2.

In terms of improving the quality of chitosan, partial autolysis plays a crucial role because it facilitates $CaCO_3$ to get exposed directly to the chemicals used during demineralization process. For the industrial practice of chitin extraction, this indicates that a shorter time and lower concentration of HCl may be adequate to demineralize so as to avoid damage due to hydrolytic action of HCl on the polymeric backbone structure of chitin. On the other hand, it was found that the protein content after treatment of autolyzed biomaterial with 2 % NaOH is similar to the protein content after standard 4 % NaOH treatment. Obviously, this study could help reduce the cost of deproteinization in industry and could help prevent the environment from pollution. The production of chitin from various sources with their different treatment processes is summarized in Table 3.

Keeping in mind the importance of chitosan as well as its economic value as an industrial product, we must pay attention on its key physical parameter,

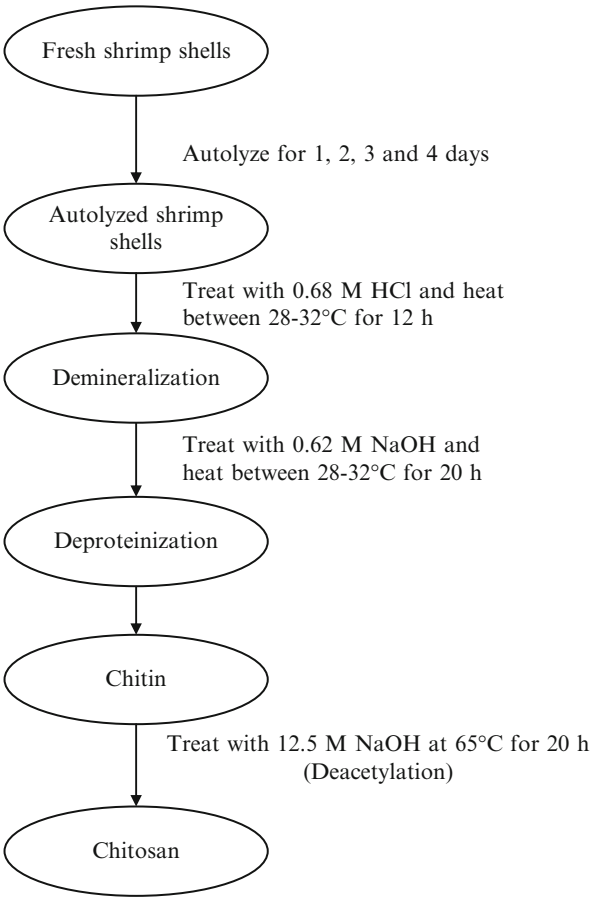

Fig. 2 Flow chart for the processing of chitin and chitosan from fresh shrimp shells

i.e., turbidity. Depending on the source, a marked difference is observed in aqueous solutions of chitosan and its derivatives in terms of their extent of turbidity (Sannan et al. 2006). Consequently, these turbid aqueous solutions of chitosan and chitosan-derived products greatly lose its commercial value. Obviously, such chitosan cannot be used as a commercial product and may have to be discarded in some instances. Therefore, the selection of the source also plays a pivotal role in the production of chitin and chitosan. In a study, Shepherd et al. (1997) reported the production of chitosan from New Zealand's arrow squid (*Nototodarus sloanii*) pens as well as the evaluation of the functional properties of this squid chitosan compared with chitosan extracted from crustacean sources. Squid pen chitin and chitosan were visibly cleaner than chitin and chitosan obtained from crab and crayfish. In addition, due to the presence of lower amount of minerals in squid pen as compared to crustacean shells, demineralization process can be skipped to extract chitin that also makes the production cost cheaper. As shown in Table 4, the squid pen chitosan is similar in composition to commercial chitosan. Further, there

Table 3 Different sources of chitin with their corresponding treatment processes

Source of chitin	Demineralization process	Deproteinization process	Reference
Crab	HCl	NaOH	Shahidi and Synowiecki (1991), No and Lee (1996)
		KOH	
Shrimp	HCl	NaOH	Gagne and Simpson (1993), No and Lee (1996), Tsaih and Chen (2003)
	CH_3COOH	KOH	
Prawn	HCl	NaOH	Acosta et al. (1993), No and Meyers (1997)
Krill	HCl	$NaHCO_3$	Synowiecki et al. (1981), No and Meyers (1995)
		KOH	
		NaOH	
Lobster	HCl	NaOH	Horowitz et al. (1957), Whistler and BeMiller (1962)
	CH_2O_2	NaOH	
Crawfish	HCl	NaOH	Acosta et al. (1993)
Squid pen	Omitted	NaOH	Shepherd et al. (1997)

Table 4 The ash, moisture, protein, nitrogen, and acetyl content of selected chitosans (Adapted from Shepherd et al. 1997)

Sample	Source	% Ash	% Moisture	% Protein	% Nitrogen	% Acetyl
Squid pen chitosan	Squid (IRL)	0.17	2.1	1.3	7.5 (7.8)	49.4
Seacure 443 chitosan	Crab/shrimp (Pronova)	0.58	11.2	1.3	7.2 (8.2)	27.2
Sigma chitosan	Crab (Sigma)	0.51	4.8	1.3	7.1 (8.2)	27.2
Profloc 340 chitosan	Crab/shrimp (Pronova)	0.40	12.9	1.4	7.1 (8.2)	–

is a substantially lower amount of ash contained in squid chitosan that makes this aqueous solution noticeably cleaner than samples of chitin and chitosan from crab and crayfish. The manufacturing process of chitin and chitosan from squid pen as well as crustacean shell is shown in Fig. 3. The traditional and commercial chitosan production process has a number of unfavorable characteristics as the process requires expensive heat energy and caustic alkali, which is a potential health hazard. The process also produces large amounts of waste, thereby necessitating significant disposal costs. In addition, the supply of shrimp or crab shells is highly dependent upon seasonal and environmental factors, leading to unpredictable limitations on production capacity (Chen et al. 2002). Therefore, low cost and available waste materials may be considered as promising sources of chitin. "Fish scales" are good sources of chitin and chitosan. The fish scales are discarded daily as waste materials from fish markets, canteens, fish processing industries, or kitchens. This abundant waste may pose environmental hazard due to easy deterioration. The use of this waste to produce valuable and biologically sustainable

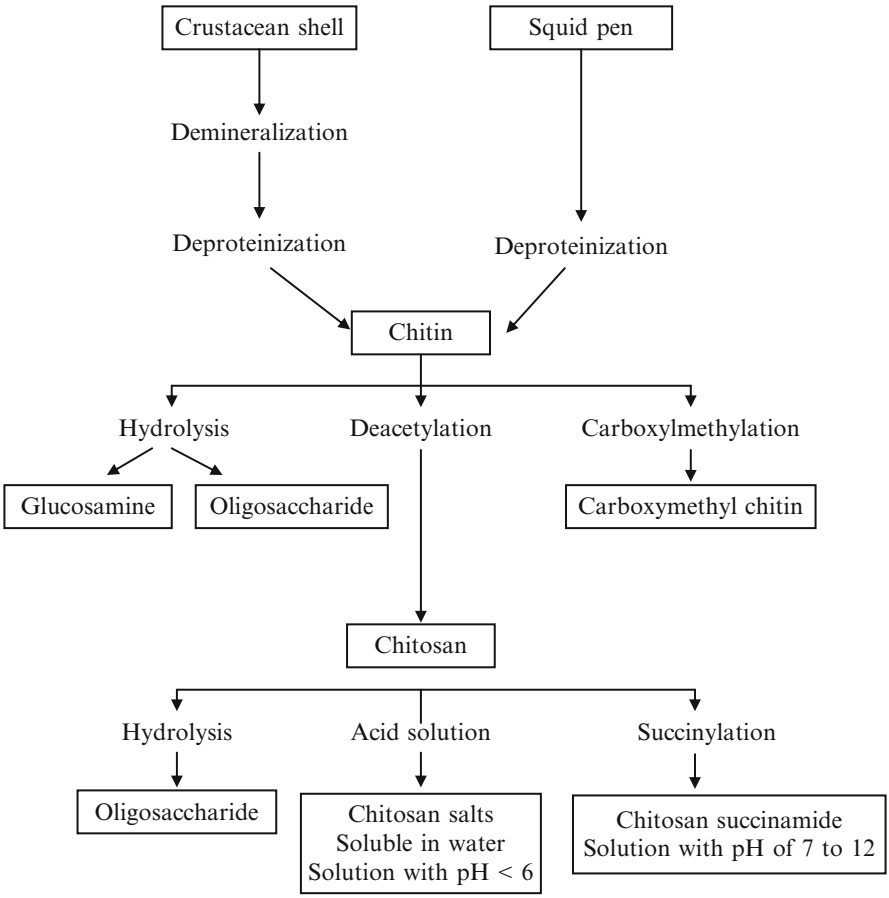

Fig. 3 A schematic diagram for manufacturing chitin and chitosan (Adapted from www.france-chitine.com/fab.e.htm)

materials is a challenge for current research and development (Zaku et al. 2011). Generally, fish scales consist of protein (type I collagen and ichthylepidin) and apatite (calcium phosphate, magnesium carbonate, and calcium carbonate) (Ikoma et al. 2003). A very few information is available on extraction of chitin and chitosan from fish scales. A study on dye-binding interaction of chitosan obtained from the fish scale of Tilapia (*Tilapia nilotica*) was reported by Uawonggul et al. (2002). Recently, a report on extraction and characterization of chitin from scales of common carp fish (*Cyprinus carpio* l) has been published (Zaku et al. 2011). In another study, Tanvir Muslim et al. (2013) demonstrated the extraction of chitin from the fish scales of *Labeo rohita* and subsequent preparation of chitosan from it by deacetylation reaction. The extraction process from the fish scales of *Labeo rohita* is shown in Fig. 4.

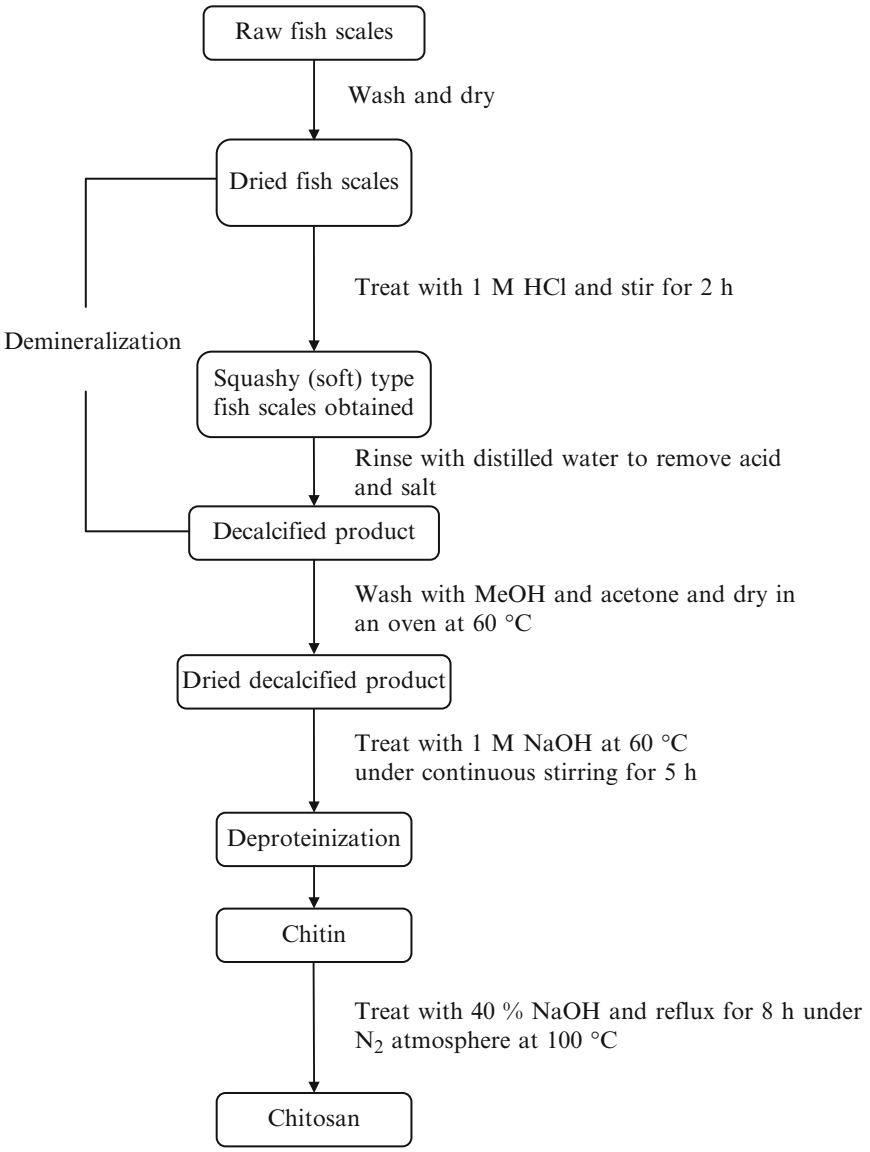

Fig. 4 Flow chart for the extraction of chitin and chitosan from "fish scales" of *Labeo rohita*

On the other hand, fungal mycelia wastes from biotechnological plants can become free and rich alternative sources of chitin and chitosan, in addition to the conventional sources of shellfish waste materials. Fungal mycelia can be cultivated throughout the year by fermentation that is rapid and synchronized and can be organized in a closed or semi-closed technological circuit to comply with modern ecological requirements. Moreover, fungal mycelia are relatively consistent in

composition and are not associated with inorganic materials. Therefore, no demineralization treatment is required to recover fungal chitin (Feofilova et al. 1996). In a study, Teng et al. (2001) investigated the concurrent production of chitin from shrimp shells and fungi by placing shrimp shells in direct contact with the fermentation of filamentous fungi in a reactor so as to avoid the use of conventional chemicals, which can lead to undue deacetylation of chitin or chain degradation of the isolated chitin. Three proteolytic *Aspergillus niger* (strains 0.576, 0.307, and 0.474) were selected from a screening for protease activity from among 34 zygomycete and deuteromycete strains. The proteolytic enzymes released by the fungi facilitate the deproteinization of shrimp powder and the release of hydrolyzed proteins. The hydrolyzed proteins in turn act as a source of nitrogen for further fungal growth, leading to a lowering of the pH of the fermentation medium, thereby further enhancing the demineralization of the shrimp shell powder. Chitin from non-fermented, fermented, and fungal mycelia were directly extracted with 5 % lithium-chloride-*N*, *N*-dimethylacetamide (5 % LiCl in DMAc), a non-degradative solvent for chitin (Austin et al. 1981). Two different problems are being solved simultaneously even without using any harsh chemicals. Fungal fermentation is a cost-effective method compared to commercial protease enzymes that only deproteinize but not demineralize the shrimp shells. Demineralization is an important step in chitin purification process from crabs also. The chemical method of demineralization includes the use of strong acid (HCl) that harms the physicochemical properties of chitin. In an attempt, Das et al. (2010) reported the usage of organic acids (lactic acid or other organic acids) produced by *Lactobacillus plantarum* as a replacement of hydrochloric acid for demineralization and proteolytic enzymes produced by *Aspergillus niger* to substitute alkaline aqueous solution for deproteinization for the extraction of chitin from trash crabs (*Podophthalmus vigil*). Although, the removal of protein entirely from crustacean shells is not yet achieved by using biological treatment during isolation of chitin, but the quality of chitin obtained by this process surely does not compromise with chitin obtained by conventional chemical treatment. The results of chitin derived from the trash crab are shown in Table 5. The study showed that the effectiveness of lactic acid for the demineralization of crustacean shells was virtually comparable to that of hydrochloric acid. For effective removal of minerals from crab shells using lactic acid, shells to acid ratio of 1:25 and temperature of 40 °C were found to be satisfactory. The use of chemicals causes depolymerization of chitin to a certain extent, and thus, its molecular weight and viscosity are affected after solubilization (Waldeck et al. 2006). But in this case, the combination of lactic acid and *Aspergillus niger* not only produces partially soluble chitin which is anticipated to increase its applications in biomedicine and pharmacy industry but also reduces the production cost of chitin. In another study, it was shown that the effectiveness of organic acids (lactic and acetic) for the demineralization of shrimp shells was also comparable to that of hydrochloric acid (Mahmoud et al. 2007). For effective removal of minerals from shrimp shells using organic acids (lactic and acetic), shells to acid ratio of 1:20, temperature of 24 °C (room temperature), and retention time of 2 h were found satisfactory. Under these conditions, the total minerals and

Table 5 Physicochemical parameters for extraction of chitin using chemical and biological treatments (Adapted from Das and Ganesh 2010)

Parameters	Chemical treatment			Biological treatment		
Concentration of acid	2 N HCl	2 N HCl	2 N HCl	69.5 g −L	69.5 g −L	69.5 g −L
Temperature	RT	RT	RT	Lactic acid 40 °C	Lactic acid 40 °C	Lactic acid 40 °C
Shells to acid ratio	1:15	1:25	1:35	1:15	1:25	1:35
Concentration of deproteinizer	1 N NaOH	1 N NaOH	1 N NaOH	5 g wet weight	5 g wet weight	5 g wet weight
Product appearance	White	Super white	Super white	Slightly brownish	White	White
Yield (g)	5.62	5.47	5.41	4.52	4.17	4.03
Yield (%)	28.1	27.35	27.05	22.6	20.85	20.15
Solubility in water	Insoluble	Insoluble	Insoluble	Partially soluble	Partially soluble	Partially soluble
pH	7.2	7.2	7.2	6.9	6.9	6.9

Conc concentration, *RT* room temperature

calcium removal efficiencies were 97.4 % and 99.11 % and 86.36 % and 85.33 % for lactic and acetic acids, respectively. Using acetic acid in the ratio of 1:20 g shells to mL acid for the demineralization of shrimp shells would result in the production of 560.38 g calcium acetate and 2.19 g potassium acetate for each 1 kg shells, provided if all the calcium and potassium present in the shrimp shells are removed. Using lactic acid in the ratio of 1:20 g shells to mL acid for the demineralization of shrimp shells would result in the production of 773.17 g calcium acetate for each 1 kg shells, provided if all the calcium present in the shrimp shells is removed. Using organic acids for the demineralization of shrimp shells would result in (a) effective removal of minerals, (b) reduction in the purification cost, (c) retention of natural chitin characteristics, and (d) production of value-added products (food preservatives and deicing agents) besides the purified chitin.

Chitosan is less commonly found in living organisms than chitin, but it can be found in the cell walls of certain groups of fungi. The traditional process of obtaining chitosan is the deacetylation of chitin using strong caustic alkali, but no significant progress has yet been made in establishing new technologies, which leads to the large-scale controlled production of chitosan. The recognition of chitin deacetylase (CDA) in several fungi and insects has given new momentum for the conversion of chitin into chitosan. In contrast to the currently used chemical procedure, the use of CDA offers the possibility of a controlled non-degradable process, resulting in the production of novel, well-defined chitosan oligomers and polymers. Indeed, the uses of biological treatment over chemical treatment for the isolation of chitin from crustacean shells as well as production of chitosan will substantially reduce environmental pollution. The use of CDA as an enzyme for the

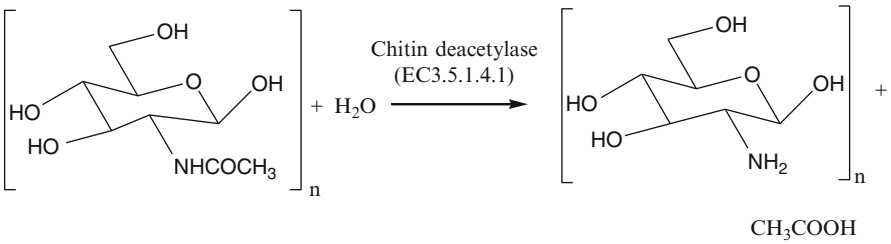

Fig. 5 Bioconversion of chitin to chitosan

preparation of chitosan polymers and oligomers offers the possibility of developing an enzymatic process that could potentially overcome most of the drawbacks as discussed earlier (Tsigos et al. 2000). As shown in Fig. 5, CDA (EC 3.5.1.41) catalyzes the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan. The presence of this enzyme has been reported in several fungi and insect species (Araki and Ito 1975; Kafetzopoulos et al. 1993; Martinou et al. 1993; Tsigos and Bouriotis 1995; Gao et al. 1995; Tokuyasu et al. 1996; Zhao et al. 2010, 2011; Song et al. 2012).

Chen et al. (2002) have patented an alternative method to produce chitin and chitosan without relying on environmentally harmful chemicals or the variable abundance of the crustacean crop. They demonstrated high-yield production of chitosan or chitin from a culture containing a *Rhizopus zygospores* or *Actinomucor taiwanensis* fungus. Further, they have shown that a medium containing corn steep liquor, glucose, yeast extract, and ammonium sulfate is capable of increasing the output of chitin and chitosan from a fungal culture. Although, the isolation of chitin from fungi is environmentally safe, but the enzymes used for the bioconversion of chitin into chitosan are required for further purification that may subsequently increase the purification cost. A selective fungal species for the isolation of chitosan are shown in Table 6. Therefore, an alternative research has been going on to find out corresponding enzymes in bacteria for the biotransformation of chitin to chitosan because bacteria are easier and faster than fungi to grow in a large-scale fermentation system. In addition, bacteria can be utilized without the necessity of purifying the enzyme.

Srinivasan (1998) have patented a method to isolate an unknown bacterium from municipal sewage. This bacterium can deacetylate chitin to the more useful, soluble chitosan. Biotransformation of chitin to chitosan by bacteria can be used in an economical and environmentally friendly process.

In another study, Yong et al. (2005) reported a novel chitinase-producing bacterium strain C4 from the soil samples. The molecular identification confirmed that strain C4 should belong to the genus *Sanguibacter*. The enzyme extracted from bacteria was able to degrade polymeric chitin from crab and shrimp shells and from insect puparium. The enzyme also displayed weak activity to chitosan.

As it is known in the earlier discussion that bacterial growth is faster than fungal growth and CDA-producing capabilities of most fungal strains are low and their

Table 6 Effect of endophytic fungal species on the percentage yield of chitosan (Adapted from George et al. 2011)

Serial no.	Species	Yield of chitosan (%)
1.	*Fusarium sp.*	–
2.	*Aspergillus flavus*	5.7
3.	*Cladosporium cladosporioides*	2.52
4.	*Phoma sp.*	3.11
5.	*Botryodiplodia theobromae*	–

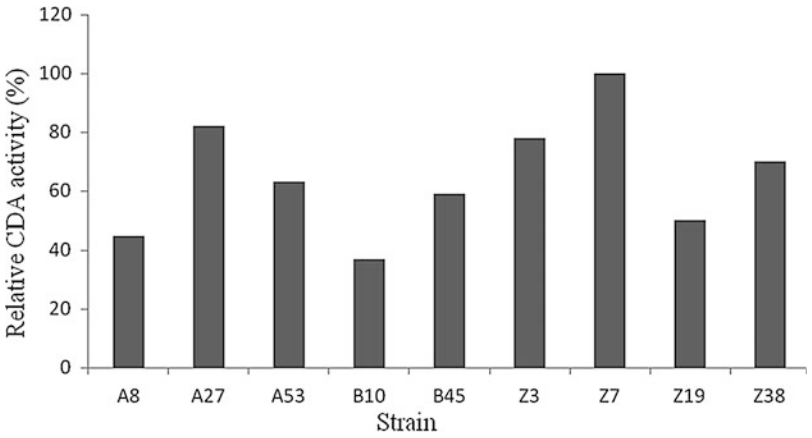

Fig. 6 The relative CDA (chitin deacetylase) activity of nine isolated bacterial strains (Adapted from Zhou et al. 2010)

fermentation requirements are complicated. In a study, Zhou et al. (2010) reported the isolation of CDA-producing bacteria from soil and optimized its fermentation process. Among 208 bacteria isolated from fresh soil samples, 9 were CDA-producing strains of bacteria, namely, A8, A27, A53, B10, B45, Z3, Z7, Z29, and Z38. Percentage of relative CDA activity of them was shown in Fig. 6. As can be seen from this figure, the Z7 strain shows the highest CDA activity, which was determined by enzyme assay screening. To enhance CDA production, various carbon sources (glucose, lactose, sucrose, starch, corn meal, and bran), nitrogen sources (beef extract, yeast extract, casein, proteose peptone, soybean meal, urea, ammonium sulfate, and sodium nitrate), and inorganic salts ($MgSO_4$, $FeSO_4$, $ZnCl_2$, $CuSO_4$, $MnSO_4$, and K_2HPO_4) were separately added to the basal formulated production medium at concentrations of 2 %, 1 %, and 0.04 %, respectively. Based on its cultural, morphological, and physiological characteristics and molecular identification, strain Z7 was recognized as *Bacillus amyloliquefaciens*. It was concluded that yeast extract at a concentration of 1 % was an ideal nitrogen source, starch at a concentration of 2 % functioned best as the major carbon source, and magnesium sulfate at a concentration of 0.04 % was an ideal inorganic salt. Ideal pH and temperature for optimum production of CDA by

Table 7 Proximate analysis of crustacean shell wastes as a percentage of dry weight (Naczk et al. 1981; Shahidi and Synowiecki 1991; Muzzarelli et al. 1997; Synowiecki and Al-Khateeb 2000; Abdulkarim et al. 2013)

Source of chitin		Parameter			
		% Ash content	% Protein	% Chitin	% Lipids
Shrimp	*Penaeus monodon*	23.0	47.4	40.4	1.3
	Pandalus borealis	34.2	41.9	17.0	5.2
	Crangon crangon	27.5	40.6	17.8	9.9
Crab	*Callinectes sapidus*	58.6	25.1	13.5	2.1
	Chionoecetes opilio	40.6	29.2	26.6	1.3
Crawfish	*Procambarus clarkii*	46.6	29.8	13.2	5.6
Krill	*Euphausia superba*	23.0	41.0	24.0	11.6
Prawn	–	29.4	61.6	33.0	1.4
Mussel[a]	–	–	9.99	23.25	–

[a]It was found in the bank of Gubi Dam in Bauchi, Nigeria

this strain were pH 6 and 37 °C. Further research is going on to find a suitable strain of CDA-producing bacteria for the bioconversion of chitin to chitosan to circumvent environmentally related problems caused during the traditional deacetylation of chitin using a harsh chemical NaOH pyrolysis method.

In another study, Kaur et al. (2012) reported the isolation of 20 strains of bacteria from soil samples collected from different beaches of Chennai, India. Of these 20 bacterial strains, only two strains (S3, S14) are potent degrader of chitin, and they are also good producer of the enzyme CDA that facilitates release of chitosan.

However, crustacean shell wastes are extensively used for the production of chitosan because the percentage availability of chitin is higher as compared to other sources. A proximate analysis of crustacean shell wastes as a percentage of dry weight is given in Table 7.

From the previous discussion, it has been known that chitosan can be produced from the mycelium, but no research has been reported on the use of fungal fruiting bodies as another source of chitin. A lot of research has been done on the extraction of chitin and chitosan from fungal cell wall. A fungus called *Ganoderma tsugae* (*G. tsugae*) was used for this purpose (Chen et al. 2007). The main components of *G. tsugae* fruiting bodies are β-1,3-D-glucan and chitin, which were found to be safe materials in enhancing wound healing in Wistar rats and accelerating proliferation. Further, it also facilitates migration of fibroblasts and keratinocytes with no cytotoxicity. George et al. (2011) carried out a study on chitosan from fungi. He used *Aspergillus flavus*, *Botryodiplodia theobromae*, *Cladosporium cladosporioides*, *Fusarium* sp., and *Phoma* sp., and these were all isolated from stem and leaf segments taken from medicinal plants. Here, the extraction of chitin from Shiitake mushroom (*Lentinus edodes*) will be discussed. Shiitake mushrooms have been known to possess a polysaccharide compound called letinan, a glucan that acts as a potent immune stimulator in the body and also simulates the production of T-lymphocytes in the body, which are important in controlling cancer and infection (Mizuno 1995; Sajoughian 2009). In a study, Senthilmohan et al. (2002)

investigated the feasibility of producing a fungal-derived chitosan by both chemical and enzymatic methods. Using the chemical method (dil. NaOH), 66 % of the proteins was removed, whereas 33 % of the proteins was removed using the enzymatic method. Deproteinization of mushroom fiber was described as follows.

First of all, 2 kg of Shiitake mushrooms was mixed with 4 L of water and refluxed at 100 °C for about 8 h. The solid and liquid fractions were separated by centrifugation at 4,000 rpm for 5 min. Both the liquid and solid fiber fractions were frozen to −20 °C and then freeze-dried until a constant mass was obtained. Fiber was deproteinized either by NaOH (1 N) or actinidin enzyme. Kiwifruit extract was prepared to obtain deproteinizing enzyme actinidin by blending the kiwifruit in a food processor. Then the extract was centrifuged and the supernatant was obtained. The fiber samples were incubated with the kiwifruit extract at room temperature (23 °C) for 48 h in the enzymatic deproteinization. In the chemical method, mixture of the fiber and NaOH was heated for 2 h under reflux at 100 °C. The samples were centrifuged at 4,000 rpm for 5 min and the fiber separated from the liquid. The deproteinized fiber was then stored for deacetylation. In essence, deacetylasing enzyme derived from the fungus *Aspergillus nidulans* was used, but it gave lower conversion compared to that of the chemical method. Despite yielding lower amount of chitosan, the enzymatic method was still encouraging enough to assure further study, because it is an environmentally friendly process alternative to the chemical treatment.

4 Preparation and Purification of Chitosan

By merely substituting secondary hydroxyl group on the alpha carbon atom of the cellulose molecule with the amino group, chitosan cannot be obtained. Chitin is the one and only precursor to prepare chitosan. Up to now, there is an open announcement pertaining to the laboratory synthesis of chitosan. If anybody is able to prepare chitosan synthetically, he or she will be rewarded a huge amount in dollars! Usually, by treating chitin with a hot alkaline concentrated solution, chitosans with various degrees of deacetylation are obtained. The conventional process for producing chitin and chitosan from crustacean shells involves grinding crustacean shells and treating the ground shells with a dilute base (e.g., sodium hydroxide) and then heating to remove protein and lipids (deproteinization). Calcium carbonate is removed by extraction with a dilute acid (e.g., hydrochloric acid) at room temperature (demineralization). Following deproteinization and demineralization, the resulting product is predominantly chitin. An optional decolorization step may be used to bleach the chitin, e.g., extraction with ethanol and ether or bleaching with sodium hypochlorite (Mukherjee 2001). Removal of acetyl groups from the chitin polymer (deacetylation) produces chitosan; deacetylation is usually performed by reacting chitin with concentrated sodium hydroxide or potassium hydroxide and heat. The deacetylation process does not remove any contaminants existing in the chitin starting material. Thus, removal of impurities only occurs during production of the chitin precursor. Chitosan is not a single, definite chemical entity, because its

composition varies depending on the crustacean species used as the starting material and the particular preparation method for extraction used as well (Quian and Glanville 2002). Substantial removal of impurities from chitosan can cause immunological reactions. Because it could be critical for chitosan intended for use as a biocompatible and biodegradable material in medical applications. However, purifying chitosan is very difficult since chitosan in solution is a highly viscous material. Producing highly pure, medical grade chitosan via the conventional techniques is very expensive since such techniques typically require costly instrumentation such as autoclaves, ultrafiltration, and molecular sieves (Jubert et al. 2009). The availability of less expensive medical grade chitosan should expand and accelerate its use in biomedical applications. Apart from this, several methods are available for purification of chitosan. Few of them are discussed here.

Chitosan may be purified from extraneous material which includes inorganic salts, proteinaceous materials, and gums (Doczi 1957). In a method, chitosan is purified by adding an excess of a soluble salicylate, preferably an alkali metal salicylate such as sodium salicylate, to an aqueous solution of a chitosan salt. The resulting mixture can then be immediately chilled in an ice-water bath where upon the chitosan will precipitate as the salicylic acid salt of it. By soluble salicylate, it is meant salicylic acid and salts of salicylic acid that are more soluble in water than chitosan salicylate. The high solubility of the sodium salicylate makes it particularly useful in this invention. The precipitate is separated by centrifugation, redissolved in water and the resulting solution is filtered and adjusted to a pH of about 9, by adding a water-soluble base (such as sodium hydroxide, ammonium hydroxide or diethylamine) to it. Finally, the resulting precipitate is collected, washed with a water miscible organic solvent and dried. Consequently, purified chitosan is obtained as desired.

Chitosan was purified according to the method described by Nasti et al. (2009). Briefly, 5 g of chitosan (batch I, batch II, and batch III) were dissolved in 400 mL of a (2 % v/v) acetic acid solution in deionized water. Complete dissolution was achieved after 16 h of stirring. The solution was then filtered through 0.22 μm pore-size filters in order to remove insoluble acid impurities and boiled for 15 min to denature and precipitate any protein content. The mixture was then centrifuged for 10 min at 4,500 rpm to remove protein; the supernatant was removed and filtered through 0.22 μm pore-size filters. The pH of the solution was then adjusted to 9 with 1 M sodium hydroxide, in order to precipitate chitosan from the aqueous phase and remove any alkali-soluble impurities.

The precipitated chitosan was then recovered by centrifugation and washed with deionized water until the pH and conductivity values reached the values of pure water. The samples were then freeze-dried and stored at 4 °C for further use.

Sometimes, a turbid appearance is found in chitosan solution but that is not favorable for its biomedical applications. Therefore, chitosan can be washed with 30 % isopropyl alcohol before processing it for any biomedical applications (Sannan et al. 2006). A small test was carried out by a group of researchers to ascertain the cause of turbidity in an aqueous solution of chitosan. First of all, chitosan was washed with isopropyl alcohol. Then, the unwashed chitosan and the

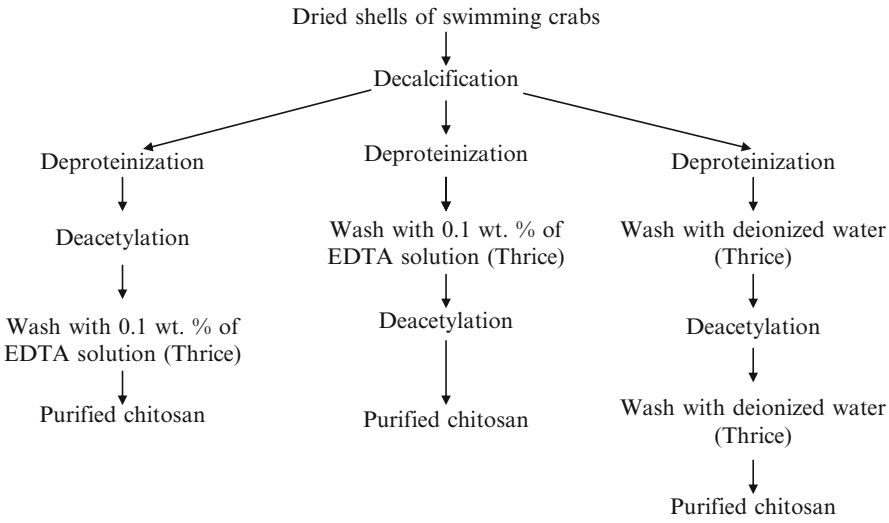

Fig. 7 Various schemes for purification of chitosan

washed chitosan were separately dissolved in aliquots of glacial acetic acid solution, and the resulting solutions were compared in terms of the extent of turbidity. As a result, it was found that the aqueous solution of the washed chitosan had much lesser turbidity. Chitosan was then subjected to extraction with isopropyl alcohol in a Soxhlet extractor, and impurities extracted in the isopropyl alcohol were analyzed by infrared spectroscopy, gas chromatography, and other methods. Those extracted impurities were found to comprise palmitic acid and oleic acid as primary components and also contain a small amount of fatty acids, such as stearic acid and myristic acid, and their corresponding salts.

As can be known from the previous discussion pertaining to the isolation of chitin that chitosan is produced from chitin in three so-called steps, i.e., deproteinization, decalcification, and deacetylation. Purified chitosan can be obtained by serial treatments of the processed crustacean shells (swimming crabs) with either 0.1 % (w/w) of EDTA or deionized water. Various schemes for purification of chitosan are shown in Fig. 7.

On the other hand, purified chitin can also be produced by washing chitin with either ethanol or isopropyl alcohol. This process is considered to be excellent because of its simple operation, it can be widely applied. Further, it has the ability to remove fatty acids and their corresponding salts present in the chitin. Using this process, the total content of fatty acids and their corresponding salts present in the chitin can be reduced to 0.2 % (w/w) or lower in terms of fatty acids. The principal cause of turbidity in an aqueous solution of a chitin is due to the formation of water-insoluble salts such as the calcium salts of fatty acids and the magnesium salts of fatty acids.

5 Pros and Cons of Chitin and Chitosan

It is noteworthy to mention that the structural difference is very important in determining many properties of chitosan, especially solubility. Chitosan obtained by a heterogeneous procedure (deacetylation at various temperatures) is not soluble in water, whereas water-soluble chitosan can be prepared by homogeneous deacetylation (at room temperature) of chitin (Sannan et al. 1976). The acetylation of highly deacetylated chitosan can also produce soluble chitosan (Kurita et al. 1989). Although, the activity of chitosan is limited to acidic conditions due to its poor solubility above pH 6.5 at which chitosan loses its cationic nature. Therefore, researchers have focused on the preparation of water-soluble chitosan derivatives in the entire range of pH. Over several decades, the studies on chitin and chitosan have been intensified as a consequence of their excellent biological properties such as biodegradation in human body, biocompatibility, non-toxicity, immunological activity, antibacterial activity, and wound-healing activity (Zhang et al. 2010). Despite the abundant availability of chitin in nature, it is no longer soluble in diluted aqueous or conventional organic solvents due to the high crystallinity of chitin, supported by strong intra- and inter-hydrogen bonds through the acetamido group. Although, chitin is soluble in hexafluoroisopropanol (HFIP), hexafluoroacetone, chloro alcohols, and chloroacetic acids in conjunction with aqueous solutions of mineral acids, *N*-methyl morpholine-*N*-oxide (NMMNO), DMAc/5 % LiCl, *N*-methyl-2-pyrrolidinone/5 % LiCl, and some other special solvents to a certain extent, but still, these solvents have limited the commercial usage of chitin (Austin 1973; Dutta et al. 1997; Ravikumar et al. 2000; Rinaudo 2006; Mark 2013). Therefore, chitin has yet to find large-scale industrial uses. No economical and suitable solvent is, however, known for the dissolution of chitin upon isolation of chitin from the exoskeletons of crustacean shells. Despite the abundance of chitin in nature and its advantages claimed (for instance, preparation of chitin films and a variety of its derivatives reported so far), satisfactory result has not always been obtained owing to its solubility related issues. Therefore, the weak solubility of chitin has become the main constraint for its usage in biomedicine and biotechnology even after possessing versatile biological activities. The various advantages of chitosan are summarized in Table 8.

6 Preparation of Selective Chitosan Derivatives

Due to the presence of amino groups as well as hydroxyl groups, chitosan can be easily customized to synthesize different derivatives of interest (Tangpasuthadol et al. 2003). Over decades, much emphasis has been given on chemical modifications of chitosan bearing in mind that chitosan is normally insoluble in solutions above pH 6 and requires acid to be protonated (Trapani et al. 2009). Chitin has two hydroxyl groups, while chitosan has three reactive groups, i.e., primary (C-6) and secondary (C-3) hydroxyl groups on each repeat unit and the amino (C-2) group on

Table 8 Various advantages of chitosan

Characteristic(s)	Action(s)
1. Chitosan behaves as a cationic polyelectrolyte in solution	1. As a result of this polycationic character, it can be employed as a flocculating agent in waste water treatment. Further, it can also be used in the food industry for the treatment of fruit juices and wines
2. Chitosan is also known as a multidentate chelating agent	2. Therefore, it is very effective for the removal of transition and posttransition metal ions
3. Chitosan shows antifungal activity	3. As a consequence of this activity, it not only facilitates promoting metabolic changes in plants but also allows influencing favorably on the development of crops and thus inducing increased germination and better yield
4. Chitosan is a biocompatible, biodegradable, nontoxic, and mucoadhesive polymer	4. These characteristics make chitosan an excellent candidate for a wide range of biomedical applications ranging from drug deliver to tissue engineering to gene delivery
5. Chitosan is a good hemostatic agent. But it is worth to mention that its sulfated derivatives exhibit anticoagulant activity	5. Due to this nature, chitosan aids in stopping bleeding
6. Chitosan shows antimicrobial, antiviral, antitumoral, and immunoadjuvant activity	6. All these interesting characteristics have led to the development of numerous applications of chitosan and its derivatives in biomedicine, such as surgical sutures, biodegradable sponges and bandages, and matrices for the delivery of drugs; in orthopedic materials; and in dentistry, among others
7. Chitosan has high specific binding capacity	7. That is why chitosan is used to remove oils, heavy metals, proteins, and fine particulate matter from waste waters. Apart from these, chitosan can also be used in affinity chromatography and for reducing cholesterol absorption
8. Chitosan shows anti-inflammatory activity	8. The ability of chitosan to inhibit an inflammatory response could be exploited in the nutraceutical industry to provide anti-inflammatory foods for the prevention and alleviation of inflammatory diseases
9. Chitosan shows anti-hypersensitive activity	9. Generally, in humans, high-salt diet increases the systolic blood pressure (SBP) and serum-associated angiotensin-converting enzyme (ACE) activity and chloride ion concentration after 1 h. It is experimentally proved that oral administration of chitosan inhibits these increases

(*continued*)

Table 8 (continued)

Characteristic(s)	Action(s)
10. Chitosan shows hypocholesterolemic action	It lowers the level of cholesterol that is also attributed to the interference with bile acid, a mechanism similar to those of dietary fiber constituents
11. Chitosan exhibits antioxidant property	Due to this property, chitosan is safe and nontoxic. Thus, offering protection from free radicals that retard the progress of numerous chronic diseases

each deacetylated unit (Sajomsang et al. 2009). This strong functionality of chitosan gives it an ample opportunity of chemical modifications. Therefore, various chemical modifications have been introduced to increase not only the water solubility of chitosan but also to chemically modify these groups for the regeneration reactions which give rise to various novel biofunctional macromolecular products having the original organization or new types of organization (Prabhakaran and Mano 2005). The chemical modification of chitosan is a powerful tool to control the interaction of the polymer with drugs and to enhance the load capability to tailor the release profile of the particles (Papadimitriou et al. 2012). Chemically modified chitosan improves its bulk properties for the preparation of sustained release systems. Different ways are there to chemically modify chitosan. Among them, chemical modification through graft copolymerization is quite promising as it provides a wide variety of molecular characteristics. In view of increasing the water solubility of chitosan, amphiphilic derivatives of chitosan have received much attention to the researchers. To prepare such type of compounds, hydrophobic moieties are introduced to the chitosan backbone via different methodologies, namely, alkylation, acylation, carboxylation, graft copolymerization, etc. Many works related to chemical modifications of chitosan have been reported in the literature (Van Luyen and Huong 1996; Kurita 2001; Jenkins and Hudson 2001; Kato et al. 2004; Zhang et al. 2004a; Zohuriaan-Mehr 2005; Muzzarelli and Muzzarelli 2005; Dutta 2013). This section will discuss about some of the chemically modified chitosan.

6.1 Chitosan Salts

The simplest form of chitosan derivative is chitosan salt, which is prepared by dissolving chitosan in an appropriate dilute acid. The most familiar chitosan salt is chitosan acetate (Fig. 8); other available salts are chitosan hydrochloride, lactate, and hydroglutamate. These salts have been extensively studied by researchers and exhibit strong antimicrobial activity against a broad spectrum of microorganism.

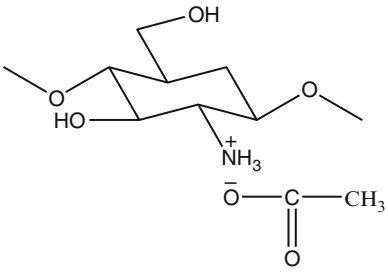

Fig. 8 Chitosan acetate salt

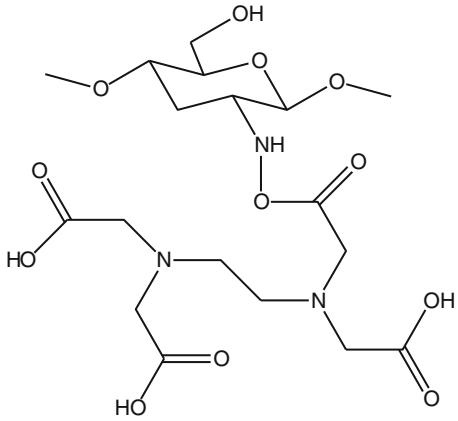

Fig. 9 EDTA-grafted chitosan

6.2 EDTA-Grafted Chitosan

Grafted chitosan presents interesting properties for wound-healing applications, because chitosan derivatives can exhibit enhanced bacteriostatic activity with respect to pure chitosan. EDTA grafted onto chitosan (Fig. 9) increases the antibacterial activity of chitosan by complexing magnesium that under normal circumstances stabilizes the outer membrane of gram-negative bacteria.

6.3 *N*-Carboxymethyl Chitosan/Glycine Glucan

The solubility of chitosan can be improved by carboxylation of chitosan. *N*-carboxymethyl chitosan (NCMC) (Fig. 10) was prepared by dissolving chitosan as a metal-chelating agent in glyoxylic acid solution followed by pH adjustment and reduction with either sodium borohydride or sodium cyanoborohydride. The NCMC was soluble in water at all pH values. The NCMC is also called glycine glucan. The derivative is water soluble and can form insoluble metal chelates after addition to transition metal ion solutions. It has good adsorption capacity for

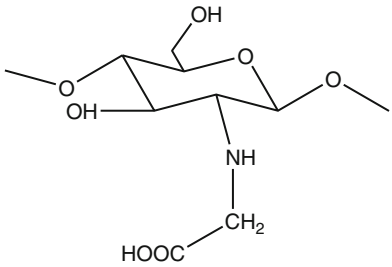

Fig. 10 *N*-Carboxymethyl chitosan/glycine glucan

Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, Co^{2+}, Cd^{2+}, and Uo^{2+} at neutral pH. The incorporation of glycine residues onto chitosan reduces the conformational rigidity of the polymer. Therefore, it shows higher adsorption for metal ions than that of chitosan and fully deacetylated chitosan even though the latter has more free amino groups.

6.4 *N*-Phthaloylated Chitosan

N-phthaloylation of chitosan was expected to be effective for solubilization since it affixes a bulky group to the rigid backbone and breaks hydrogen atoms on the amino groups to prevent hydrogen bonding. Fully deacetylated chitosan was treated with phthalic anhydride in dimethylformamide (DMF) to give *N*-phthaloylchitosan. It was readily soluble in polar organic solvents. The proposed mechanism for the synthesis of phthaloylchitosan is shown in Fig. 11.

6.5 Alkylated Chitosan

The primary amino groups of chitosan undergo a Schiff reaction with aldehydes and ketones to yield aldimines and ketimines, respectively. Further, they are converted to their corresponding *N*-alkyl derivatives by reduction with sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$). A synthesis scheme for the preparation of *N*-alkyl chitosan is shown in Fig. 12 (Bobu et al. 2011). In brief, chitosan was dissolved in 0.1 M acetic acid solution, and then octanal ($C_8H_{16}O$) and reducing agent ($NaBH_3CN$) were added into it under continuous stirring at room temperature. The reaction was allowed to continue for a period of 20–24 h, and then the pH of the resulting solution was adjusted between 8 and 10 with a NaOH solution so as to form precipitation. The precipitate was separated by centrifugation followed by washing initially with ethanol/water mixtures in the volume ratios either 50/50 or 70/30 and finally only with ethanol. After washing, the precipitate was dried at 50 °C under air circulation. The dried *N*-alkyl chitosan was dissolved in 0.1 M HCl solution to obtain its water-soluble salt. The *N*-alkyl chitosan

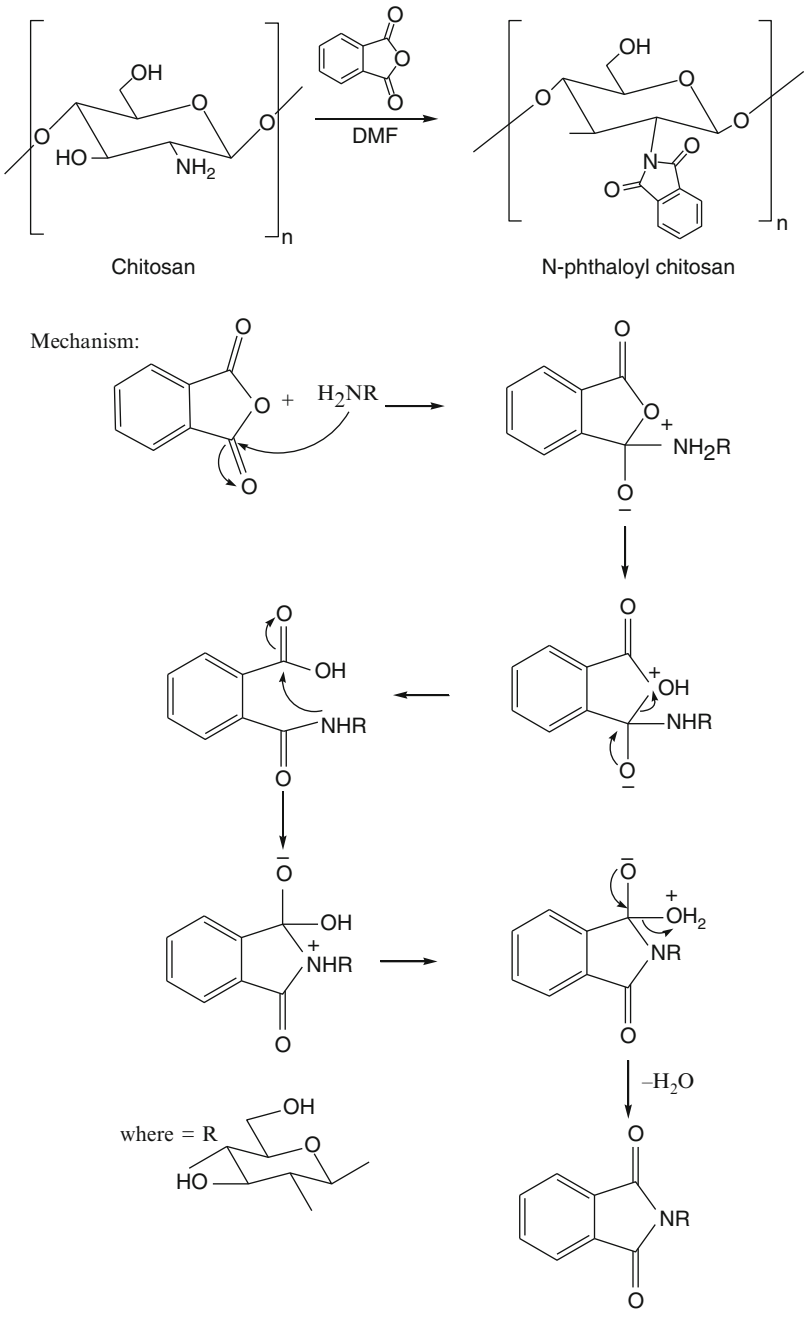

Fig. 11 A plausible mechanism for the synthesis of *N*-phthaloylchitosan

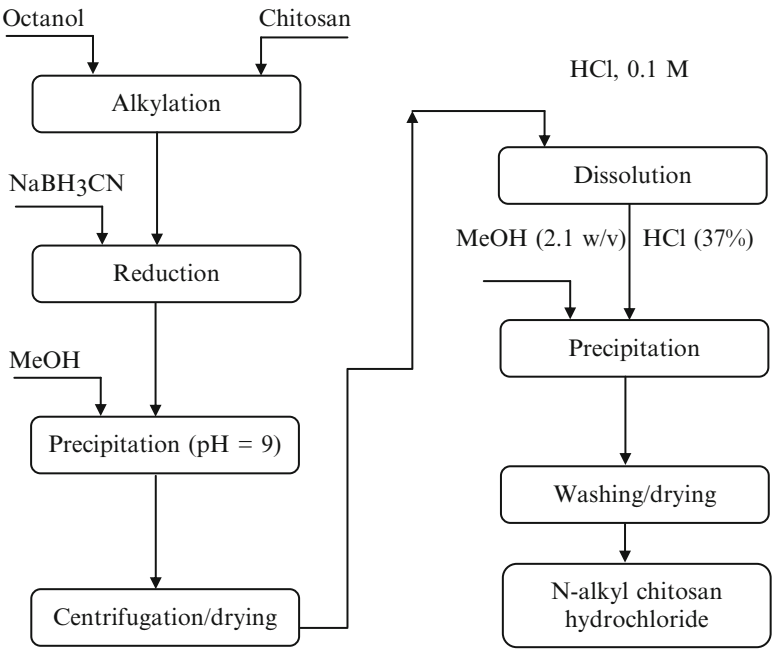

Fig. 12 A synthesis scheme for the preparation of *N*-alkyl chitosan

hydrochloride was precipitated by adding methanol (2:1 v/v) and then HCl (37 %), corresponding to 1.02 mol −L HCl to the final volume. The precipitate was centrifuged and dried afterward so as to obtain the final compound.

6.6 Thiolated Chitosan

According to the method developed by Schnurch et al. (2003) for the chemical modification of chitosan with 2-iminothiolane, Prabhakaran and Mano (2005) reported synthesis of thiolated chitosan. In brief, 500 mg of chitosan was dissolved in 50 mL of 1 % acetic acid. In order to facilitate reaction with thioglycolic acid (TGA), 100 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added to the chitosan solution. After the dissolution of EDAC, 30 mL of TGA was added and the pH was adjusted to 5 with 3 N NaOH. The reaction mixture was stirred and was left at room temperature for 3 h. To eliminate the unbound TGA and to isolate the polymer conjugates, the reaction mixture was dialyzed against 5 mM HCl five times (molecular weight cutoff 10 kD) over a period of 3 days in the dark and then two times against 5 mM HCl containing 1 % NaCl to reduce ionic interactions between the cationic polymer and the anionic sulfhydryl compound. The reaction scheme is shown in Fig. 13.

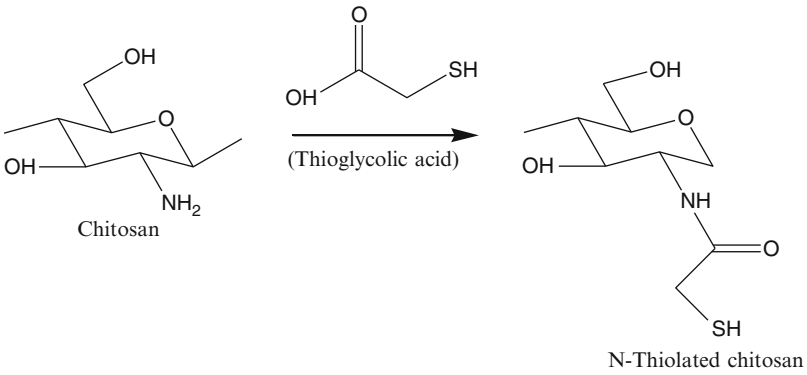

Fig. 13 A synthetic pathway for the formation of *N*-thiolated chitosan

7 Nanotechnological Applications of Chitin and Chitosan

Due to the inherent characteristics of chitin and chitosan such as biodegradability, biocompatibility, non-toxicity, etc., they have found a large variety of biomedical and industrial applications. The development of well-organized carrier systems in the nanoscale range for the biological substances for various biomedical applications has been a major challenge to the worldwide researchers who have been particularly working in the biomedical domain (Arayne et al. 2007; Sahoo et al. 2007; Jong and Borm 2008; Singh et al. 2010; Diebold and Calonge 2010; Wu et al. 2010). In light of developing efficient carrier systems, multifunctional polysaccharide-based nanomaterials combining diagnostic and therapeutic use have recently attracted intensive interests (Meyers et al. 2008). Polysaccharides are a class of biological macromolecules that play important roles in a wide variety of biochemical and biomechanical functions (Andrade et al. 2011). Among the several classes of polymeric materials including polysaccharides that have become the important tools for the biomaterial scientists, chitosan possesses unique properties that have recently been used in a wide variety of biomedical applications. Chitosan also offers possibility of modifying different chemical groups in its structure (Boddohi and Kipper 2010). In order to realize full biochemical and biomechanical potential, biomaterial scientists are exploring ways to engineer chitosan and chitosan-based nanostructures that can be used to tune the response of biological systems, namely, cells, tissues, etc. to these materials (Zhang et al. 2007). Chitosan nanoparticles offer a unique potential not only for clinical and biological applications due to low immunogenicity, low toxicity, and high biocompatibility but also for industrial applications, since due to its polycationic nature, it is capable of binding negatively charged molecules. In this section, chitosan-based nanomaterials and their corresponding applications are summarized in Table 9.

Table 9 Selective chitosan-based nanomaterials and their applications

Chitosan-based nanomaterials	Methods of preparation	Applications	Reference
Chitosan and starch-coated magnetic nanoparticles	Coprecipitation method	To use as a therapeutic modality for treating malignant tumors	Chan et al. (1993), Kim et al. (2009)
Plasmid DNA-loaded chitosan nanoparticles	Complex coacervation method	To use for nasal immunization against hepatitis B	Khateri et al. (2008)
Paclitaxel-loaded chitosan-PEG-cholesterol nanoparticles	–	It is most effective in suppressing tumor growth with a high dose of paclitaxel, while the same at equivalent dose is less effective to suppress tumor growth	Nah et al. (2004)
CS-g-PEG nanoparticles	Ionic gelation method/self-assembly	Protein delivery	Papadimitriou et al. (2012)
Heparinized chitosan/poly(g-glutamic acid) nanoparticles	Self-assembly	To facilitate multifunctional delivery of fibroblast growth factor and heparin	Tang et al. (2010)
Chitosan-dextran sulfate nanoparticles	Self-assembly	To protect protein in an oral insulin delivery application	Sarmento et al. (2007)
Chitosan-cyclodextrin nanoparticles	–	To facilitate nasal drug delivery	Teijeiro-Osorio et al. (2009)
Chitosan-heparin polyelectrolyte complex nanoparticles	–	Protein delivery	Liu et al. (2007)
Chitosan-DNA nanoparticles	–	Liver-targeted gene delivery	Dai et al. (2006)
Monodisperse chitosan nanoparticles	–	Mucosal delivery	Zhang et al. (2004b)
Folate-conjugated pluronic F127/chitosan core-shell nanoparticles	Self-assembly	Breast cancer treatment	Manaspon et al. (2012)
N-succinyl chitosan nanospheres	Self-assembly	Sustained drug delivery system	Aiping et al. (2006)
Magnetic chitosan nanospheres	Coprecipitation followed by ionic gelation	Bioseparation	Hrictu et al. (2009)
PEI-PEG-chitosan copolymer-coated iron-oxide nanoparticles	Chemical coprecipitation	Gene delivery	Kievit et al. (2009)
Folic acid-modified carboxymethyl chitosan nanoparticles	Sonication	Drug carrier for tumor-specific drug delivery	Tan and Liu (2011)

(*continued*)

Table 9 (continued)

Chitosan-based nanomaterials	Methods of preparation	Applications	Reference
Chitosan/chondroitin sulfate/nano-SiO_2 composite scaffold	Lyophilization	Promising for bone tissue engineering	Kavya et al. (2012)
Chitosan-coated ZnS and Zns:Mn^{2+} quantum dots	Radiation technology	Due to low cytotoxicity and high biocompatibility, it could be useful for various biomedical applications	Chang et al. (2011)
Chitosan-coated magnetic nanoparticles for high-efficient cellular imaging	Chemical coprecipitation	These new magneto-fluorescent nanoagents have the potentiality for future medical use	Ge et al. (2009)
Both folic acid (FA)- and methoxypoly(ethylene glycol) (mPEG)-conjugated chitosan nanoparticles	Ionic gelation and chemical cross-linking method	For targeted and prolong anticancer drug delivery system	Hou et al. (2011)
Carboxymethyl chitosan nanoparticles	Ionic gelation method	To inhibit the proliferation of keloid fibroblast	Feng et al. (2011)
Water-soluble chitosan nanoparticles	Ionic gelation	Protein delivery	Chun et al. (2007)
N-hexanoyl chitosan-stabilized magnetic nanoparticles	–	For cellular labeling and magnetic resonance imaging	Bhattarai et al. (2008)
Vitamin C-loaded chitosan nanoparticles	Ionic gelation method	To enhance antioxidant effects because of the continuous release of vitamin C from chitosan nanoparticles in food processing	Jang and Lee (2008)
Chitosan nanorod	Cross-linking method	It is an excellent material as a biosorbent to remove chromium from water	Sivakami et al. (2013)

8 Conclusions

Despite being the second most abundant natural polysaccharide, next to cellulose, chitosan has not yet been completely cherished by the scientific community. Over decades, the deployment of crustacean, mollusk shell waste streams, fungi, etc. for conversion to value-added products has led to significant research and development efforts to investigate innovative uses for chitinous materials via novel modification reactions. In this regard, it is quite noteworthy to mention that depending on the source, various isolation processes (chemical and biological processes), and, obviously, different types of conversion methods, the properties of chitosan are drastically changed that leads to specific applications. In other words, it can be said that one

particular chitosan (depending on its source) used for a particular application may not be suitable for other applications and vice versa. Therefore, it can be suggested that it must be mandatory to mention the source of chitin and chitosan while using chitosan for a particular purpose so as to avoid gross confusion. In view of this, various sources of chitin and chitosan and their extraction processes are explicitly discussed here. Chitosan not only possesses versatile properties but also offers a wealth of biological, biochemical, and biomechanical functionality that can be effectively utilized to develop new biomaterials. In order to understand its full functional potentiality, selective chemical modifications of chitosan as well as various types of chitosan-based nanomaterials along with their possible nanotechnological applications are also discussed. Therefore, it is hoped that this chapter will provide an excellent basis for the researchers that will not only enable them to isolate chitin from various sources but also stimulate them to think in a different way to further conduct research in the new directions by means of nanotechnology for bettering mankind.

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Enzyme-Assisted Extraction of a Marine Algal Polysaccharide, Fucoidan and Bioactivities

37

H. H. Chaminda Lakmal, Ji-Hyeok Lee, and You-Jin Jeon

Contents

Abstract

Gellan gum is an anionic extracellular bacterial polysaccharide identified in 1978 by CP Kelco (San Diego, USA). It is available in two forms in normal commercial production, native gellan gum and deacylated gellan gum. For deacylated gellan gum, acyl groups present in the native polymer are removed by alkaline hydrolysis resulting deacylated polymer which is known generically as "gellan gum." Gellan gum can form gels at low concentrations when hot solutions are cooled in the presence of gel promoting cations which provides it as a texture modifier to control the viscoelasticity in foods, as delivery vehicle in pharmaceuticals, and

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as cell encapsulation material in tissue engineering etc. Gellan gum has been extensively used as a culture medium in microbiology, as a rheology modifier in foods and cosmetics industries since the early stage of its discovery. Recently, gellan gum as a natural polymer and its hydrogel show a wide range of application perspective in drug delivery and tissue engineering. Gellan gum is nontoxic, biocompatible, biodegradable and the resulting hydrogel is transparent and stable. However, gellan gum-based hydrogels have intrinsic defects such as lack of toughness and tissue tolerance as tissue engineering materials that restrict their use in biomedicine field. In order to solve these problems, quite a large number of studies on chemical modification of gellan gum have been carried out. In this chapter, the gelation behaviors, mechanism as well as various modification methods of gellan gum are summarized. Applications of gellan gum and modified gellan gum in food and biomedicine are highlighted.

Keywords
Enzyme-assisted extraction • Sulfated polysaccharides • Fucoidan • Carrageenan • Bioactivity • Antioxidant • Anticancer

1 Introduction

Living organisms contain a vast variety of biomolecules, some of which are important for the survival of living organisms. For example, carbohydrates, lipids, proteins, and nucleic acids are primary metabolites, and these compounds are important in producing essential cellular building blocks, such as cell walls and membranes, DNA, etc., in living organisms. Some biomolecules, known as secondary metabolites, have a much more limited distribution in nature and are encountered only in specific groups of organisms. Secondary metabolites are synthesized for specific reasons: some provide toxic materials that protect certain animals against predators and allow others to catch prey, some are coloring agents that attract other organisms or warn away predators.

Nearly 70 % of the earth's surface is covered by ocean, a rich ecological system containing a vast diversity of marine organisms, microorganisms, vertebrates and invertebrates, higher plants, and algae (Wijesekara et al. 2011). Algae are widely diverse plant species and are classified according to their size into unicellular algae, microalgae, and macroalgae, the latter of which can grow to 100 or 150 f. in length (Witvrouw and De Clercq 1997). Algae are also divided into three main groups according to the presence of various types of pigments in their cells: green algae (Chlorophyceae), brown algae (Phaeophyceae), and red algae (Rhodophyceae) (Wijesinghe and Jeon 2012). Some types of algae are considered edible; therefore, populations in some nations consume these edible algae in their regular diets.

Algal polysaccharides, especially sulfated polysaccharides, are useful materials with health benefits and are considered as functional foods and nutraceuticals. Various protocols are used to extract sulfated polysaccharides from the algal matrix. These procedures include hot-water, ultrasound-assisted, microwave-assisted,

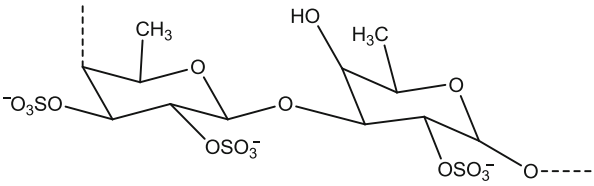

Fig. 1 Repeating unit of fucoidan

and enzyme-assisted extraction (Wijesekara et al. 2011). This chapter primarily discusses enzyme-assisted extraction (EAE) of marine algal sulfated polysaccharides and evaluates their various bioactivities.

2 Sulfated Algal Polysaccharides

Terrestrial plants contain polysaccharides such as celluloses and starches. Marine macroalgae contain quite different types of polysaccharides, which contain a sulfate group in their molecular structure and hence are known as sulfated polysaccharides called carrageenans, xylomannan sulfates, fucoidans, and galactan sulfate. Carrageenans are isolated from red seaweed and contain kappa- (κ-) and lambda- (λ-) carrageenan. *Eucheuma cottonii*, a red alga, is a source for κ-carrageenan isolation (Witvrouw and De Clercq 1997). Kappa-carrageenan consists of sulfated D-galactose and 3,6-anhydro-D-galactose in approximately equimolar amounts (Rosa 1972). Lambda-carrageenan is extracted from *Gigartina aciculaire* and *G. pistillata* and contains sulfated D-galactose. Identification of κ-carrageenan and λ-carrageenan is based on the position of the sulfate group and the presence or absence of anhydrogalactose. A repeating unit of 4-sulfate-β-galactopyranosyl (1 → 4)-linked-3,6-anhydro-α-D-galactose is found in κ-carrageenan. In addition, (1 → 3)-linked disaccharide of 2-sulfate-β-D-galactopyranosyl (1 → 4)-α-D-galactose-2, 6-sulfated is the repeating unit of λ-carrageenan (Witvrouw and De Clercq 1997).

Among these sulfated polysaccharides, fucoidans have been well studied over the past 100 years. Fucoidans are found in brown seaweeds and sea cucumbers. They contain a substantial percentage of the L-fucose and sulfate groups and exist as l-fucose-4-sulfate. Fucoidan polymers show special pharmacological activities due to the presence of the sulfate ester group (Yu et al. 2014; Yang et al. 2014). Fucoidans have been reported for a broad spectrum of bioactivities due to their antioxidant, anti-inflammatory, antitumor, antiproliferative, anticoagulant, and antiviral properties (Zhu et al. 2010). Figure 1 shows the repeating unit of fucoidan.

3 EAE of Algal Polysaccharides

Marine algae synthesize various metabolites, including primary and secondary metabolites, for their survival, and some exist for special reasons, such as defense mechanisms. These algal materials have for the potential for human benefits.

Some compounds extracted from marine algae are used in the pharmaceutical, food, and cosmetic industries. Some compounds have excellent pharmacological properties and provide promising pharmacological efficacy. Hence, most natural product research laboratories and researches tend to find novel natural compounds from plant materials, especially from marine algae. Marine alga is a reliable source that satisfies the increasing demand for natural marine bioactive compounds. However, the amount and availability in plant cells of these useful bioactive compounds is very low (Puri et al. 2012).

To isolate these small quantities of useful compounds from the plant matrix, several types of extraction protocols have been adopted: organic solvent, supercritical, microwave-assisted, ultrasound-assisted, cold press, and EAE (Herrero et al. 2006; Puri et al. 2012). In relation to the isolation of various bioactive compounds from plant matrixes, extraction protocols play the most important role in the process. An ideal extraction protocol should be quantitative, nondestructive, and time efficient (Yang et al. 2011). Some conventional protocols, such as water, hot-water, and organic solvent extraction, have several drawbacks: they are time consuming and laborious, have low selectivity and low extraction yield, and employ toxic solvents that produce environmental pollution (Herrero et al. 2006). Also, organic solvent extraction techniques have limited use in the food industry due to their safety hazards and toxic effects, as traces of organic solvents remain after the extraction process, ultimately decreasing product quality. Furthermore, extraction yields and efficiencies of bioactive compounds are reduced due to plants' cell walls. Therefore, there is great demand for useful, optimized, nontoxic, environmentally friendly extraction protocols to safely recover a higher yield from plant material (Puri et al. 2012).

EAE is an environmental friendly extraction process to which researchers are paying increased attention. During this process, cell walls and membranes are disrupted by particular enzymes, thus releasing bioactive components into the medium (Puri et al. 2012) in higher yields.

Several types of enzymes are used for the extraction of polysaccharides from marine algae. Each enzyme shows the highest efficiency under optimum conditions (Heo et al. 2005) (Table 1). Some of these enzymes are derived from microorganisms for commercial production and are available in markets. According to its commercial producer, Novozymes®, Viscozyme is produced by a select strain of the fungus *Aspergillus aculeatus*. Viscozyme is a mixture of carbohydrases, including cellulase, β-glucanase, hemicellulase, and xylanase. Viscozyme assists in the extraction of cell-wall materials by breaking down pectin-like substances in the algal cells. Celluclast is an enzyme of cellulase produced by a selected fungal strain, *Trichoderma reesei*. It catalyzes the breakdown of cellulose in algal cells into glucose, cellobiose, and longer glucose polymers. Celluclast shows optimum activity at pH 4.5–6.0 and temperatures between 50 °C and 60 °C. The commercial enzyme AMG is a type of amyloglucosidase isolated from *A. niger*. AMG breaks down starches consisting of the 1,4 and 1,6 linkages. The commercial enzyme Termamyl is a type of heat-stable α-amylase isolated from a genetically modified bacterial strain of *Bacillus licheniformis*. Termamyl hydrolyzes starch at

Table 1 Commercially available carbohydrases: origins, enzyme compositions, and optimum digestion conditions

Name of the enzyme	Origin[a]	Optimum condition		Enzyme composition
		pH	Temperature	
Viscozyme	*Aspergillus aculeatus*	4.5	50	Arabinose, cellulase, β-glucanase, hemicellulase, xylanase
Celluclast	*Trichoderma reesei*	4.5	50	Cellulase
AMG	*Aspergillus niger*	4.5	60	Exo-1,4-α-glucosidase
Termamyl	*Bacillus licheniformis* (genetically modified)	4.5	60	Heat-stable α-amylase
Ultraflo	*Humicola insolens*	7	60	Heat-stable, multiactive β-glucanase

[a]Origin of enzymes according to commercial producer, Novozymes (Adapted from Heo et al. 2005)

1,4-α-glycosidic linkages, and its optimum activity is observed at pH 7.0 and 90 °C. Ultraflo is a heat-stable, multiactive β-glucanase isolated from the fungus *Humicola insolens*.

Crude polysaccharide was extracted from the seaweed *Ecklonia cava* by Athykorala et al. using enzymatic hydrolysis and evaluated for antiproliferative and antioxidant activity (Athukorala et al. 2006b). During the study, lyophilized *E. cava* was ground and sifted through a 50-mesh standard testing sieve to obtain small particles. Since surface areas contacted by enzymes increase when the algal particle size is smaller, this method produces higher extraction yields. Water is added to ground algal samples to produce the desired medium, and a desired enzyme is added to digest algal samples at optimum pH and temperature. Some studies report that the desired extraction time is 12 h (Athukorala et al. 2006a; Wijesinghe et al. 2011) whereas others indicate 24 h (Kang et al. 2011; Lee et al. 2011).

In EAE processing, reactants are shaken during the extraction process to increase extraction efficiency under the mixing of enzymes and substrate algae. This increases the collision of enzyme and substrate algae. The enzyme is inactivated by boiling at 100 °C for 10 min to stop further hydrolysis (Athukorala et al. 2006a). The extract is centrifuged at 3,000 rpm for 20 min at 4 °C, and unwanted cell debris and unhydrolyzed residue are deposited as a pellet at the bottom and discarded in the final processing step (Heo et al. 2005). The filtered supernatant is subjected to ultrafiltration and passed through several filters with different membrane pores sizes, such as 5, 15, and 50 kDa, and the extracted water-soluble sulfated polysaccharide is separated according to molecular sizes (Kang et al. 2011; Siriwardhana et al. 2004). Ethanol is added to each filtrate after concentration and the crude polysaccharide is precipitated. Most sulfated polysaccharides have a negative charge due to the sulfate group in their structure. Dielectric constant is reduced when adding ethanol, which is a less polar solvent

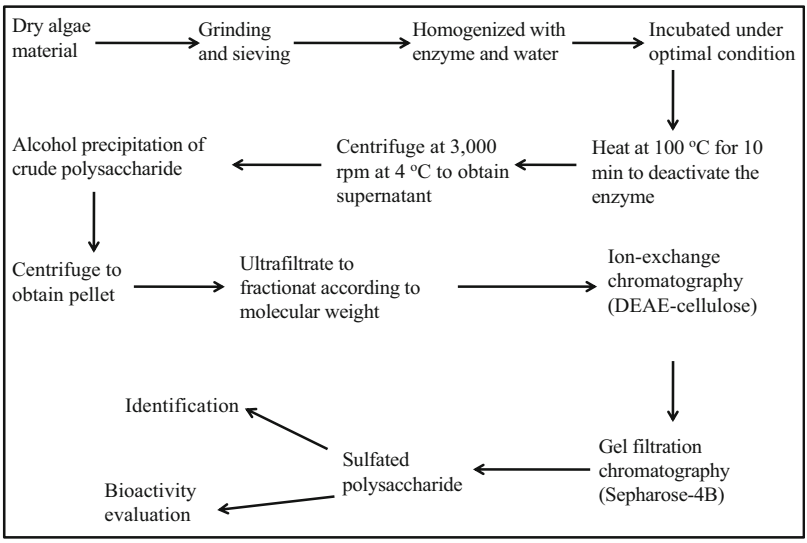

Fig. 2 Preparation, purification, and isolation of sulfated polysaccharides

in contrast to water. Negatively charged sulfated polysaccharide is formed by the ionic bond with cations available in extract, and the sulfated polysaccharide is precipitated. The precipitated extract is centrifuged at 10,000 rpm for 20 min at 4 °C to recover as much as possible of the precipitated sulfated polysaccharide (Kang et al. 2011; Chihara et al. 1970). Figure 2 shows the essential steps involved in all extraction procedures for the isolation and purification of sulfated polysaccharides.

4 Purification and Isolation of Algal Polysaccharides

Sulfated polysaccharides are negatively charged molecules that hence can be separated using ion-exchange chromatography. Diethylaminoethyl cellulose (DEAE-cellulose), a positively charged resin, is used to separate sulfated polysaccharides. Sulfated polysaccharides in crude polysaccharide extract are separated while running through the resin according to the affinity between anionic resin and sulfated polysaccharides. The column is eluted with different types of buffer systems with different pH values, and fractions are collected into small containers such as test tubes. Polysaccharide content is quantified by phenol–sulfuric acid (H_2SO_4) carbazole reaction to identify polysaccharide distribution throughout the column-eluted fractions (Athukoral et al. 2006).

Gel-filtration chromatography is another method for the isolation and purification of polysaccharides depending on their molecular weights. Sepharose is a chromatographic material used for the separation process. Sepharose is a bead consisting of agarose-based crosslinked materials and is available in agarose

contents 2, 4, and 6 % and named 2B, 4B, and 6B, respectively. They have different pore sizes depending on the available agarose amount. The Sepharose column is eluted with water after loading the desire polysaccharide sample and is separated according to sizes. The polysaccharide content is quantified by phenol–H_2SO_4 carbazole reaction to identify the polysaccharide distribution throughout the column-eluted fractions (Kang et al. 2011).

5 Identification of Algal Polysaccharides

Infrared (IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy techniques are applied for molecular identification. IR spectroscopy use electromagnetic radiation at the infrared region and IR radiation is associated with the bond vibration of the molecule. The molecular bond is vibrated after absorbing the frequency specific for the particular molecular bond. This allows researchers to identify the functional groups in the molecular structure depending on the absorbed frequency. Melo et al. reported an identified polysaccharide from *Gracilaria cornea* and characterized the sulfated polysaccharide using IR spectroscopy (Melo et al. 2002). NMR spectroscopy is used for characterizing molecules and promises to provide the accurate molecular structure. Melo et al. used NMR spectroscopy to characterize the isolated sulfated polysaccharide from *G. cornea* (Melo et al. 2002). Ruperez et al. studied an edible brown algae *Fucus vesiculosus* to isolate sulfated polysaccharide and used IR and NMR spectroscopic to determine the structure (Ruperez et al. 2002).

6 Bioactivities of Algal Sulfated Polysaccharides

Sulfated polysaccharides possess a broad spectrum of essential bioactivities, such as antioxidant, anti-inflammatory, anticancer, and other bioactivities (Table 2).

7 Antioxidant Activity

Usually, cellular aerobic respiration contributes to the production of reactive oxygen species (ROS) such as superoxide, hydroxyl, peroxyl, alkoxyl, hydrogen peroxide, and singlet oxygen, as well as reactive nitrogen species (RNS), including peroxynitrite, which are formed by reaction between nitric oxide and superoxide in living cells in an oxygen-rich environment. Exogenous factors, such as ultraviolet (UV) radiation, smoking, and metabolites of foreign materials, also contribute to the production of ROS (Yoon et al. 2011). ROS have a greater potential to damage cellular components such as DNA, proteins, and lipids and cause inflammatory lesions and, furthermore, diseases such as cancers, aging, arteriosclerosis, rheumatoid arthritis, and allergies. Recent studies have reported that the sulfated polysaccharide isolated from marine algae show antioxidant properties. Several types of

Table 2 Selected bioactivities of marine algal sulfated polysaccharides

Sulfated polysaccharide	Source	Biological activity	References
Fucoidan	*Laminaria japonica*	Angiogenesis induction	Kim et al. 2014a
Porphyra	*Porphyra yezoensis*	Apoptosis induction in gastric cancer cells	Kwon and Nam 2006
Fucoidan	*Laminaria japonica*	Thrombin-induced platelet aggregation	Zhu et al. 2010
Crude extract	*Ecklonia cava*	Anticoagulant activity	Athukorala et al. 2006a
Fucoidan	*Ecklonia cava*	Anti-inflammatory activity	Kang et al. 2011
		Anticoagulant effect in Wistar rats	Wijesinghe et al. 2011
		Anti-inflammatory activity	Lee et al. 2012
		Anti-inflammatory activity in zebrafish	Lee et al. 2013
		Protective antioxidant activity in zebrafish	Kim et al. 2014b
		Antiproliferative activity on cancer cells	Athukorala et al. 2006b
		Antioxidant property	
Crude extract	*Sargassum hornei*	Antioxidant property	Heo et al. 2005
Fucoidan	*Saccharina japonica*	Improve innate immunity in yellow-fin catfish	Yang et al. 2014
	Sargassum horneri	Antioxidant property in yellow fin catfish	
	Fucus vesiculosus		
Lambda carrageenan	*Gigartina acicularis*	Antioxidant property	Souza et al. 2007
	Gigartina pistillata	Antioxidant property	
Fucoidan	*Padina gymnospora*	Antioxidant property	

assays, such as chemical assay, electron spin resonance (ESR) spectroscopy, and cell culture technique are used to evaluate the antioxidant property of the desire sulfated polysaccharide. Different types of ROS species were used to identify antioxidant sulfated polysaccharides, such as 1,1,-diphenyl-2-picrylhyrazyl (DPPH), hydrogen peroxide, hydroxyl, and alkyl. Siriwardana et al. reported antioxidant activity of the enzymatic extract of *Hizikia fusiformis*. The five carbohydrases were used to extract the crude polysaccharides, and antioxidant activity was evaluated by chemical assay, including DPPH and hydrogen peroxide. All enzyme hydrolysates were shown to have $>$ 70 % DPPH radical scavenging ability at 2 ml extract and enzyme hydrolysates of AMG, Termamyl, and Ultraflo exhibited

strong scavenging abilities for hydrogen peroxide (Siriwardhana et al. 2004). Heo et al. screened carbohydrate hydrolyses of seven brown seaweeds (*E. cava*, *Ishige okamurae*, *Sargassum fulvellum*, *S. coreanum*, *S. thunbergii*, *and Scytosiphon lomentaria*) for radical scavenging activity using different ROS, including DPPH, superoxide anion, hydroxyl, and hydrogen peroxide. Hydrogen peroxide scavenging activity was more prominent than for other ROS species, and Ultraflo enzyme hydrolytic extract of *S. horneri* indicated the highest activity in a dose-dependent manner (Heo et al. 2005). Moreover, fucoidan is well studied and well characterized: Kim et al. used fucoidan from extracted *E. cava* by digestion with Celluclast, and in vitro antioxidant activities were evaluated using the ESR technique, as well as on Vero cells (monkey fibroblast kidney cells). Also, in vivo antioxidant activity was evaluated in zebrafish in which oxidative stress was induced using 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH). According to the results with ESR, IC_{50} values were 0.73 mg/ml^{-1} for DPPH and 0.48 mg/ml^{-1} for peroxyl radicals. Intracellular ROS scavenging activity was done using 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay on Vero cells; IC_{50} value was 75 μg/ml^{-1}. Furthermore, in vivo antioxidant activity was investigated using a zebrafish model; results showed a higher survival rate in fucoidan-treated zebrafish and a reduction of ROS levels compared with the AAPH-treated group (Kim et al. 2014b). De Souza et al. evaluated the antioxidant capacity of several sulfated polysaccharides, including iota- (ι-), κ-, and λ-carrageenan, as well as fucoidan, and then the IC_{50} values were 0.058, 0.112, 0.332, and 0.046 mg/ml^{-1} of fucoidan, κ-, ι-, and λ-carrageenan, respectively (De Souza et al. 2007).

8 Anticancer Activity

Cancer is caused by abnormal cell growth and has become a major public health problem around the world (Jemel et al. 2008). Chemotherapy is a heavily used treatment for cancer. However, chemotherapeutic drugs are toxic to somatic cells and exhibit serious adverse effects. There is a greater demand for natural nontoxic anticancer therapies (Anastyuk et al. 2012). Fucoidan is a well-studied sulfated polysaccharide, and Athukorala et al. reported that the sulfated polysaccharide isolated from the enzymatic extract of *E. cava*, specially fucoidan, exhibited antiproliferative effects on murine colon carcinoma (CT-26), human leukemia monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60), and mouse melanoma (B16) cell lines in a dose-dependent manner (Athukorala et al. 2006b; Athukorala et al. 2009). Sulfated polysaccharides are the main components of the porphyrins. They showed anticancer activity on human gastric adenocarcinoma cancer cells (AGS) by inhibiting DNA synthesis. They then reduced cancer cell growth and induced apoptosis (Kwon and Nam 2006). Enzyme-digested fucoidan extract from *Cladosiphon novae-caledoniae* Kylin also produced antitumor activity on human fibrosarcoma HT-1080 cells by increasing oxidative stress by ameliorating intracellular ROS levels and suppressing the expression of angiogenesis. It also suppressed the secretion angiogenesis factor for

vascular endothelial growth factor (VEGF), resulting in the inhibition of vascular tube formation of tumor cells (Ye et al. 2005).

Antiangiogenic therapy is another effective treatment for tumor inhibition. Liu et al. reported that fucoidan derived from *Undaria pinnatifida* significantly inhibited cell proliferation and migration and tube and vascular network formation on human umbilical vein endothelial cells (Liu et al. 2012). Zhang et al. reported enhanced anticancer activity of the chemotherapeutic agents cisplatin, tamoxifen, and paclitaxel by fucoidan extracted from *C. novae-caledoniae* Kylin after digestion with glycosidases (Zhang et al. 2013). Furthermore, the study mentioned that the combination of fucoidan and chemotherapeutic agents induced cell growth inhibition, apoptosis, and cell cycle modifications in MDA-MB231 and MCF-7 breast cancer cells.

9 Anti-inflammatory Activity

Inflammation is a bodily response to pathogenic bacteria, viruses, and other pathogens, being a defensive response generated by the host (Kang et al. 2011). Some host-originated inflammatory mechanisms worsen a disease (proinflammatory), but some anti-inflammatory activities help heal the disease processes. Some host-originated anti-inflammatory mechanisms cause harmful effects to the host, such as fever, inflammation, and tissue destruction (Dinarello 2000). Therefore, there is a greater demand for anti-inflammatory drugs. Many studies are ongoing to identify natural compounds with anti-inflammatory activity. Natural marine products play an important role in this field of natural medicinal products. Recent studies show that many researchers have isolated sulfated polysaccharides with anti-inflammatory activity from marine algae. One study reported that the purified polysaccharide, fucoidan, isolated from AMG-assisted extract of *E. cava*, exhibited anti-inflammatory activity on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells by inhibiting nitric oxide (NO) production, and prostaglandin E2 production via inhibition of NO synthase (iNOS) and cyclooxygenase-2, respectively (Kang et al. 2011). Another study reported the isolation of fucoidan by enzymatic extraction and purification using ion-exchange chromatography. The isolated fucoidan exhibited a significant inhibition of NO production on LPS-stimulated RAW 264.7 cells and down-regulated proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and IL-1β (Lee et al. 2012). Evaluating bioactivity on zebrafish is the latest technique among in vivo activity assays. It is an advance technique and gives promising results. A recent study in a zebrafish model reported that fucoidan isolated from *E. cava* has anti-inflammatory effects (Lee et al. 2013): according to the study, tail-cutting and LPS treatment significantly increased ROS levels and NO production; fucoidan administration significantly inhibited NO production and reduced ROS levels. In addition, fucoidan showed protective effects in zebrafish embryos in which inflammation was induced by LPS treatment. These results imply that fucoidan is a potential candidate for developing anti-inflammatory drugs.

10 Other Bioactivity

Angiogenesis is the important physiological process that produces new blood vessels from existing blood vessels during some physiological processes, such as reproduction, development, and tissue repair. Kim et al. evaluated the effect of fucoidan isolated from *Laminaria japonica* on human umbilical vein endothelial cells (HUVECs). The observed results suggested that fucoidan was capable of inducing the cell proliferation and migration and tube formation (Kim et al. 2014a). Furthermore other studies reported that sulfated polysaccharide isolated from marine algae has anticoagulant properties: Wijesinghe et al. reported that sulfated polysaccharide isolated from *E. cava* showed anticoagulant properties in both in vitro and in vivo studies (Wijesinghe et al. 2011). In that study, fucoidan isolated using AMG-assisted extraction was evaluated in Wistar rats. Both in vivo and in vitro assays were used to evaluate activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT). The results indicated that fucoidan inhibited coagulation in a dose- and time-dependent manner.

11 Conclusion

The EAE technique is an environmentally friendly and effective method for isolating sulfated polysaccharides from algal matrix. Marine algal sulfated polysaccharides have tremendous pharmacological activities, such as anticancer, antioxidant, anti-inflammatory, anticoagulant, and other bioactivities. Research will open new pathways to new natural pharmacological compounds for various medicinal purposes.

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Microwave-Synthesized Polysaccharide Copolymers

38

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Contents

Abstract

Known since the end of the nineteenth century, microwave radiation (Zlotorzynski 1995) was used to carry out a chemical reaction for the first time in the 1960s, but in these experiments, irradiation was used for the production of electric arc discharge only in which some organic transformation has been carried out (Streitwieser and Ward 1962). The very first "microwave synthesis," in full sense of the word, was performed in the 1980s of the last century, when Gedye et al. (1986) conducted the microwave-assisted hydrolysis of amides in a substantially shorter time than the corresponding reaction at conventional conditions. A few years later, in 1992, Mingos showed the possibility of heating liquid beyond their boiling points at microwave conditions

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(Mingos and Baghurst 1991). The experiment was a milestone in the development of microwave synthesis. Since then a sharp increase is microwave synthesis in organic synthesis observed in interest in many parts of chemistry of both low and high molecular compounds.

Keywords
Copolymerization • Microwave • Polysaccharide

1 Introduction

Known since the end of the nineteenth century, microwave radiation (Zlotorzynski 1995) was used to carry out a chemical reaction for the first time in the 1960s, but in these experiments, irradiation was used for the production of electric arc discharge only in which some organic transformation has been carried out (Streitwieser and Ward 1962). The very first "microwave synthesis," in full sense of the word, was performed in the 1980s of the last century, when Gedye et al. (1986) conducted the microwave-assisted hydrolysis of amides in a substantially shorter time than the corresponding reaction at conventional conditions. A few years later, in 1992, Mingos showed the possibility of heating liquid beyond their boiling points at microwave conditions (Mingos and Baghurst 1991). The experiment was a milestone in the development of microwave synthesis. Since then a sharp increase is microwave synthesis in organic synthesis is observed in many parts of chemistry focusing on both low and high molecular compounds.

In contrast to the conventional synthesis (transport of energy into the reaction mixture by means of conduction and convection, i.e., from the source to the reaction mixture), distribution of microwave energy which occurs from within the heated sample has at least several advantages such as:

(a) Direct supply of the energy to the system, while the reaction mixture has no contact with source of heat
(b) Volumetric heating method, leading to a much larger heating rate as compared with conventional methods
(c) Different temperature distribution (reverse temperature gradient)
(d) For heterogenic mixtures, selective heating compounds susceptible to the effects of microwave radiation
(e) Possibility of heating liquids to temperatures much above their boiling temperature (from a few to several degrees)

1.1 The Nature of Microwaves

Microwave radiation is part of electromagnetic radiation from the range of 300 MHz to 30 GHz, which is equal to a wavelength of 1–0.01 m (Kappe et al. 2009). According to definition, it is a kind of energy propagating in space with the speed of light as a

Table 1 Comparison of the radiation energy delivered by the different type of interactions

Interactions	Energy, eV
μν	1.24×10^{-6}–1.24×10^{-3}
C–C dissociation (Morrison and Boyd 1992)	4.0
Hydrogen bonding (Atkins and De Paula 2010)	2.1
van der Waals interactions (Coulson and McWeeny 1979)	2.0
Brownian motion (Morrison and Boyd 1992)	2.70×10^{-3}

form of periodic variation of the electric and magnetic field (Knight 2013). Energy transmitted through this type of radiation is about 1.24×10^{-6}–1.24×10^{-3} eV (calculated using Planck relation: $E = h{\cdot}\nu$) (Berlan 1995). Comparing those values to the power of intra- and intermolecular interactions shown in Table 1, one should note that microwaves cannot be an activating force in organic reactions, because the amount of energy supplied together with the radiation is comparable to Brownian motion only. According to that one can see that interaction of microwave irradiation with matter had to have a complete different character. In fact, it is a result of the reorientation of charged particles of matter (ions, dipoles, etc.) in pulsed electromagnetic field of high frequency; however other mechanisms including so-called nonthermal effects are postulated as well.

Going into details we can say that for dielectrics, if the external static electric field is stopped at the time $t = 0$, the polarization caused by the field is going to diminish exponentially (Davies 1965). The time needed to establish the polarization at the level of 1/e of the initial value describes the relaxation time. Like the constant electric field, the application of alternating fields may cause the phenomenon of polarization in the sample. When acting on the matter, the alternating electromagnetic field with microwave frequencies, dipole reorientation may occur slower than changes in the field. In the extreme case, it may lead to a situation where the dipoles can be oriented contrary to the operating electric field. Such a phenomenon results in energy absorption, and hence, substantial temperature increase of the whole volume of the system is observed. The rate of reorientation of the dipoles is a characteristic of the substances and may be varied with the frequency of irradiation and temperature. As a result different amounts of heat are generated by the system. This energy may be easily estimated using the parameter of dielectric loss factor ε'' – see Eq. 1:

$$\varepsilon'' = \frac{\varepsilon'_0 - \varepsilon'_\infty}{1 + \omega^2 \cdot \tau^2} \omega \cdot \tau \tag{1}$$

where ε'_0 is called static dielectric permittivity, ε'_∞dielectric permittivity in the electromagnetic field with frequencies in visible light range, ω electromagnetic field frequency, and τ relaxation time (Kingston and Haswell 1997).

The mechanism of converting electromagnetic radiation energy into heat for conductors (metals, metal oxides, graphite, other types of carbon, etc.) is substantially different from that described for dielectrics. This is due to the presence of free electrons with conductive material that are nonpermanently linked with specific atomic

structures. Under the influence of an applied electric field, the electrons can move along the guide in the direction of the applied external field. The observed energy dissipation occurs when collisions with other conduction electrons or themetal ions in the atomic network of crystal material takes place (Kingston and Haswell 1997).

Another factor that may be responsible for increasing of the temperature in systems consisting of at least two polarizable components may be interfacial polarization, i.e., the Maxwell–Wagner effect (Baghurst and Mingos 1992). Heating effect in such systems is the result of the differences in dielectric permeabilities and conductivity of the individual components of the system. Observed dielectric properties (dielectric permittivity) of the medium, after the introduction of the second component of described mixture, may increase up to tenfold, which has a substantial effect on the rate of temperature increase in the mixture, when irradiated by microwaves.

1.2 Influence of the Microwaves on Chemical Transformations

Reactions under microwave irradiation allow significant reduction in the total time of synthesis, modification, or distribution of products and also increase in the overall yield of the reaction. Increase in microwave-assisted process efficiency can be explained using the discussed relaxation processes occurring in dielectrics (dielectric dispersity) and conductivity, leading to rapid heating and bulk of reaction mixture, i.e., thermal effects of microwave action. Additionally some specific microwave effects with nonthermal origin have been postulated as well.

1.2.1 Specific Thermal Effects

Microwave thermal effects are directly related to the volumetric heating of the reaction mixtures subjected to microwave irradiation. Considering the effects of this kind, usually one can focus on much faster achieving of appropriate temperature of the irradiated system, as well as efficient mixing of the reactants in the reaction medium and the effect of interfacial diffusion (Kingston and Haswell 1997). In general the heating rate of the mixture irradiated using microwaves may be described as follows (Kingston and Haswell 1997):

$$\frac{\delta T}{\delta t} = \frac{\omega \cdot \varepsilon_0 \cdot \varepsilon'' \cdot E^2}{\rho \cdot C_p}$$

where:

ω – field frequency
ε_0 – dielectric permittivity
ε'' – loss factor
E – electromagnetic field
ρ – density of the mixture
C_p – specific heat

Factors appearing in the equation as dielectric losses and specific heat are temperature dependent. This causes the complete solution to the mathematical problem of the temperature rise of the substance subjected to microwave irradiation to be very complicated.

But the most important factor that is responsible for the acceleration of reactions carried out under microwave conditions seems to be the possibility of achieving temperatures much higher than in conventional conditions. Such a phenomenon can occur in the whole volume of the mixture or solution (due to overheating) (Berlan 1995) or in certain locations only (called hot spots) (Zhang et al. 1999).

Nucleation of gas bubbles, which takes place during the boiling of the liquid, most often occurs on the inner surface of the vessel of heated liquid. During a conventional process when the temperature rises, the bubbles can expand as the process of evaporation will occur at a place where the surface tension is lower and with minimal inner energy. When using microwave radiation, the surface of the reaction vessel does not heat up and is lower than the temperature of the heated liquid. In such a system boiling can occur only if the energy delivered is able to exceed the intermolecular interactions in the liquid. The observed higher boiling point in microwave processes is reached only when the solution is not stirred or in the absence of additional nucleating sources (Baghurst and Mingos 1992). The observed boiling temperature is higher than a few to a few tens of degrees (100 °C vs. 105 °C at μν for water, 82 °C vs. 120 °C for acetonitrile, or 67 °C vs. 103 °C for THF) (Perreux and Loupy 2001; Kingston and Haswell 1997).

The phenomenon of overheating may occur on a local scale also. Subjecting the liquid or solid to microwave irradiation results in local hot spots. Temperature gradients in such mixtures may be up to several dozens of degrees, which enables local reaction rate constants to increase several times. Observation of hot spots, however, is extremely complicated, and their existence has not been conclusively proven.

1.2.2 Nonthermal Effects

The rate of chemical reactions is described using the Arrhenius equation, in which the rate constant of the process depends among others on the activation energy and factors hidden in a pre-exponential coefficient. The electromagnetic field with microwave frequencies can, as it turns out, affect both of these factors, thereby altering the rate of reaction.

Pre-exponential coefficient depends, mainly, on the probability of particles collisions in a chemical reaction. It is postulated that microwave radiation may be influenced on this factor, increasing the possibility of intermolecular collisions of reagents (Langa et al. 1997).

On the other hand, the free enthalpy of activation (defined as $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$), in a field at frequencies in the microwave range, can decrease, and this is due to increased ordering in the system, resulting from polarization. The phenomenon leads to an increase in the entropy factor ($T\Delta S^{\neq}$) (Lewis et al. 1992).

It is generally accepted that nonthermal, microwave-specific effects depend on the reaction mechanism, especially since the composition and polarity of the transition state complex (Loupy 2003).

2 Radical Processes Under Microwave Condition

Nowadays, there is growing interest in organic syntheses mediated by free radicals (Tōgō 2004). The methodology has been adapted in thousands of chemical transformation with both low molecular or high molecular (polymer) compounds (Moad et al. 2006). The very first stage of those processes, i.e., initiation of free radicals, may be generated from unstable compounds using different mechanisms including homolytic dissociation after irradiation with electromagnetic field (visible or ultraviolet light) or using chemical processes. In fact, in most cases an introduction of an initiator is needed to start the process. The initiator serves as a source of primary radicals that are generated using several techniques including thermal processes. Because the microwave irradiated may serve as an excellent "heat generator" (especially in the case of polar reaction systems), there are also a growing number of research dealing with employing of microwaves in free radical organic synthesis.

According to that one can state that the general advantage of microwave-assisted radical processes is allowing a reaction mixture to heat rapidly (very often beyond its boiling point). The reduction of total reaction time as well as simple reaction setup and increase in product yield may also be treated as a benefit of the protocol (McBurney et al. 2006). In order to enhance the reaction efficiency or enhance the impact of microwaves on the process, systems containing polar solvents are usually in use. In cases in which low or nonpolar solvents are needed, the heating effect may be obtained using strongly microwave-absorbing additives.

As it was discussed above, the energy linked with microwave irradiation is too low to make any significant changes in chemical bonds. As a result the thermally initiated radical processes are the most common. Next stages of the process include propagation and termination which are also microwave sensitive. In the case of propagation, the high temperature, e.g., overheating that may be observed locally or globally is a key factor for increasing the rate of this process stage. On the other hand, the influence of microwaves on termination is much lower. It is a result of the low activation energy of this step. However in the case of a sterically overcrowded recombination or disproportionations, the microwave effect may be more significant (McBurney et al. 2006).

The microwave grafting techniques allow formation of free radicals on the polysaccharide backbone by means of two different methodologies: microwave-initiated technique that uses microwave irradiation only to initiate grafting reaction or techniques that use a combination of microwaves and chemically initiated free radicals for grafting reaction.

3 Graft Copolymerization

First graft copolymer was synthesized by Alfrey and Bandel in 1950 (Alfrey and Bandel 1951). They polymerized vinyl acetate in the presence of styrene and vinylidene chloride. This idea of graft copolymers was promoted by H.F. Mark; in 1953 he has announced the new graft copolymers (Mark 1953). Generally graft

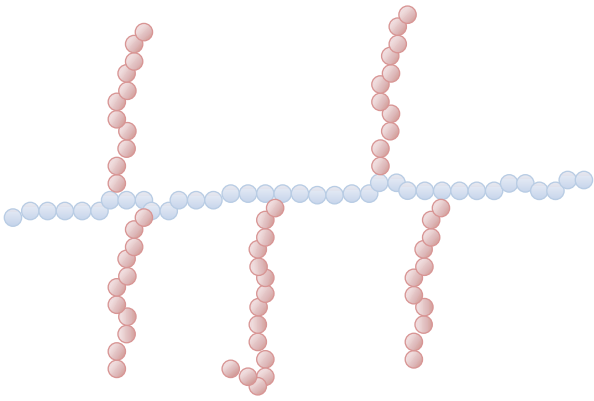
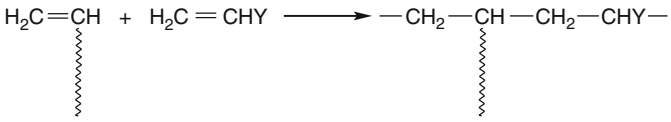


Fig. 1 Structure of typical grafted polymer

copolymers consist of a preformed polymeric linear backbone of one composition to which other polymeric chains are randomly attached. There is no general rule, but both the backbone and the side chain can be homo- or copolymers. It is believed that in graft copolymer, molecules contained in the polymeric side chain have different molecules or structure from those in the polymeric backbone (Ring et al. 1985). Structure of the common graft copolymer is shown in Fig. 1; monomer X is grafted onto the backbone of the polymer which is built from monomer Y units.

Most methods of synthesizing of graft copolymers base on radical polymerization mechanism. When polycondensation process is involved, non-vinyl graft copolymerization is possible. However this method has not been widely used for preparing polysaccharide grafted copolymers due to low thermal resistance of the polysaccharide backbone as well as harsh conditions which are common for polycondensation reaction. Graft copolymerizations are carried out in heterogeneous as well as homogeneous systems. Polymer chains are grafted through three main strategies:

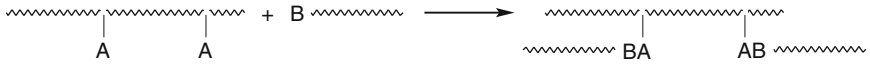
(a) *Grafting through* involves the polymerization (or copolymerization) of a macromonomer, usually a vinyl macromonomer:



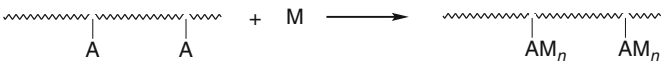
Grafting through method is one of the simplest ways to synthesize graft copolymers with well-defined side chains. In a typical process a low molecular weight monomer is radically copolymerized with a vinyl-functionalized macromonomer (polysaccharide in this case) (Tizzotti et al. 2010).

(b) *Grafting onto* involves the reaction between functional groups on two different polymers. In this technique presynthesized end-functionalized linear polymeric chains are subsequently covalently bonded to the polysaccharides. In most cases copolymers obtained with "grafting onto" strategy are characterized by

low grafting percentage (due to steric hindrance) and complicated polymerization procedures, thus significantly limiting its development.



(c) *Grafting from* technique involves the growth of grafted polymer chains directly from the polysaccharide backbone. The primary requirement for a successful "grafting from" reaction is a macromolecule with distributed initiating functionality. In the case of polysaccharides, radicals can be easily generated along the polysaccharide backbone in the presence of chemical or photochemical initiators or by using irradiation. This technique has been extensively investigated due to the fact that it is possible to obtain densely grafted copolymers. However during radical reaction the homopolymer, which is not chemically bonded to the polysaccharide backbone, can be produced, depending on the experimental conditions.



3.1 Chemical Initiation of Grafting

The grafting is achieved by generating free radicals on the polysaccharide backbone; after that these radicals serve as *macroinitiators* for the vinyl monomer. Free radicals can be generated in a conventional, chemical initiation. The most often, radical/redox initiators are used such as ammonium persulfate (Nishioka and Kosai 1981; Nishioka et al. 1983; Toti et al. 2004), ceric ammonium nitrate (Thimma et al. 2003; Kim and Mun 2009), ceric ammonium sulfate (Wan et al. 2007), ferrous ammonium sulfate/potassium persulfate (Yazdani-Pedram et al. 2000), potassium persulfate (da Silva et al. 2007) or in combination with other coinitiators (Singh et al. 2005b; Singh 2006; Shi and Zhang 2007). Those strategies of initiation of polysaccharide modification have been extensively investigated in recent years. Although this method is not without drawbacks such as inert working conditions, complicated and time-consuming procedures and as mentioned previously, homopolymer formation, which is not chemically bonded with polysaccharide, may occur, thus decreasing the grafting efficiency.

3.2 Radiation-Induced Grafting

Another technique of free radicals generation includes radiation-induced grafting. By careful selection of dose and rate of irradiation, it allows a high degree of

control over the number and length of grafted chains. The radiation techniques advantage is to maintain the purity of the product as it is free from contamination. These features of radiation methods make it a preferred technique of graft copolymerization.

High-energy radiation (γ-rays (Geresh et al. 2004; Liu et al. 2004; Wang et al. 2008) or electron beam (Vahdat et al. 2007)), low-energy radiation (Bertolini et al. 2001; Carlmark and Malmström 2002; Coskun and Temuz 2005; Zhou et al. 2005) (UV radiation and visible light), and microwave irradiation (Kaith et al. 2007; Kumar et al. 2009; Singh et al. 2012; Luo et al. 2012; Işıklan and Küçükbalcı 2012) are used to initiate the grafting process. The irradiation of the polysaccharide backbone with low-energy radiation results in homolytic fission, and free radicals are generated on the polymeric backbone. In the presence of low-energy radiation, grafting may occur with or without using a photoinitiator. High-energy radiation like γ-rays or electron beam has been used for graft copolymerization. The most widely used source of γ-rays is Co-60.

Another important energy source and effective tool for graft copolymerization is microwave radiation that has an advantage in the rapid transfer of energy into the reaction mixture which results in specific interaction of reagents. Moreover using microwave irradiation reduces the use of toxic solvents as well as reaction time. Microwave-synthesized polysaccharide copolymers are characterized with better properties for commercial exploitation in comparison with copolymers synthesized in a conventional way (Singh et al. 2012).

3.3 Other Grafting Methods

In some cases conventional procedures may lead to degradation of the polysaccharide backbone and to formation of a significant amount of homopolymer. Those procedures are characterized by very limited control over graft molecular weight and graft molecular weight distribution. These difficulties can be overcome by controlled/living radical polymerization techniques to obtain graft-functionalized polysaccharide-based macromolecular materials. The most common techniques for controlled/living radical polymerization include nitroxide-mediated polymerization, atom transfer radical polymerization, and reversible addition–fragmentation chain transfer. All mentioned techniques are moisture tolerant and can be performed with a large range of polymers having different functional groups. The properties of polysaccharide-based copolymers can be easily tuned with graft length, the chemical composition, and topology of polymers. All controlled/living radical polymerization techniques base on significant reducing the concentration of propagating radical chain ends. In that way possibility to terminate radical reaction is minimized. Such phenomenon is possible by addition of species with possibilities to reversibly trap the ‘active’ propagating species. This is done on the basis of reversible termination or reversible transfer.

4 Polysaccharides

4.1 Starch

Starch and cellulose belong to the group of the most common and easy to recover biopolymers. According to that both of them are the subject of much research. In fact only a few purposes have been found for raw, so-called "native" starch. Most of the material is modified by various methods, i.e., physical, enzymatic, or chemical processes including grafting. Starch may be found in almost all known plants where it plays a role as a storage material that can be used by the plant in a depolymerized form, i.e., glucose, the only monomer for both starch and cellulose. In fact, starch is not a homogeneous material. It is composed of two different fractions, i.e., linear amylose (AM) and branched amylopectin (AMP). Depending on the botanical origin of starch, the AM/AMP ratio may vary from 1:99 (waxy starches) to about 80:20 for high amylose starches. The most common AM/AMP ratio for nonmodified plants is about 20–30 AM to 80–70 AMP. For both of them glucose is a monomer; however the anhydroglucose is known as a repeated unit in starch. This is the result of starch polymerization in plant. The process may be simplified as the polycondensation of glucose with water as a low molecular side product. In AM anhydroglucose units (AGU) are linked together by α-1-4 glycosidic bond. On the other hand, in AMP, the main backbone is built in a similar manner, but branching points are located at C_6 of AGU which means that α-1-6 glycosidic bond appears at those places. In each case the glycosidic bond is formed between the hemiacetal group of one AGU and the hydroxyl group of another AGU unit (Fig. 2).

As it was mentioned before, starch is one of the most common polysaccharide in the plant kingdom. Due to different concentrations in plants, the occurrence as well as technological capabilities of commercial starch is mainly isolated from: cereals (wheat, corn, rice, etc.), tubers (e.g., potato), or seeds of leguminous plants (e.g., soybean). Alternative sources of starch are also known, but their commercial significance is strongly limited.

Although the native starch is rather uncommon for wider application, the product of its modification finds a lot of attraction. It also includes the chemical modification of AGU by means of etherification, esterification, oxidation, etc. Most of the mentioned processes have also been successfully carried out using microwaves especially when focusing on oxidation in paste, catalytic, and enzymatic esterification. The wide possibility of starch modification is the result of three hydroxyl groups in the AGU molecule that may react with different types of chemicals.

Microwaves were also used for grafting polymers onto the starch backbone. For now several starch grafted copolymers were obtained using microwaves that include: acrylic derivatives or caprolactone (Fig. 3).

In the case of caprolactone, the ring-opening polymerization of the monomer results in the polyester PCL that in the presence of polymers with reactive hydroxyl may be grafted on its backbone (Labet and Thielemans 2009). The ring-opening polymerization of caprolactone in the presence of pea (Yu et al. 2008;

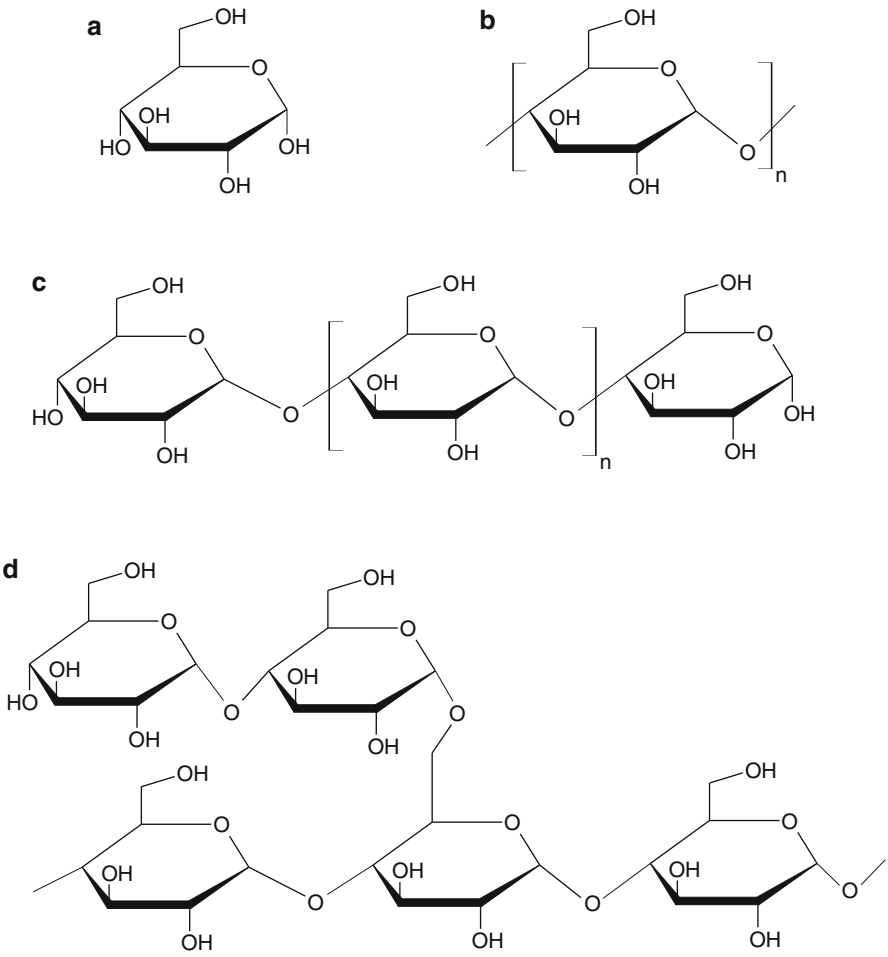


Fig. 2 Molecular structure of starch: (**a**) glucose monomer; (**b**) anhydroglucose unit – AGU; (**c**) amylose chain; (**d**) branching point of amylopectin

Chang et al. 2009a, b) and potato starch (Koroskenyi and McCarthy 2002) has been successfully carried out using microwaves. As a catalyst, tin(II) octoate was used. During the research no comparison to conventional conditions was done; however the total reaction time for PCL grafting was about 3 min (255 W). The structure of the obtained polymer was proven by means of detection of ester (carbonyl) signal formation observed using FTIR. Due to simple reaction setup (no group protection in this case; see chitosan grafted with PCL), the substitution degree and localization in starch chain remains unknown, but as in the case of all starch modifications, all three hydroxyls may be involved in this process.

On the other hand, many of the acrylic derivatives were applied to modify starch by means of free radical grafting (see Fig. 4) (Singh 2007; Singh et al. 2006;

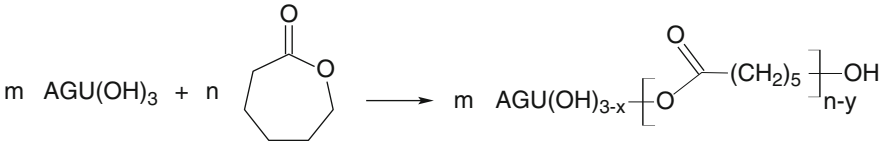
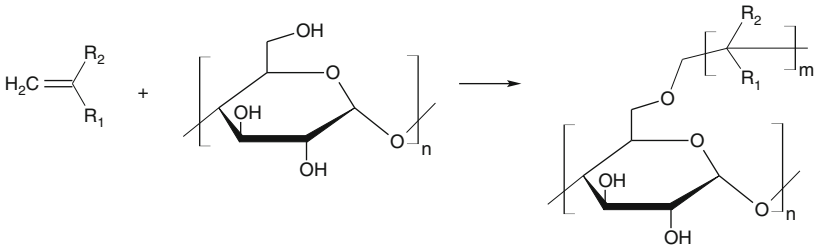


Fig. 3 Ring-opening polymerization of caprolactone in the presence of starch



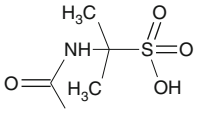
R_1	R_2	Acrylic compound name	Ref.
H	CN	acrylonitrile	Singh 2007
	$COONH_2$	acrylamide	Singh et al. 2006, Adhikary and Krishnamoorthi 2013, Singh and Nath 2013, Wang et al. 2012, Wang et al. 2011, Sorour et al. 2013, Singh et al. 2006
	COONa	sodium acrylate	Xu et al. 2005, Tong et al. 2005
		acrylamido-2-methyl-propanosulfonic acid	Singh et al. 2006
Me	$COOC_4H_9$	butyl methacrylate	Da-Wei et al. 2011
Me	$COOC_2H_5$	ethyl acrylate	Singh and Maurya 2010

Fig. 4 Acrylic monomers used for starch grafting at microwaves

Zheng et al. 2005; Xu et al. 2005; Chen et al. 2002). All those reactions were carried out in a very short time, i.e., 60 and 70 s for acrylamide and acrylonitrile, respectively. For sodium acrylate and acrylamido-2-methyl-propanosulfonic acid, the observed reaction time was a little bit longer and takes 10 and 7 min, respectively. In the case of acryloamide, the obtained material may be characterized by grafting percentage of about 160 %, while in the case of acrylonitrile, the value of 225 % was obtained. In those two experiments soluble starch was a starting material. Sodium acrylate and acrylamido-2-methyl-propanosulfonic acid were

grafted on corn starch. In those examples the superabsorbent products were obtained with the swelling ratio of about 520–620 g/g (for starch-*g*-sodium acrylate). On the other hand, only the branched fraction of starch, i.e., amylopectin, was grafted using acrylamide with shortening of the reaction time and grafting yield when compared with conventional conditions using cerium salts (Adhikary and Krishnamoorthi 2013). In other research the sago starch was grafted with acrylamide under microwave conditions as well (Singh and Nath 2013). The resulting polymer was successfully used as a controlled release polymeric carrier for drug delivery.

Acrylamide was also used in cationic starch modification in microwaves (Wang et al. 2011; Wang et al. 2012), while the corn starch was also modified using butyl methacrylate (Da-Wei et al. 2011). Generally speaking acrylamide is one of the most common grafting monomer. Under microwave condition it was used also for grafting polysaccharide blends containing starch, chitosan, and alginate (Sorour et al. 2013). In the last case the specific process environment was used that was called rheological phase reaction. The grafting occurs in microwaves, but the starch was first pregelatinized in high temperature of about 60 °C. In fact, the rheological phase reaction method is a kind of process in which products are formed in a solid–liquid rheological mixture in which the solid particles and liquid substance are distributed uniformly (Sun et al. 1999). Such kind of process environment in the presence of microwaves may result in a bigger influence of Maxwell–Wagner effects on interphase polarization. Experiments conducted by the authors show the phenomenon of radiation time on the viscosity of the grafted starch. The longer the radiation time was, the lower the viscosity of the product was observed. Additionally microwaves have also an influence on grafting efficiency with the maximum radiation time of 3.5 min, initiator content of 4.55 %, at which the grafting ratio reached 19.57 %.

Interesting research on starch grafting was also performed using ethyl acrylate (Singh and Maurya 2010). The authors have shown that however the efficiency of grafting without initiator is quite high, the presence of potassium persulfate–ascorbic acid initiating system improves grafting yield. The starch grafting may be also performed on starch derivatives including carboxymethyl starch grafted by acrylamide (Sen and Pal 2009). Additionally it was shown that polysaccharide blends containing starch (corn), chitosan, and alginate may be successfully grafted with acrylamide using combined microwaves and UV irradiation (Sorour et al. 2013). Obtained grafted blend may be treated as a new hydrogel with a swelling affected by pH due to ionic interactions between highly polar groups incorporated in the grafted material or by an external hydronium ion concentration.

The mechanism of acrylic polymer grafting on starch under microwave irradiation was proposed by Banerjee et al. (2012; Mishra et al. 2011). According to the authors highly electrophilic cerium ions may attack the lone pair of oxygen in the hydroxyl group. In anhydroglucose unit, the attack can be at the secondary C_2–C_3 or at the C_6 hydroxyl. Under conventional condition, C_2–C_3 attack is preferred because of higher electron density. On the other hand, when microwave irradiates the sample, the exothermic option is less preferable and binding at

C_6 hydroxyl occurs. According to that in conventional conditions, the cleavage of the carbon–carbon single bond occurs at C_2–C_3. Grafting of the acrylic monomer takes place at one of those carbons, while the second one is only oxidized to the form of carbonyl. At microwaves it takes place at the C6 position without any changes in the glucopyranose skeleton.

4.2 Seaweed Polysaccharides

Seaweed and seaweeds polysaccharide are paid a lot of attention by means of its commercial utilization in many branches of industry. In fact, most of them are well known and applied since ages, especially in the Far East Asian countries. But today there is a growing interest to apply them in many branches of science and industry. Very often the properties of the native polysaccharide do not meet the requirements of application. In those cases modification allows the change of properties. The most common plant polysaccharides acquired from the sea are alginates, agar, and carrageenan.

The term "carrageenans" describes a family of linear biopolymers obtained by water or alkali solution extraction from red seaweed found on the coast of North and South America, Asia, and Africa (Fig. 5). At the beginning of the last century, they were called as Irish moss extract (BeMiller and Whistler 2007; Glicksman 1986).

There are three main carrageenan fractions – fractions ι and κ, forming gels, and λ fraction, which does not gel at all. All these fractions are composed of D-galactose and 3,6-anhydro-D-galactose units, further esterified with sulfuric acid. Carbohydrate units are alternately connected by α-(1,3) and β-(1.4) bonds. Fractions κ and ι have similar compositions except that the fraction ι, except sulfate groups at the fourth carbon atom of D-galactose, has also a sulfate group at the second carbon atom of the 3,6-anhydro-D- galactose. On the other hand, λ fraction does not contain a 3,6-anhydro-D-galactose unit, and the sulfate groups are attached to every second unit of D-galactose, at the position of C_2 and C_6 (BeMiller and Whistler 2007; Thành et al. 2002; Necas and Bartosikova 2013). On the other hand, agar is a polymer composed of agarose and agaropectin. Agar consists of a mixture of agarose and agaropectin. Agarose, the predominant fraction, is a linear polymer, composed of anhydrogalactose and 3,6-anhydro-L-galactopyranose units.

Agaropectin fraction occurs in lesser amounts and is made up of alternating units of anhydro-D-galactose and anhydro-L-galactose heavily modified with such side groups as sulfate and pyruvate.

In recent research both polymers, i.e., carrageenan and agar, were successfully grafted with different side groups. At microwave conditions the most common grafting polymer for seaweed polysaccharide is polyvinylpyrrolidone (PVP). Hydrogels of carrageenan-g-PVP and agar-g-PVP were obtained at neutral pH with 125 % and 131 % of grafting, respectively (Prasad et al. 2006a). Both reactions have been carried out using potassium persulfate as radical initiator for about 2 min at 95 °C. As it was proved using HNMR and CNMR analysis, the grafting occurs simultaneously with cross-linking of polysaccharide chains using PVP bridges.

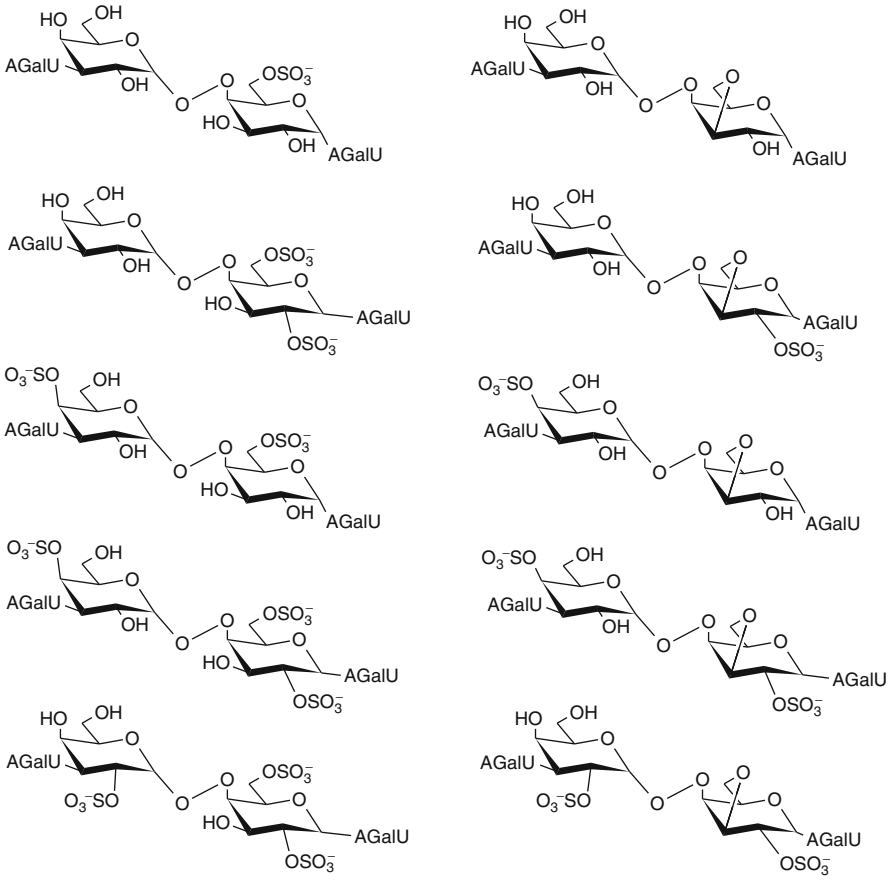


Fig. 5 Carrageenan family

As a result a higher water-holding capacity for grafted polymers was observed when compared with starting carbohydrate polymers. Microwaves were also applied for methyl methacrylate grafting on the carrageenan backbone (Prasad et al. 2006b, 2008; Meena et al. 2006). As a result a hydrophobic material was obtained with interesting improved barrier properties. As it was described for PVP grafting, methyl methacrylate is also able to cross-link two or more carbohydrate chains (Prasad et al. 2006b); however the presence of oxygen, "peroxide-like" bridges has not been proved by the authors. Meena et al. have also synthesized a carrageenan grafted methacrylate using microwaves, but in this case no specific molecular structure has been shown (Meena et al. 2006). It is also worthy to point out those specific short chains or low molecular organic compounds may also be grafted on carrageenan and agar forming interesting polysaccharide derivatives. Microwave may also be useful in this case as it was shown for the nucleobase cytosine (Oza et al. 2012a). In the reaction potassium persulfate was used as an initiator.

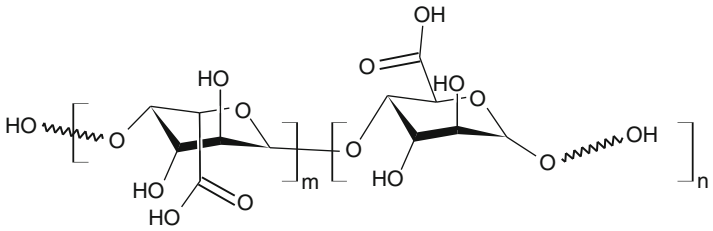


Fig. 6 Alginic acid structure

The reaction was carried out in water solution for about 3 min at 85 °C. Obtained products exhibited good fluorescence emissions. Similar results were obtained when adenine was grafted on carrageenan and agar in the microwave-assisted reaction performed in 2 min (Oza et al. 2012b).

Alginates are the group of seaweed polymers with different molecular constitutions when compared to carrageenan and agar. In this group sodium alginate is one of the most principal commercial water-soluble salts of alginic acid. Alginates are block copolymers of two carbohydrate subunits (Fig. 6).

Those building blocks are D-mannuronopyranosyl and L-guluronopyranosyl units. The ratio of those subunits is the key factor determining the alginate properties. As in the case of other polysaccharides, grafting of alginate skeleton may lead to a new group of polymers with designed properties. Some of those modifications have already been performed at microwave conditions. In fact, most alginates are grafted using acrylic polymers including methyl methacrylate (Rani et al. 2013), acrylamide (Sorour et al. 2013; Rani et al. 2013), or *N*-isopropylacrylamide (Işıklan and Küçükbalcı 2012). Methyl methacrylate has been grafted on agar using ceric ammonium nitrate. Microwave irradiation of about 800 W was used periodically in order to not exceed the temperature of 65 °C (g-PMMA 5, SAG-g-PMMA 6). During the research it was stated that the grafting process occurs in a most effective way at methyl methacrylate concentration of 7.5 g/1 g of alginate. The ceric ammonium nitrate concentration should be fixed as of 8 mg per 1 mL of the reaction mixture, and microwave power is maintained at 800 W (Rani et al. 2013). Obtained alginate-g-polymethyl methacrylate with highest observed intrinsic viscosity has been shown to possess a good flocculation efficacy against standard coal suspension. Alginate-g-acrylamide was obtained in water solution of alginate with no chemical initiator. The reaction conditions were set to 900 W of microwave power and the temperature do not reach the 70 °C. The total reaction time was 1–4 min; however the highest grafting efficiency was observed at 3 min (Sen et al. 2010b). Interesting results were obtained when *N*-isopropylacrylamide was grafted on alginate using different chemical initiators (Işıklan and Küçükbalcı 2012). The product of modification at microwaves was a pH- and temperature-responsive polymer that (in one case) exhibited temperature-induced phase transition behavior. It was proposed to apply alginate-g-*N*-isopropylacrylamide as an active drug-release matrix.

Besides acrylic polymers grafted on alginate, also an *N*-vinyl-pyrrolidone was successfully applied for grafting at microwave conditions (Yiğitoğlu et al. 2014). As in a former case, the resulted copolymer was shown to be able to play a role of a drug-release system, e.g., ibuprofen as a standard active substance. In described research azobisisobutyronitrile was used as an initiator. The process was carried out with a broad range of time from 2 min up to 2 h. It was shown that grafting yield and efficiency are rising with time and reach 57 % and 25.6 %, respectively. On the other hand, the viscosity average molecular mass of the product decreases about two times with time.

4.3 Gums

Gums belong to the group of polymers obtained mostly from the so-called higher plants or microorganisms. There are several gums that are important by means of technological application. In the group a variation in chemical constitution is observed. As a result different properties and industrial functions of those hydrocolloids may be detected. Due to chemical differences also the grafted polymers will vary by means of synthetic route and final properties.

4.3.1 Xanthan Gum

Xanthan gum is produced by microorganisms and has a complicated structure (Fig. 7). The macromolecule of xanthan is built from a cellulosic backbone in which the individual repeating units are combined together by β-1,4 glycosidic bonds. Every second unit, however, is substituted with short side chains of mannopyranosyl-D-(2→1)-β-D-glucuronic acid-(4→1)-β-D-mannopyranose which is linked to the main chain using α-1,3 glycosidic bond (Palaniraj and Jayaraman 2011). About 40 % of the terminal mannose groups form a cyclic ester of pyruvic acid (at positions 4, 6), and the inner mannose unit in most cases is acetylated at position C^6.

With the aid of microwaves, several xanthan grafted derivatives were obtained. Acrylamide was grafted on xanthan by Kumar et al. (2009). Xanthan-g-poly(acrylamide) was prepared employing microwave-assisted grafting and ceric-induced grafting. It was shown that relative grafting efficiency increases about three times (conventional method vs. 100 W of microwaves operating for 100 s) together with yield of grafting (more than three times for the same conditions). It is worthy to point out that a domestic microwave oven was used during experiments. The obtained polymer was characterized and used as a matrix for drug release. The study revealed faster release of the drug from graft copolymer matrix tablets as compared with the ungrafted gum. The release rate increased with the increase in percentage grafting (Kumar et al. 2009). Similar experiments on grafting were performed with ethyl acrylate (Pandey and Mishra 2012). During the research the comparison was done on the influence of microwaves on grafting. Microwave-assisted processes conducted in a domestic microwave were compared with a conventional one (water bath). In both cases as a result of the experiment, a series

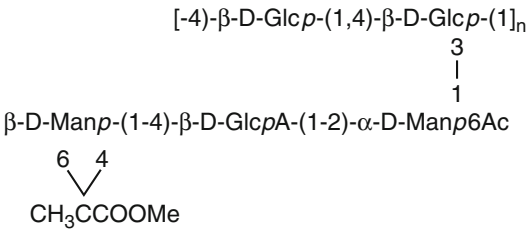


Fig. 7 Simplified xanthan gum structure

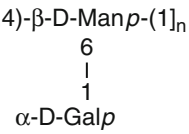
of grafted xanthan was obtained; however at the same monomer concentration, higher %G could be obtained under microwave conditions indicating grafting of more/larger monomer. The optimum %G was obtained by exposing a reaction mixture (25 mL) containing ethyl acrylate to microwaves for 15 s. During the research it was shown that microwave-assisted chemical grafting of the xanthan gum increased the adsorption capacity of lead ions from aqueous solution more than four times compared to the native xanthan gum.

4.3.2 Guar Gum

Guar gum is a polysaccharide that is extracted from plant seeds. Guar or cluster bean is the only source of that polysaccharide. Guar gum is an example of galactomannan, which is widely distributed in the plant kingdom (Cerqueira et al. 2011). Galactomannans are heterogeneous polysaccharides in which the backbone is built of mannopyranose units to which some galactopyranose units are attached as side groups. Various galactomannans found in nature differ in mannose/galactose ratio (Mulimani and Prashanth 2002). By means of chemical structure, the single macromolecule of guar contains anhydrogalactose and anhydromannose units. The backbone of the polymer is a linear chain of β-1,4-linked anhydromannose to which galactose residues are linked at C_6 position. The galactose units are presented at every second anhydromannose and forming short side branches (Srivastava and Kapoor 2005). As a result mannose/galactose ratio is about 2:1 (Fig. 8).

Guar gum has been widely used as a matrix for grafting acrylic monomers in microwaves including acrylonitrile (Singh et al. 2004), acrylamide (Pal et al. 2011; Sen et al. 2010; Shahid et al. 2013; Singh et al. 2004), ethyl acrylate (Singh et al. 2009), methyl methacrylate (Mishra and Sen 2011; Mundargi et al. 2006), etc. In the case of acrylonitrile, it was shown that grafting efficiency as well as percent of grafting is higher when microwaves are applied during the synthesis. It clearly shows the advantages of microwaves for grafting on guar; however there was a big difference in temperature observed at conventional and microwave-assisted processes (35 °C vs. 95 °C). Comparing polyacrylonitrile grafting guar is controversial in this case. During the described research it was also shown that grafting with microwaves results in higher molecular mass and viscosity of the product. It may be the result of shortening of the reaction time from 90 to 1.66 min. Also the factor of water absorption increases significantly when microwaves are applied. During the described research the mechanism of microwave-assisted

Fig. 8 Simplified guar gum structure



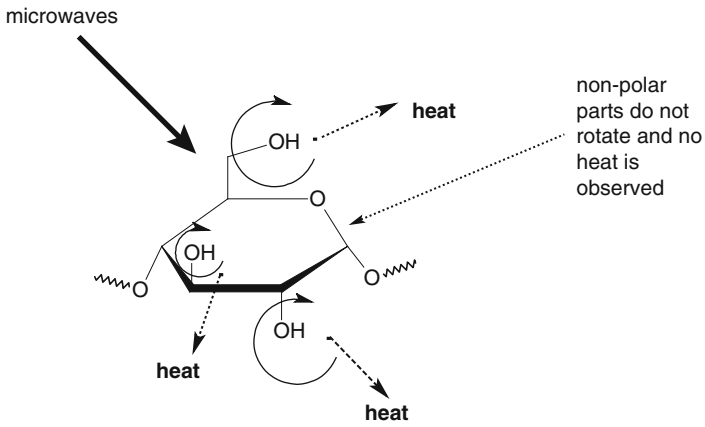


Fig. 9 Mechanism of microwave heat generation on polysaccharide chain

grafting onto guar gum was also proposed as an example of a polysaccharide matrix possessing high concentration of highly polar hydroxyl groups. According to that it was believed that dielectric (microwave) heating may be involved in rapid energy transfer from those "antennas" to neighboring molecules, i.e., monomer and solvent and water. In the polysaccharide chains there are large numbers of –OH groups (at least three for each anhydroglucose unit), so the energy dissipated to the environment seems to be crucial for the process (Singh et al. 2004) (Fig. 9).

Microwave-assisted grafting of acrylamide on guar gum has been performed in several studies (Table 2). Comparing all those data one can see that ternary initiating system allows to obtain the product with highest percent of grafting and grafting efficiency. The temperature of the processes does not exceed 70 °C and the total reaction time 4 min.

It is worthy to emphasize that under conventional conditions, no grafting product was observed in the absence of an initiator even when at high temperature. It clearly shows that radicals in the reaction system are of "microwave origin" (Singh et al. 2004). Except research on grafting and product characterization, also some application for acrylamide grafted guar gum has been proposed including flocculation processes (Pal et al. 2011) and drug release (Sen et al. 2010). The same application as well as synthetic protocol was used to graft guar gum with methyl methacrylate (Mishra and Sen 2011).

Microwaves were also employed in guar gum grafting using ethyl acrylate (Singh et al. 2009). In this case a microwave-induced emulsion copolymerization was performed in a very short time (15 s.). By varying with the ratio of gum and

Table 2 Grafting of guar gum using acrylamide

Initiator	Substrate	Solvent	Temperature °C	Time min	Power W	% grafting	% efficiency	Lit
PSA	Carboxymethyl guar gum	Water	70	1–3	1.64 W/cm^{2a}	84–96	No data	Pal et al. 2011
PSA/ascorbic acid/Ag^{+}	Guar gum		60	0.22	960	190	67	Singh et al. 2004
–			60	0.33	840	120	42	Singh et al. 2004
–			No data	2–4	900	23–58	No data	Sen et al. 2010; Mishra and Sen 2011

[a]Power density

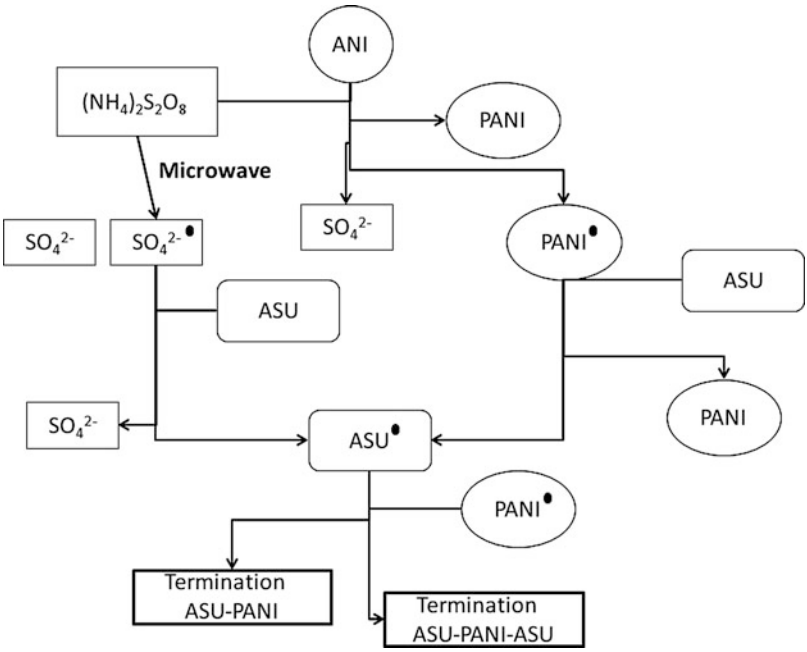


Fig. 10 Oxidative polymerization of aniline and grafting of polyaniline on polysaccharide backbone (*ANI* aniline, *PANI* polyaniline, *ASU* repeating unit of polysaccharide)

acrylate, a series of products was obtained with different grafting percents that reach up to 295 %G. The process was done in the absence of any redox initiator. Obtained copolymers were shown as capable to bind and remove cadmium ions from water solution (Singh et al. 2009).

Despite the most popular xanthan and guar gum, also some other gums have been successfully grafted in the presence of microwaves. During an interesting study acacia gum (gum Arabic) was grafted using polyaniline (Tiwari and Singh 2008). Arabic gum, in fact, is a complicated and undefined mixture of polysaccharides and glycoproteins (Phillips and Williams 2000). Although there is no strict information on the direct place where polyaniline chain is grown, the comparison between the conventional and microwave-assisted process clearly shows that grafting is more efficient when microwaves are operating especially by means of shortening of the reaction time. Because the polymerization of aniline is not only a radical process but a so-called oxidative polymerization, the mechanism of grafting looks different.

According to Tiwari et al., the primary radicals are generated from peroxydisulfate ions under the action of microwaves (Fig. 10). On the other hand, secondary radicals are forming on growing polyaniline chain. The grafting process is a result of the recombination of two macroradicals, i.e., the free radical on polysaccharide chain and secondary radical on polyaniline backbone (Tiwari and Singh 2008). Because polyaniline is one of the promising examples of conducting

polymers, the described synthetic protocol shows that microwaves may also be involved in natural compound modification that led to conductive substances. The obtained material is interesting also due to the phenomenon of pH switching conducting behavior that was dependent on the grafting extent. The occurrence is typical for polyaniline polymers but rather untypical in the case of biopolymers or polysaccharides. The observed controlled multifunctional electronic properties of the material might be usefully exploited for many technological applications.

Another example of gum grafting is ghatti gum that has been grafted using acrylamide (Rani et al. 2012). Ghatti gum is one of the oldest gums known. It is composed of L-arabinose, D-galactose, D-mannose, D-xylose, and D-glucuronic acid in a molar ratio of 10:6:2:1:2 and traces of 6-deoxyhexose (Whistler 1973). During the research ceric ammonium nitrate was used as an initiator and the process was performed in water solution. Microwave irradiation of the reaction mixture takes about 3 min at 800 W of microwave power. In order to avoid overheating of the reaction mixture, irradiation was done in cycles of heating (irradiating) and cooling. The obtained product was shown to serve as an effective flocculent for purifying water (Rani et al. 2012).

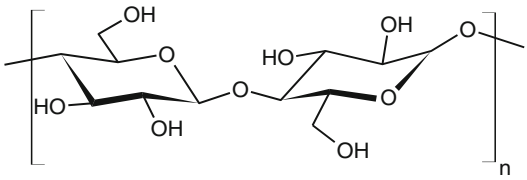
Additionally it is also worthy to point out that some rare gums have also been investigated as matrixes for grafting at microwave conditions. It includes gum kondagogu-g-poly(acrylamide) (Malik and Ahuja 2011), marginata gum (Singh et al. 2007, 2008, 2010), or *Cassia javanica* (Singh et al. 2010).

5 Cellulose

Cellulose is a naturally occurring polymer and is one of the most common organic polymers representing about 1.5×10^{12} tons of the annual biomass production. Additionally it is a very promising material due to its plenitude, availability, and low cost of acquisition. Cellulose is a linear polysaccharide and consists of β-D-glucopyranose units joined together by β-1,4 linkages (Fig. 11). In each repeating unit there are methylol and two hydroxyl groups as functional groups. In the structure of cellulose, there are no side chains or branching; therefore chains of polymer can exist in very ordered structure resulting in the existence of semicrystalline structure. In fact, the structure of cellulose is represented by areas of both high order (crystalline) and low order (amorphous). Although it is a linear polymer and contains a large amount of hydrophilic groups, e.g., two primary and secondary hydroxyl groups in each β-D-glucopyranose unit, it does not dissolve in water and in many organic solvents. It is caused by the existence of extensive hydrogen bonding system and van der Waals forces that are between polymer chains (Klemm et al. 2005).

The chain length of cellulose, expressed as the number of constituent units, varies with the origin and treatment of the raw material. In the case of wood, typical values are between 300 and 1,700, cotton and other plant fibers are in a 800–10,000 range. Partial chain degradation occurs on regenerating cellulose fibers (Phillips and Williams 2000).

Fig. 11 Cellulose formula



Cellulose has many crystal forms: cellulose I, which contains chains in a parallel orientation, and cellulose II, which is the most stable structure of cellulose and contains antiparallel-oriented chains. Cellulose has other polymorphic forms – cellulose III and cellulose IV (Klemm et al. 1998).

The properties of cellulose cannot be modified by conventional copolymerization methods, but they can be changed by some physical or chemical treatment, e.g., swelling in concentrated NaOH solution and then regeneration. During this process cellulose I is converted to cellulose II, and physical properties are changed. On the other hand, chemical properties of cellulose can be changed by (i) preparing ester or ether derivative cellulose, (ii) cross-linking cellulose chains, and (iii) grafting various monomers onto cellulose by copolymerization.

Graft copolymerization is among the widely used chemical modification methods for cellulose in order to improve its properties. During grafting, the side chains are covalently bonded to the main polymer structure to form a copolymer which has a branched structure. Graft copolymers have many useful properties different from those which each polymer has alone. There is extensive scientific literature available on the grafting modification of cellulose (Roy et al. 2009).

5.1 Microwave-Assisted Method

Cellulose has been modified to increase adsorption performance for copper(II) (Bao-Xiu et al. 2006). Partially neutralized acrylic acid (AA) and acrylamide (AAm) were grafted under microwave conditions. Potassium persulfate and sodium thiosulfate were used as initiators of radical reaction and *N*,*N*′-methylenebisacrylamide (MBA) as a cross-linker. Before grafting reaction cellulose was smashed into small particles and remains water immiscible and forms dispersion in the aqueous solution of vinyl monomers, cross-linker and initiator. Therefore grafting reaction takes place in heterogeneous conditions in the presence of redox system initiator. The radicals produced under microwave irradiation are generated more easily than in conventional heating conditions. Shortening of reaction time under microwaves is observed for several minutes at 360 W power. Alternatively reaction time for conventional heating was 10 h. Due to the presence of MBA in reaction medium, the resin obtained after grafting was also slightly cross-linked. The adsorption of Cu^{2+} by the optimum sample was 99.2 % and adsorption capability was 49.6 mg of Cu^{2+} per gram of grafted cellulose. Resin could be regenerated by using 8 % ammonia, regeneration ratio could achieve 85 %, and when recycled up to seven times adsorption ratio still kept 90 % after each cycle.

Graft copolymerization of *N*-isopropylacrylamide (NIPAM) and methyl acrylate (MA) on cellulose in heterogeneous conditions was carried out. Cellulose was dispersed in nitric acid, and an initiator system consists of cerium ammonium nitrate (CAN) and potassium persulfate (KPS). Series of reactions were done varying the microwave power as well as the reaction temperature to determine their influence on the grafting efficiency of polymers onto cellulose (Matahwa et al. 2007). The reaction time in microwave-assisted grafting was reduced to 10 min. The microwave power had only a small effect on grafting percentage, which was 36.86–49.14 %, depending on used microwave power. CAN/KPS initiation system is not suitable for microwave reactions in temperatures above 60 °C, only homopolymer is formed and cross-linking is highly enhanced.

Other sources of cellulose like mercerized cellulosic pine needles were used for grafting (Thakur et al. 2013a). Graft copolymerization synthesis of butyl acrylate (BA) was carried out in the presence of ferrous ammonium sulfate (FAS)/potassium persulfate initiator system. The reaction was carried out at selected doses of microwave radiation and for different time intervals. The optimum conditions for maximum percentage of grafting (28.18 %) were 90 mL of water, monomer 2.95×10^{-1} mol/L, time 400 s, FAS/KPS ratio 1:0.390, and microwave power 70 %. When reaction time is longer than 400 s, homopolymerization reaction dominates the graft copolymerization. When analyzing the influence of microwave power on grafting percentage, increasing of power up to 70 % causes increasing in grafting percentage, further increase in microwave power resulted in decreased grafting. In such high microwave power, homopolymerization reaction or decomposition reaction of graft copolymers occurs.

Bamboo cellulose was used in graft polymerization of methyl methacrylate (MMA); cerium ammonium nitrate was used as an initiator of polymerization reaction (Wan et al. 2011). Reaction was carried out under microwave radiation upon dispersion of cellulose in an aqueous medium in 3 min repeated period of time. The cellulose was pretreated with Ce^{4+} ions to form active reaction centers, and after that monomers were added. During pretreatment in the initiator of microwave-assisted grafting method, breaking of C–C bond in AGU unit occurs to form a carbonyl group and radical which is able to react with monomers to form grafted cellulose. The grafting reaction was confirmed with FTIR (characteristic band at 1,730 cm^{-1} corresponding to the stretch vibration of carbonyl group) and XRD spectra. Increasing of initiator concentration, grafting percentage (%G) and grafting efficiency (%GE) had the maximum at around 0.004 mol/L of the initiator, which caused more reaction sites on the cellulose backbone at low CAN concentration, and formation of the growing grafted chains or initiation of homopolymerization reactions at concentrations of CAN higher than 0.004 mol/L. %G and % GE initially increased with the increasing of microwave power up to 160 W, which is caused by more macroradicals generation. Whereas at microwave power higher than 160 W homopolymerization reaction is preferred, some decomposition of the graft polymer occurs. The maximum value of %G and %GE was reached after 9 min of reaction; further increasing of reaction time caused intensified interactions

between the initiator, monomer, and cellulose backbone and resulted in more generation of free radical sites both on the backbone and methyl methacrylate chains.

Microwave irradiation is one of the very effective methods for activating chemical reactions (Varma 1999; Elander et al. 2000; Perreux and Loupy 2001). Taking into consideration cellulose availability and biodegradability, glycidyl methacrylate grafted cellulose adsorbent for mercury was prepared, using microwave-initiated method (Kumar et al. 2013). Such initiation is possible due to the fact that during microwave irradiation, the localized rotations on an almost immobile OH group arising as a result of the dielectric heating involve energy transfer to solvent molecules, whereas the rupture/cleavage of the C–C bonds leads *t*o the formation of free radicals and then ensuing interaction with glycidyl methacrylate (GlyMA) to form grafted cellulose (Galema 1997). The grafting with GlyMA onto the cellulose backbone was confirmed with FTIR, XRD, and SEM analysis. FTIR spectra shows the O–H and C–H stretching peaks which appear at 3,343 and 2,895 cm^{-1}. Shifting of O–H and C–O peaks to 3,356 and 1,720 cm^{-1}, respectively, is observed after adsorption of mercury.

5.2 Microwave-Initiated Method

Microwave radiation was used to initiate grafting reaction. In that type of process, there were no other chemical substances which could initiate radical reaction. The nature of microwave radiation shows that the radiation beams cause "selective excitation" of only polar bonds, which results in their cleavage and leading to the formation of free radical sites. The C–C backbone of the polymer, which is relatively nonpolar, remains unaffected by the microwave radiation. Using this technique polyacrylic acid chains (PAA) were grafted on the carboxymethylcellulose (CMC) backbone. To initiate grafting reaction, microwave radiations that cause excitation of O–H bonds following free radicals were generated on the cellulose backbone. After that typical propagation reaction, with the participation of monomer (acrylic acid), according free radical mechanism occurs to build poly (acrylic acid) chain onto the CMC backbone. Authors suggests that in cellulose, free radical sites are expected to be generated at the primary hydroxyl group; however in carboxymethylated cellulose, part of those groups are unavailable due to the fact that they were substituted. Therefore when higher degree of substitution of cellulose, less of primary O–H sites will be available and hence and percentage grafting will be lower. To avoid competing homopolymer formation reaction and to prevent any thermal damage to the backbone polymer chain, the microwave irradiation was paused periodically whenever the reaction mixture starts boiling, i.e., at about 65 °C. The optimized reaction conditions have been determined, and it has been found that the highest grafting percentage (15.5 %) was at acrylic acid concentration of 10 g in the reaction mixture and 800 W of microwave power during 5 min.

Table 3 Optimized reaction conditions for microwave grafting of vinyl monomers onto flax fibers (Kaith and Kalia 2007, 2008; Kaith 2008)

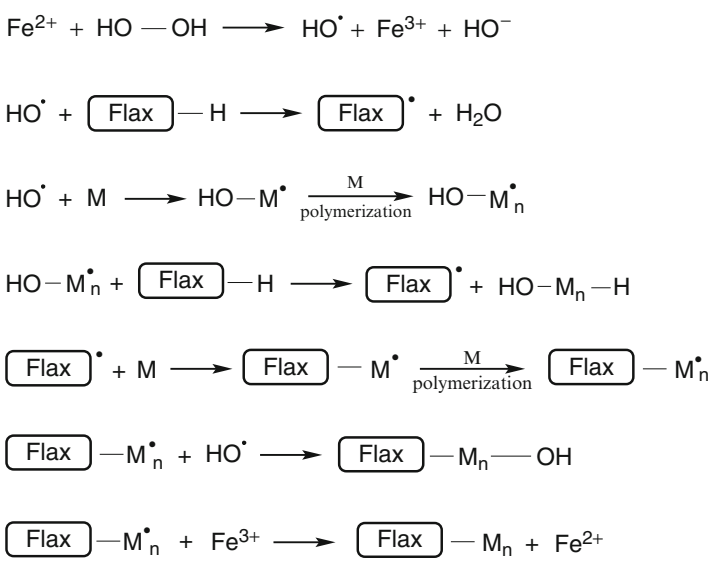
Monomer(s)	Monomer concentration $\times 10^3$ [mol/l]	% grafting
MMA	1.96	24.64
MMA/EA	2.3	51.2
MMA/AN	2.27	17.8
MMA/AA	2.91	12.08

5.3 Grafting onto Cellulose Fibers

Kaith et al. (Kaith and Kalia 2007; Kalia and Kaith 2008; Kaith 2008) report the grafting of methyl methacrylate or binary vinyl monomers such as MMA/ethyl acrylate(EA), MMA/acrylonitrile(AN), and MMA/acrylic acid onto flax fiber under different reaction conditions. In this technique of modification of natural fiber properties, the radiation technique reduces the extent of physicochemical stresses to which the fibers are exposed when modification occurs in conventional way. Properties of natural fibers treated under microwave radiation are even better than properties of fibers grafted through conventional technique (Freddi et al. 1996). Flax fibers grafted with vinyl monomers are excellent material as reinforcement to prepare of phenol-formaldehyde composites. Grafting reaction was carried out at microwave oven at 210 W microwave power, temperature not higher than 60 °C, reaction time 20–60 min. To initiate radical reaction Fenton reagent (ferrous ammonium sulfate/hydrogen peroxide) was used (Table 3).

The basis of the Scheme 1 hydroxyl radicals can be generated in the reaction between Fe^{2+} ions and hydroxy peroxide. OH radicals are able to react with flax fibers to form active sites onto the polymeric (cellulose in that case) backbone. Vinyl monomer can react with them to form graft copolymers as a concurrent homopolymerization reaction of vinyl monomer occurs. Percentage of grafting increases with increase on monomer concentration to about 2×10^{-3} mol/L (grafting percentage 24.64 %) for methyl methacrylate; further increase of monomer concentration prefers homopolymerization than graft copolymerization. Analyzing the influence of initiator concentration on grafting percentage, it was observed that maximum was observed when FAS–H_2O_2/monomer molar ratio was 1:6. Higher concentration of Fenton reagent could produce more Fe^{3+} ions, but they are rather consumed in termination reaction than in propagation reaction of copolymer chain. The optimum found reaction time was 30 min. Grafting reaction under microwave radiation results in less surface defects and crystal lattice of the fibers. The structure of fibers did not change very much during grafting which leads to better strength of flax-g-copolymer reinforced phenol-formaldehyde composites (Kalia and Kaith 2008).

Methyl methacrylate was grafted onto *Grewia optiva* fibers under microwave radiation where ferrous ammonium sulfate and potassium persulfate were used as a redox initiator system (Thakur et al. 2013b). *Grewia optiva* fibers consist of linear chains with repeating β-D-glucopyranose units joined together by β-1,4 linkages. To create free radical sites onto the cellulosic backbone, fibers were pretreated with



Scheme 1 Mechanism of grafting reaction onto flax fibers

initiator (FAS-H_2O_2). Generated free radicals may react with C_2, C_3, or C_6 hydroxy groups and C–H sites to form active sites for grafting of methyl acrylate. The optimum conditions for maximum grafting percentage (36.73 %) were as follows: solvent 110 mL, monomer (3.05×10^{-1} mol/L), time (350 s), FAS/KPS 1:0.250 mol/L, and microwave power at 70 %. The percentage of grafting increases with the increase in monomer concentration as initially more and more free radicals react with the cellulosic backbone of *Grewia optiva* fibers. However homopolymerization reaction dominates over graft copolymerization when monomer concentration is above optimum value resulting in decreasing of grafting percentage.

5.4 Hemicellulose

Another approach is to use tamarind kernel polysaccharide derived from the seeds of the commercially important tree in South Indian states and also in other Southeast Asian countries – *Tamarindus indica* (Ghosh et al. 2010). Tamarind seeds contain mainly xyloglucans, which have β-1-4-linked D-glucan backbone partially substituted with short side chains containing fucosyl, arabinosyl, galactosyl, and xylosyl residues (Fry 1989).

Tamarind kernel polysaccharide was grafted on acrylamide by three different techniques: (i) conventional technique with ceric ammonium nitrate as initiator, (ii) microwave-assisted method with the same initiator, and (iii) microwave-initiated method. When the third method was used, microwave energy was absorbed by the water molecules and then quickly transferred to the acrylamide causing dielectric

heating, which resulted in severing of double bonds in acrylamide, and producing of free radicals. The intrinsic viscosities and molecular weights of grafted products,were higher compared to the base polysaccharide. Such phenomenon was due to the presence of grafted polyacrylamide chains. As it was reported when microwave-initiated method is used, some grafting is observed, increasing molecular weight from 6.05×10^5 g/mol to 3.81×10^6 g/mol (grafting percentage 53 %); however it is not as effective as microwave-assisted method where molecular weight ranges to 5.12×10^6 g/mol and grafting percentage is 87 %.

6 Chitin and Chitosan

6.1 Chitin and Chitosan Chemistry

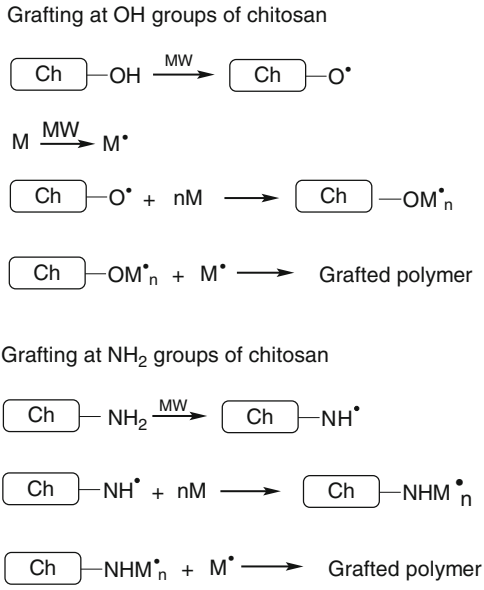
Chitin as a biopolymer is the most abundant organic compound of nitrogen in nature and is estimated to be at a level of about 10^{11} tons annually. It represents the major structural component of the skeletal material of invertebrates. At least 10^{13} kg of chitin is constantly present in the biosphere (Heinze 2005). It occurs in nature as ordered crystalline microfibrils forming structural components of exoskeletons, peritrophic membranes, and cocoons of insects. However only limited attention has been paid to chitin, mainly due to its intractable bulk structure, but chitin and its derivatives have high potential in many fields, e.g., pharmaceutical, cosmetics, agriculture, and environmental protection (Shahidi et al. 1999; Ravi Kumar 2000; Rinaudo 2006).

Chitin is structurally very similar to cellulose, but it has acetamide groups at the C-2 position. It is a highly ordered linear copolymer of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose (Scheme 2). Chitin occurs naturally partially deacetylated; the occurrence of amino groups in chitin (5–15 %) is highly advantageous for providing biological reactions. The solubility of chitin is remarkably poorer than that of cellulose, because of high crystallinity supported by hydrogen bonds, which are formed mainly through the acetamido groups. Therefore chitin is insoluble in all the usual solvents. The insolubility is a major problem that confronts the development of processing and uses of chitin.

The most important derivative of chitin is chitosan (Fig. 12), obtained in two main processes: (i) (partial) deacetylation of chitin in the solid state under alkaline conditions (concentrated NaOH) or (ii) by enzymatic hydrolysis in the presence of a chitin deacetylase. Chitosan is the most important chitin derivative in terms of applications, due to fact that when the degree of deacetylation of chitin reaches about 50 %, polymer becomes soluble in aqueous acidic media. Therefore the degree of acetylation determines whether the polymer is called chitin or chitosan. Generally chitosan is used to name deacetylated chitin (deacetylation degree above 70 %). Chitosan, unlike chitin, is soluble in dilute acetic acid.

Taking into account the fact that chitin is poorly soluble and the reactions under heterogeneous conditions are accompanied by various problems, much attention had been paid to chemical modification of chitosan rather than chitin.

Scheme 2 Mechanism of copolymer grafting onto chitosan backbone under microwave irradiation



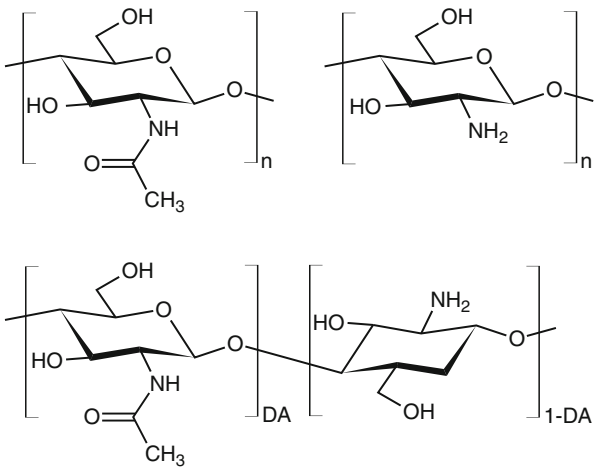


Fig. 12 Chemical structure of (**a**) chitin (**b**) chitosan (**c**) structure of partially acetylated chitosan

6.2 Graft Copolymerization of Chitosan

Graft copolymerization is expected to be the most promising approach to synthesize a wide variety of materials based on bio- and synthetic polymers. Vinyl grafted chitosan has many of its properties enhanced, and grafted chitosan materials behave as efficient flocculants (Hon and Tang 2000; Babel 2003; Chen and Park 2003; Guibal 2004), paper strengtheners, drug carrier (Calvo et al. 1997; Giunchedi et al. 1998; Wang et al. 2009; El-Sherbiny and Smyth 2010), etc. Most of the

chitosan copolymers are prepared through graft polymerization of vinyl monomers onto the polysaccharide backbone. But there are other approaches to synthesize grafted chitosans, e.g., cyclic monomers graft copolymerization via ring-opening method.

6.2.1 Vinyl Graft Copolymerization

Grafting reactions of vinyl monomers onto the chitosan backbone are mainly achieved by radical polymerization. In the beginning free radicals are generated on the polysaccharide backbone, and then they serve as macroinitiators for vinyl monomers, and propagation reaction of vinyl polymer onto the chitosan backbone occurs. Free radicals could be generated with microwave irradiation or with using different redox initiators, mainly ceric ions, potassium persulfate, and silver nitrate/potassium bromate.

Among different methods of initiation, the vinyl monomer graft copolymerization in microwaves onto chitosan *microwave initiation method* is the most frequently used (Table 4). In such conditions chitosan as quite a large molecule with pendant –OH and $–NH_2$ groups may interact with microwaves. Those groups anchored to an immobile backbone may rotate locally in the presence of microwaves, as a result dielectric heating of reaction medium is observed. In chitosan molecules there are large amounts of those pendant groups; therefore the amount of energy produced is expected to be very high, resulting in O–H and N–H bond breaking to produce radical sites on oxygen or nitrogen atoms of the chitosan molecule. Moreover as it was reported in literature, microwave irradiation may lower the Gibbs energy of activation of the reaction (Galema 1997). It was also reported (Singh et al. 2006a) that due to higher bond energy in hydroxyl group compared with N–H, more N · radicals will be produced; thus more grafting is expected at N–H sites.

Grafting of acrylamide (Singh et al. 2006a), methyl methacrylate (Singh et al. 2006b), and acrylonitrile (Singh et al. 2005) on chitosan has been done in the absence of any chemical initiator in aqueous conditions. Reactions were carried out in a very short time. In all abovementioned experiments, reactions were carried out in homogenous conditions, solution of chitosan in formic acid, in an open-glass vessel using domestic microwave oven without temperature control. The temperature in Table 4 was measured by inserting thermometer in the reaction mixture after the microwave exposure. Maximum MW power used was 80 % (960 W) due to the fact that further increase in microwave power leads to the decrease in grafting. This phenomenon was explained with increasing of homopolymerization process and partial degradation of the grafted polymer at such high microwave power.

During microwave-initiated grafting of acrylamide onto chitosan, it was found that the grafting percentage was increased with an increase in the initial concentration of monomer; thus more macroradicals were produced, which in turn may generate more grafting sites on chitosan (Singh et al. 2006a). When MW power was increased above 80 % (960 W), yield of the grafting starts decreasing due to the fact that some decomposition occurs and

Table 4 Reaction conditions and grafting parameters of vinyl polymers grafted onto chitosan with the use of microwave irradiation

Substrate	Monomer	Initiator	Reaction conditions					%G	Ref
			Initiator concentration	Monomer	Temp., °C	MW Power, W	Time, s		
Chitosan	AM[b]	–[a]	–	0.016 M/L	95	960	70	169	(Singh et al. 2006a)
Chitosan	MM[c]	–[a]	–	0.017	95	960	120	160	(Singh et al. 2006b)
Chitosan	AN[d]	–[a]	–	0.028	95	960	90	170	(Singh et al. 2005)
Chitosan	BA[e]	–[a]	–	0.00375	–	1,200	180	87.5	(Santhana Krishna Kumar et al. 2014)
Chitosan	AA[f]	CAS[g]	8.3×10^{-4} mol/L		60	120	1,800	89.6	(Huacai et al. 2006)
SChA blend[h]	AM[b]	KPS[i]		0.2	35		600	148	(Sorour et al. 2013)

[a]Microwave irradiation was used to generate radicals
[b]Acrylamide
[c]Methyl methacrylate
[d]Acrylonitrile
[e]*n*-Butylacrylate
[f]Acrylic acid
[g]Cerium ammonium sulfate
[h]Blend consists of chitosan, corn starch, and alginic acid sodium salt
[i]Potassium persulfate

homopolymerization reaction occurs. Under optimal grafting conditions for synthesis of chitosan-*graft*-poly(acrylamide), 169 %G was observed at 960 W microwave power after 70 s of reaction (Table 4).

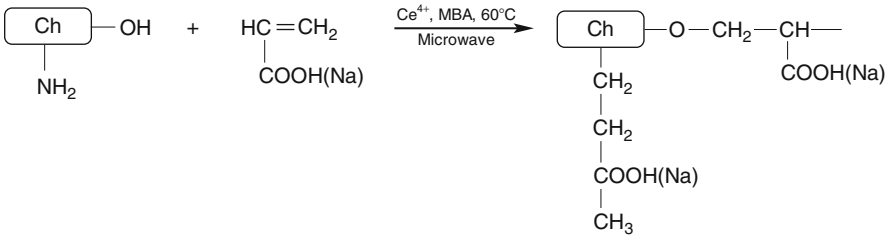
Grafting of acrylonitrile (Singh et al. 2005) was made in similar conditions and optimal parameters were found for grafting. When 960 W of MW power during 90 s was used, the copolymer sample having 170 %G was obtained. As it was in the case of acrylamide (Singh et al. 2006a) grafting, increasing of microwave power above 80 % (960 W) decreases grafting percentage and grafting efficiency.

Optimum conditions for microwave-initiated graft copolymerization of methyl methacrylate (MMA) on chitosan having 160 %G were as follows: under 80 % of microwave power after 2 min of exposure, using MMA in such amount, the concentration in the solution was 17×10^{-2} mol/L, 0.1 g of chitosan. It was found that in all cases mentioned above, grafting percentage was much higher and reaction time was much shorter, when compared with grafting in conventional way (redox initiator, without microwave irradiation) (Singh et al. 2005, 2006a, b).

Rajesh et al. tried to use a similar technique to graft *n*-butyl acrylate on chitosan in homogeneous conditions using microwave irradiation to initiate radical reaction (Santhana Krishna Kumar et al. 2014). They obtained grafted polymer after 3 min of reaction with 87.5 grafting percentage. Structure of copolymer was confirmed by FTIR spectra.

The microwave-assisted synthesis of partially neutralized acrylic acid grafted onto chitosan has been reported (Huacai et al. 2006). Cerium ammonium sulfate was used as an initiator in the presence of cross-linker *N*,*N*′-methylenebisacrylamide. Reaction was carried out in modified domestic microwave oven to control the temperature at 60 °C and radiated by microwave for 30 min. The grafting efficiency has been found to strongly depend on the amount of chitosan and, to a lesser extent, depends on the irradiation power. In optimum conditions 0.3 g of chitosan, 2.5 mL of 0.01 mol/L initiator, 5 mL of 0.01 mol/L of cross-linker, and microwave power 120 W over 30 min. were used, and grafting percentage was 89.6 % (Scheme 3).

The graft copolymerization of acrylamide on natural polysaccharide blend consisting of corn starch, chitosan, and alginic acid has been done (Sorour et al. 2013) in the presence of *N*,*N*′-methylenebisacrylamide that acts as a cross-linker and potassium persulfate as initiator. Reaction was carried out in domestic microwave oven without temperature control at different concentrations of acrylamide and polysaccharide blend. Due to the presence of cross-linker during grafting, cross-linking reaction occurs to form water-swelling hydrogel. Maximum swelling ratio was increased with increasing of acrylamide/blend weight ratio to reach a maximum value of 32 g/g. Such phenomenon is initially originating from the greater availability of monomer molecules in the vicinity of polysaccharide macroradicals; additionally higher acrylamide content enhances hydrophilicity of hydrogel. Further increasing of acrylamide/blend ratio causes decreasing of swelling ratio, due to intensification of homopolymerization reaction, increasing the viscosity of reaction medium and enhancing chances of chain transfer reaction to monomer molecules.



Scheme 3 Synthesis of microwave-assisted chitosan-*graft*-poly(acrylic acid)

6.2.2 Cyclic Monomer Graft Copolymerization: Ring-Opening Method

Graft copolymerization of ε-caprolactone onto chitosan has been done via three-step reactions involving (a) phthaloyl protection of amino groups of chitosan, (b) microwave-assisted graft copolymerization of ε-caprolactone in heterogeneous environment in the absence of solvent with the presence of tin(II) octoate as catalyst, and (c) deprotection to regenerate amino groups (Liu et al. 2005). The introduction of phthaloyl groups caused destruction of crystalline structure of chitosan as well as increasing of hydrophobicity of chitosan. This will cause better affinity of chitosan with hydrophobic ε-caprolactone and hydroxyl groups of chitosan (Koroskenyi and McCarthy 2002). At the microwave power 450 W, both the yield and grafting percentage were greatly improved, compared to conventional heating method. The optimum grafting percentage at level 120 % was found when feed ratio of ε-caprolactone monomer to phthaloylated chitosan was 1:2 and exposed for 12.5 min at 450 W microwave power. At MW power above 450 W, discoloration of the final product occurs due to overheating. Due to the fact that grafting reaction occurs only on hydroxyl groups of chitosan, after deprotection there are large amounts of free amino groups. Due to the presence of amino groups and hydrophobic polycaprolactone side chains, the obtained chitosan-*graft*-polycaprolactone could be used as degradable amphoteric material having extensive potential applications in biomedical materials (Fig. 13).

A similar procedure was used to synthesize thermoformable bionanocomposites based on chitin whisker-*graft*-polycaprolactone (Feng et al. 2009). Grafting of ε-caprolactone on chitin whisker was carried out through "graft from" strategy, to obtain long and dense "plasticizing" tails of polycaprolactone on chitin whisker surface, which are key issue of thermoforming. The reaction was carried out in the presence of tin(II) octoate under microwave irradiation of 255 W for 3 min. Authors observed that with an increase of polycaprolactone content, the strength and elongation as well as the hydrophobicity of the nanocomposites were increased.

Chitosan-*g*-poly(D,L-lactide) copolymers have been synthesized by ring-opening polymerization of D,L-lactide with the presence of tin(II) octoate as catalyst (Luo et al. 2012). Modified chitosan with pendant hydroxyl groups was used as the polymer backbone to weaken the intramolecular and intermolecular hydrogen bonds and to enhance reactivity of chitosan. The optimal conditions for the microwave-assisted copolymerization were as follows: 10 W of microwave

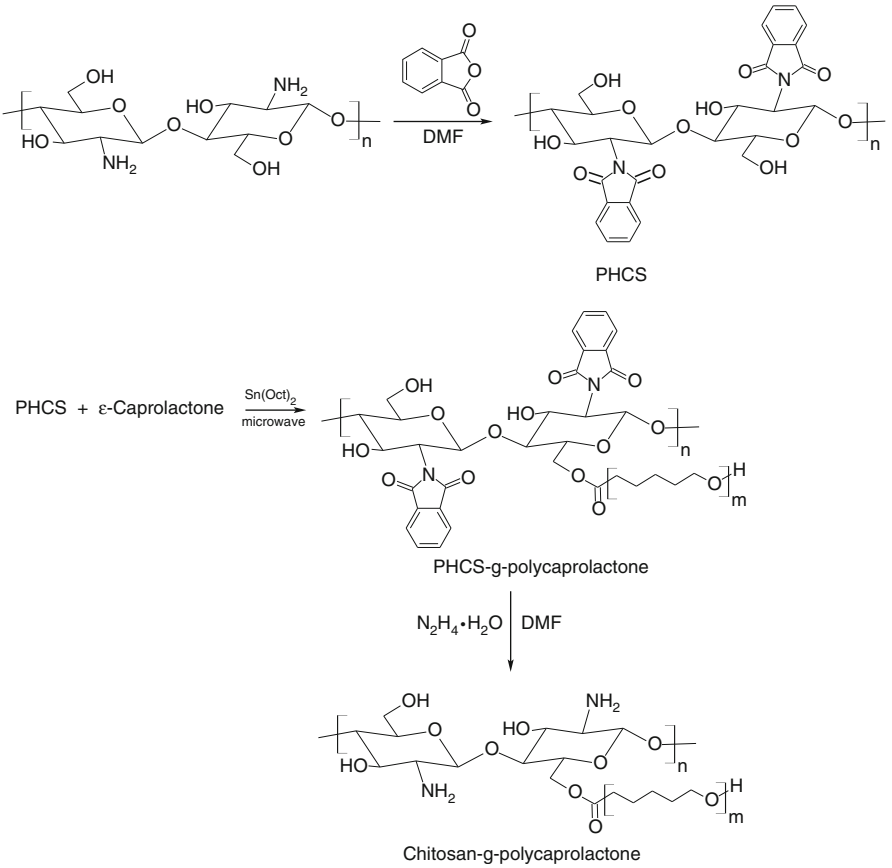
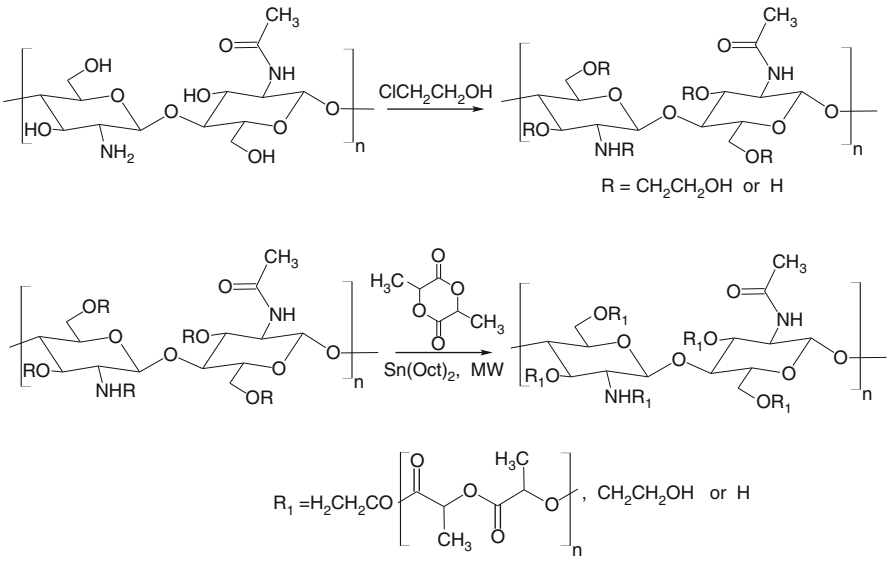


Fig. 13 Preparation of chitosan-g-polycaprolactone by ring-opening method with phthaloyl protection of amino groups

power for 15 min, reaction temperature 130 °C, and catalyst concentration 0.05 %. In those conditions grafting percentage reached 229.4 %. When microwave power is above 10 W, temperature higher than 130 °C, or reaction time longer than 15 min, the degradation of poly(D,L-lactide) side chains occurs, which led to a lower grafting percentage. The grafting percentage of the copolymer could be adjusted by control of the ratio of D,L-lactide to aminoglucoside units (Scheme 4).

7 Conclusions

Desirable properties can be imparted to natural polysaccharides through synthetic polymer grafting in order to obtain products with requirements needed in specialized applications. Polymer grafting under microwave irradiation is a clean method for altering properties of numerous biopolymer (polysaccharides) backbones and



Scheme 4 Synthesis of chitosan-*g*-poly(D,L-lactide) copolymer

can be a powerful strategy for the development of the valuable derivatives with diversified properties. It may also be used for various scientific and industrial applications. Microwave heating has many advantages over conventional thermal heating, and it offers an environmental friendly, cleaner, greener approach resulting in greater control and reproducibility of the final product, which is suitable for commercial production. There are some easy to see difficulties with commercialization of microwave synthesis of grafted polysaccharides, of which the most important could be the scale-up, because higher energy input is required for larger batch. Polysaccharide grafting reactions under microwave irradiation offers shortening in reaction time, solvent less/free or aqueous conditions, fast heating rate, and possibility of high-temperature reactions with modified selectivity. However microwave-based grafting reactions still require very careful control of reaction parameters to obtain polysaccharides with suitable properties and grafting efficiency. Current research in the area of polysaccharide grafting under microwave irradiation generated awareness that materials, with desirable properties using a natural polymers, can be produced through such processes with much simpler, cleaner, and quicker technologies than the conventional ones.

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Analytical Methods for Lignocellulosic Biomass Structural Polysaccharides

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Contents

Abstract

The use of lignocellulosic biomass has been postulated as a potential pathway toward diminishing global dependence on nonrenewable sources of chemicals and fuels. Before a specific feedstock can be selected for biochemical conversion into biofuels and bio-based chemicals, it must first be characterized to evaluate the chemical composition of the cell walls. Polysaccharides, specifically cellulose and hemicellulose, are often the focal point of these appraisals, since these constituents are the dominant substrates converted into monomeric sugars like glucose and xylose. These monosaccharides can be transformed, using microorganisms like yeast, into substances such as ethanol. Plant species containing abundant polysaccharides are highly desirable, as higher quantities of sugars should translate into larger end-product yields. Given the vast pool of potential feedstocks, qualitative and quantitative analytical methods are needed to assess cell wall polysaccharides. Many of these tools, such as wet chemical and chromatographic techniques, have been ubiquitously used for some time.

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Shortcomings in these analyses, however, prevent their usage in screening large sample sets for quintessential, high-yield, fuel-producing traits. This chapter briefly summarizes how analytical spectroscopy can lessen some of these limitations and how it has been utilized for polysaccharide analysis.

Keywords
Lignocellulosic biomass • Analytical instrumentation • Spectroscopy • Polysaccharides • Cellulose • Hemicellulose

1 Introduction

The analytical assessment of polysaccharides continues to be a prevalent research topic. Many applications previously developed for or applied to the food or textile industry have been transferred to the characterization of lignocellulosic biomass for the production of biofuels and other bio-based chemicals. This chapter seeks to provide an overview of the spectroscopic analytical methods employed for evaluating polysaccharides, encompassing qualitative (structural) and quantitative analyses applied to lignocellulosic biomass.

Lignocellulosic biomass conversion to biofuels is one possible pathway to supplanting global dependence on fossil fuels. In a 2011 supplement to the 2005 US Department of Energy *Billion Ton Report*, Perlack et al. projected that the existing biomass from agricultural and forest crops including wastes plus dedicated energy feedstocks, in the USA, is between 400 and 600 million tons priced at $60 per dry ton or less (Perlack 2005, 2011). Before a specific plant can be isolated for biofuel or bio-based chemical production, it must first be characterized using an array of analytical tools. Structural polysaccharides represent one of the dominant plant cell wall fractions evaluated in lignocellulosic biomass.

Structural polysaccharides are polymers of monosaccharides such as glucose or xylose and include cellulose, hemicellulose such as arabinoxylans, and pectin. Cellulose, the most abundant biopolymer on earth, comprising 15–30 % of primary walls and larger proportions of secondary walls, is a linear chain of β-D-glucopyranosyl units, connected via 1,4-glycosidic linkages. Hydrogen bonding between chains allows for the formation of tightly bound cellulose microfibrils. Measuring cellulose both qualitatively and quantitatively has been of considerable interest in the biofuel community, as higher cellulose contents provide a larger quantity of fermentable sugars following hydrolysis. Hemicelluloses are heteropolymers of arabinose, galactose, glucose, rhamnose, mannose, and xylose monomeric units and are classified as xyloglucans, xylans, mannans, glucomannans, and β-(1→3,1→4)-glucans (Scheller and Ulvskov 2010). They represent a supplemental source of cell wall sugars, in addition to cellulose, for conversion to biofuels and bio-based chemicals. The primary role of hemicelluloses is to strengthen the plant cell wall through interactions with cellulose and lignin (Scheller and Ulvskov 2010). Hemicellulose chains are long enough to interlace through the cellulose microfibrils, forming a complex network. Arabinoxylans are

similar to hemicelluloses in that they are predominantly chains of xylose, substituted with a few arabinose units. Pectin, considered to be the most structurally complex biopolymer, is a polymer of 1,4-linked α-D-galactosyluronic acid units (Mohnen 2008). Until recently, pectin had not received much attention as a potential feedstock for biofuels, despite the fact that pectin comprises approximately 2–35 % of plant primary walls depending on the type of tissue (Mohnen 2008; Edwards and Doran-Peterson 2012).

Analytical methods for characterizing polysaccharide structure and content include a variety of wet chemical degradation reactions and separation instrumentation such as capillary electrophoresis (CE) (Goubet et al. 2002; Suzuki and Honda 2003; Gamini et al. 2008; Persson et al. 2011; Li et al. 2013), high-performance liquid chromatography (HPLC) (Hausalo 1995; Rohrer 2003; Willfoer et al. 2009; Sluiter et al. 2010; Rohrer et al. 2013; Dvorackova et al. 2014), gas chromatography (GC) (Collings and Yokoyama 1979; Brummer and Cui 2005; Cui 2005; TAPPI 2009; Willfoer et al. 2009; Deutschmann and Dekker 2012; Sarossy et al. 2012), gel permeation chromatography (GPC) (White 1999), and size-exclusion chromatography (SEC) (Gillespie and Hammons 1999; Eremeeva 2003; Brummer and Cui 2005; Isogai and Yanagisawa 2008); mass spectrometry (MS) (Kelley et al. 2004; Cui 2005; Beecher et al. 2009; Ralet et al. 2009; Foston and Ragauskas 2012; Hounsell 2013); pyrolysis (py) (Kelley et al. 2004); thermogravimetric analysis (TGA) (Cozzani et al. 2008; Hatakeyama and Hatakeyama 2008; Serapiglia et al. 2008, Singh et al. 2009a; Carrier et al. 2011; Freda et al. 2012); spectroscopic tools such as mid-infrared (MIR) (Liang and Marchessault 1959a, b; Reeves 1997; Kacurikova et al. 1998; Kacurakova et al. 2000; Moore and Owen 2001; Workman 2001; Atalla and Isogai 2005; Choi et al. 2010; Coimbra et al. 2010; Persson et al. 2011; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013), near-infrared (NIR) (Reeves 1997; Hames et al. 2003; Kelley et al. 2004; Poke and Raymond 2006; Ye et al. 2008; Hames 2009; Templeton et al. 2009; Wolfrum and Sluiter 2009; Liu et al. 2010; Tyson et al. 2010; Deutschmann and Dekker 2012; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013), nuclear magnetic resonance (NMR) (Atalla et al. 1980; Atalla and VanderHart 1999; Ferrier et al. 2002; Maunu 2002; Atalla and Isogai 2005; Cui 2005; Beecher et al. 2009; Harris et al. 2010; Park et al. 2010; Persson et al. 2011; Deutschmann and Dekker 2012; Foston and Ragauskas 2012; Li et al. 2012; Kim et al. 2013; Lupoi et al. 2013; Nieto and Jiménez-Barbero 2013), Raman (Wiley and Atalla 1987a, b; Kenton and Rubinovitz 1990; Ona et al. 1997; Ona et al. 1998; Agarwal 1999; Schenzel and Fischer 2001; Workman 2001; Ona et al. 2003; Atalla and Isogai 2005; Schenzel et al. 2005; Agarwal 2008; Beecher et al. 2009; Schenzel et al. 2009; Agarwal and Atalla 2010; Agarwal et al. 2010; Choi et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Kim et al. 2013; Lupoi et al. 2013), and ultraviolet/visible (UV/Vis) (Brummer and Cui 2005; Masuko et al. 2005; Chundawat et al. 2008; Foster et al. 2010; Lupoi et al. 2013); X-ray diffraction (XRD) (Segal et al. 1959; Thygesen et al. 2005; Beecher et al. 2009; Agarwal et al. 2010; Harris et al. 2010; Park et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Kim et al. 2013); and microscopic methods such as atomic force microscopy (AFM) (Brant 1999; Abu-Lail and Camesano

2003; Stenius and Kolijonen 2008; Beecher et al. 2009; Funami 2010; Harris et al. 2010; Ding and Liu 2012; Foston and Ragauskas 2012) and scanning and transmission electron microscopy (SEM and TEM, respectively) (Atalla and Isogai 2005; Harris et al. 2010; Fazilova et al. 2011). Although this chapter will place emphasis on common or cutting-edge spectroscopic techniques for polysaccharide analysis, Table 1 provides a quick reference guide for a more complete breakdown of all methodologies. A recent review of how spectroscopic techniques have been employed for assessing lignocellulosic biomass illustrated the most contemporary and high-throughput methodologies (Lupoi et al. 2013).

1.1 Structural Analysis of Polysaccharides

1.1.1 Vibrational Spectroscopy

Raman spectroscopy is a noninvasive, nondestructive analytical tool that measures the scattering of photons when probed with an excitation source, commonly a laser (Carey 1982; Smith and Dent 2005). Approximately one per every one million photons will inelastically scatter with concomitant energy shift representing characteristic signatures of specific vibrational modes (Smith and Dent 2005). Thus, although Raman spectroscopy is a relatively weak phenomenon, it presents a versatile instrumental technique that has been routinely applied to measurements of polysaccharide structure, predominantly for cellulose analysis (Mathlouthi and Koenig 1986; Wiley and Atalla 1987a, b; Kenton and Rubinovitz 1990; Kacurikova et al. 1998; Agarwal 1999; Schenzel and Fischer 2001; Workman 2001; Atalla and Isogai 2005; Agarwal 2008; Schenzel et al. 2009; Agarwal and Atalla 2010; Agarwal et al. 2010; Choi et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Lupoi et al. 2013). Infrared and Raman spectroscopies are often described as complementary techniques, due to the difference in selection rules. In infrared spectroscopy, a change in the dipole moment of a bond is necessary to be infrared "active," while in Raman spectroscopy, there must be a change in polarizability. The C-C and C-O bonds composing cellulose have high polarizabilities resulting in quality Raman spectra showcasing strong, spectrally resolved peaks.

The application of Raman spectroscopy was significantly advanced through the work of Atalla and coauthors in the 1970s and 1980s (Wiley and Atalla 1987a, b; Atalla and Isogai 2005). The innovative instrument combined an optical microscope with a standard spectrometer. By using a microscope, the excitation source could be focused down to a smaller spot size, facilitating morphological analysis. The authors analyzed a variety of cellulose preparations, including fibers extracted from algae, deuterated algal and bacterial fibers, and ramie cellulose. The excitation light polarization was varied to further elucidate key cellulose vibrational modes. This study demonstrated the utility of Raman spectroscopy for cellulose structural analysis and provided a foundation with which future researchers could progress upon.

Cellulose polymorphism was also evaluated using the Raman microprobe. Cellulose is known to have two major conformations, celluloses I and II.

Table 1 Methods for analyzing polysaccharides

Polysaccharide component	Wet chemical methods	Chromatographic methods	Thermochemical methods	Spectroscopic methods	Microscopy and others
Cellulose content	Acid hydrolysis (Cui 2005; Foyle et al. 2007; Hames 2009; Sluiter Justin et al. 2010)	CE (Persson et al. 2011)	MALDI-MS (Foston and Ragauskas 2012)	FTIR (Li et al. 2010, 2011; Reeves 2012; Lupoi et al. 2013)	Raman imaging (Lupoi et al. 2013)
	Monoethanolamine (Foyle et al. 2007)	DASH (Li et al. 2013)	PyroMBMS (Kelley et al. 2004)	FT-Raman (Ona et al. 1997; Lupoi et al. 2013)	
	Updegraff (Updegraff 1969)	GC-FID (Willfoer et al. 2009)	TGA (Cozzani et al. 1997; Serapiglia et al. 2008, Singh et al. 2009a, Carrier et al. 2011; Freda et al. 2012)	NIR (Kelley et al. 2004; Poke and Raymond 2006; Ye et al. 2008; Hames 2009; Templeton et al. 2009; Wolfrum and Sluiter 2009; Liu et al. 2010; Tyson et al. 2010; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013)	
	Van Soest (Van Soest 1963; Van Soest et al. 1991)	GCMS (Willfoer et al. 2009)	TOF-SIMS (Foston and Ragauskas 2012)	UV/Vis (Masuko et al. 2005; Chundawat et al. 2008; Foster et al. 2010; Selig et al. 2011)	
		HPAE-PAD (Rohrer 2003; Willfoer et al. 2009)			
		LC (International 2007; Sluiter Justin et al. 2010)			
		PACE (Goubet et al. 2002; Persson et al. 2011)			

(*continued*)

Table 1 (continued)

Polysaccharide component	Wet chemical methods	Chromatographic methods	Thermochemical methods	Spectroscopic methods	Microscopy and others
Cellulose crystallinity				FT-Raman (Schenzel et al. 2005; Beecher et al. 2009, Agarwal et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Kim et al. 2013; Lupoi et al. 2013)	XRD (Segal et al. 1959; Thygesen et al. 2005; Beecher et al. 2009, Agarwal et al. 2010; Harris et al. 2010; Park et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Kim et al. 2013)
				Infrared (O'Connor et al. 1958; Akerholm et al. 2004; Beecher et al. 2009; Harris et al. 2010; Siroky et al. 2010; Kljun et al. 2011; Kim et al. 2013; Xu et al. 2013)	
				NMR (Beecher et al. 2009; Foston and Ragauskas 2010; Harris et al. 2010; Park et al. 2010; Cao et al. 2012; Foston and Ragauskas 2012; Kim et al. 2013; Lupoi et al. 2013)	
				SFG (Kim et al. 2013)	

Monosaccharide content	Methylation (Cui 2005)	DRFC-GC (Lu and Ralph 1997; Brummer and Cui 2005)	TGA (Cozzani et al. 1997; Serapiglia et al. 2008; Freda et al. 2012)	FT-Raman (Ona et al. 1997, 1998, 2003)	
	Sulfuric acid (Brummer and Cui 2005; Cui 2005; TAPPI 2009; Sluiter Justin et al. 2010)			FTIR (Rodrigues et al. 2001)	
	TFA (Collings and Yokoyama 1979; Fengel and Wegener 1979; Brummer and Cui 2005; Foyle et al. 2007; Lygin et al. 2011)	Gas-LC (TAPPI 2009)		NIR (Smith-Moritz et al. 2011)	
		GC-FID (Willfoer et al. 2009)		NMR (Alves et al. 2010; Gjersing et al. 2013)	
		GCMS (Willfoer et al. 2009; Sarossy et al. 2012)			
		HPAEC (Brummer and Cui 2005)			
		LC (Sluiter Justin et al. 2010; Dvorackova et al. 2014)			
		PACE (Goubet et al. 2002; Persson et al. 2011)			

(*continued*)

Table 1 (continued)

Polysaccharide component	Wet chemical methods	Chromatographic methods	Thermochemical methods	Spectroscopic methods	Microscopy and others
Noncellulosic polysaccharide content	Acid hydrolysis (Cui 2005; Hames 2009)	DASH (Li et al. 2013)	PyroMBMS (Kelley et al. 2004)	FT-Raman (Ona et al. 1997, 1998, 2003; Lupoi et al. 2013)	
	Ethanol extraction (Lygin et al. 2011)	HPLC (Deutschmann and Dekker 2012)	TGA (Carrier et al. 2011; Freda et al. 2012)	NIR (Kelley et al. 2004; Ye et al. 2008; Hames 2009; Templeton et al. 2009; Wolfrum and Sluiter 2009; Liu et al. 2010; Tyson et al. 2010; Deutschmann and Dekker 2012; Lupoi et al. 2013; Xu et al. 2013)	
	KOH extraction (Lygin et al. 2011)	Gas-LC (Collings and Yokoyama 1979)		NMR (Alves et al. 2010)	
		GC-FID (Willfoer et al. 2009)			
		HPAE-LC (Hausalo 1995; Rohrer 2003; Willfoer et al. 2009; Deutschmann and Dekker 2012)			
		GCMS (Willfoer et al. 2009; Deutschmann and Dekker 2012; Sarossy et al. 2012)			

Cellulosic and noncellulosic polysaccharide structure	Chemical characterization (Atalla and Isogai 2005; Cui 2005)	CE (Gamini et al. 2008)	MALDI-TOF (Cui 2005; Persson et al. 2011)	CARS (Foston and Ragauskas 2012; Pohling et al. 2014)	AFM (Brant 1999; Abu-Lail and Camesano 2003; Stenius and Kolijonen 2008; Beecher et al. 2009; Funami 2010; Harris et al. 2010; Ding and Liu 2012; Foston and Ragauskas 2012)
		DASH (Li et al. 2013)	MS (Cui 2005; Ralet et al. 2009; Hounsell 2013)	CD (Gekko 2013)	Confocal microscopy (Harris et al. 2010)
		DRFC-GC (Cui 2005)	SIMS (Beecher et al. 2009)		CRS microscopy (Zeng et al. 2012)
		GCMS (Cui 2005)	TGA (Hatakeyama and Hatakeyama 2008)	CoMPP (Persson et al. 2011)	EM (Atalla and Isogai 2005; Harris et al. 2010; Fazilova et al. 2011)
		HPAE-PAD (Rohrer 2003; Willfoer et al. 2009; Persson et al. 2011; Hounsell 2013)	TOF-SIMS (Tokareva et al. 2007)	Fluorescence (Castellan et al. 2007; Lupoi et al. 2013)	Fluorescence imaging (Castellan et al. 2007; Liu et al. 2009; Singh et al. 2009b; Wang et al. 2010, 2012; Sun et al. 2013)
		Methylation (Harris et al. 1984)		FTIR (Liang and Marchessault 1959a, b; Mathlouthi and Koenig 1986; Kacurikova et al. 1998; Moore and Owen 2001; Workman 2001; Atalla and Isogai 2005; Choi et al. 2010; Coimbra et al. 2010; Persson et al. 2011; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013)	Raman imaging and microscopy (Agarwal 2006; Hamad 2008; Singh et al. 2009b; Sun et al. 2010; Lupoi et al. 2013; Sun et al. 2013)

(*continued*)

Table 1 (continued)

Polysaccharide component	Wet chemical methods	Chromatographic methods	Thermochemical methods	Spectroscopic methods	Microscopy and others
		PMAA (Cui 2005)		NMR (Atalla et al. 1980; Atalla and VanderHart 1999; Ferrier et al. 2002; Maunu 2002; Atalla and Isogai 2005; Cui 2005; Balakshin et al. 2008; Yelle et al. 2008; Hedenstroem et al. 2009; Rencoret et al. 2009; Persson et al. 2011; Yuan et al. 2011; Deutschmann and Dekker 2012; Foston and Ragauskas 2012; Foston et al. 2012; Li et al. 2012; Mazumder et al. 2012; Du et al. 2013; Lupoi et al. 2013; Miyagawa et al. 2013; Nieto and Jiménez-Barbero 2013; Du et al. 2014)	SAXS (Brant 1999)

		TLC (Hounsell 2013)		Raman (Mathlouthi and Koenig 1986; Wiley and Atalla 1987a, b; Kenton and Rubinovitz 1990; Kacurikova et al. 1998; Agarwal 1999; Kacurakova et al. 2000; Kacurakova and Wilson 2001; Schenzel and Fischer 2001; Workman 2001; Atalla and Isogai 2005; Schenzel et al. 2005; Agarwal 2008; Schenzel et al. 2009; Agarwal and Atalla 2010; Choi et al. 2010; Lupoi and Smith 2012; Lupoi et al. 2013)	
Total carbohydrates	Anthrone-sulfuric acid (Brummer and Cui 2005)	Gas-LC (Collings and Yokoyama 1979)		FT-Raman (Lupoi et al. 2013)	
	DNS (Miller 1959)	ME (Suzuki and Honda 2003)		FTIR (Lupoi et al. 2013)	
	Phenol-sulfuric acid (Brummer and Cui 2005; Masuko et al. 2005)			NIR (Lupoi et al. 2013)	
	TFA (Fengel and Wegener 1979)			UV/Vis (Lupoi et al. 2013)	

Cellulose I is the native structure, while cellulose II is produced from mercerization or dissolution and subsequent regeneration of the fibers. To investigate whether cellulose I possessed internal polymorphism, dependent on the source, algal and ramie celluloses were measured. Spectral regions below 1,600 cm^{-1} were found to be most sensitive to conformational changes in the cellulose backbone, while the spectral contributions above 2,700 cm^{-1} were more sensitive to hydrogen bonding. By comparing the spectra of different native celluloses, the authors determined that any polymorphic structures belonging to cellulose I must be similar in conformational structure, as relatively few spectral differences were elucidated. The analysis of native celluloses at the higher wavenumber spectral regions revealed significant differences in cellulose hydrogen bonding. Schenzel et al. explored the use of Fourier transform (FT)-Raman spectroscopy to monitor the transformation of cellulose I into cellulose II and compared the Raman spectral data with that obtained using XRD and NMR (Schenzel and Fischer 2001; Schenzel et al. 2005, 2009). Raman microscopy can also be used to evaluate the deformation of cellulose (Hamad 2008). Shifts in characteristic Raman vibrational modes have been detected that coincide with the application of stress. For example, the 1,095 cm^{-1} mode has been measured to shift to lower wavenumbers and decrease in intensity as the strain on the cellulosic fibers increases.

Raman spectra of polysaccharides can be partitioned into distinct vibrational modes as depicted in Table 2. The most intense Raman peaks are typically near 1,091 and 1,117 cm^{-1} and correspond to C-C and C-O stretching (Wiley and Atalla 1987a, b; Agarwal 1999; Lupoi and Smith 2012). Other intense vibrational modes occur at 380, 435, 458, 520, 896, 1,002, 1,033, 1,074, 1,147, 1,268, 1,338, 1,376, and 1,460 cm^{-1}. Figure 1 depicts NIR, dispersive Raman spectra of microcrystalline cellulose and a commercially available hydrolytic lignin preparation (Lupoi and Smith 2012). A comparison of these spectra revealed that the main lignin vibrational modes are near 1,600 cm^{-1}, isolated from the aforementioned cellulose peaks. This can facilitate the analysis of solid biomass, at least in regard to the identification of peaks stemming from lignin and polysaccharides, as seen in Fig. 2, for a diverse assortment of potential biofuel feedstocks. In this figure, for example, a variety of hard- and softwoods and herbaceous feedstocks were grouped according to spectral similarity. Xylan structure has also been evaluated using FT-Raman spectroscopy (Kacurikova et al. 1998). As can be seen from Table 2, many of the vibrational modes of xylan and cellulose have overlapping or proximate peaks, due to their structural similarity. However, the analysis of the less intense vibrational modes of glucan and xylan can often reveal unique spectral signatures. Figure 3 illustrates this technique, as developed by Shih et al., depicting the simultaneous quantitation of glucose and xylose in a complex saccharification matrix, enabled by the identification of distinctive vibrational modes from the measurement of glucose and xylose standards using a spectral peak deconvolution method (Shih et al. 2011). The malleability of this method should allow its extension to the qualitative assessment of glucan and xylan polymers.

Raman imaging has enabled researchers to localize plant cell wall constituents such as cellulose and hemicellulose, as exemplified in Fig. 4, where black spruce

Table 2 Characteristic Raman vibrational modes for polysaccharides

Approximate peak location	Polysaccharide	Primary assignment
311	Cellulose II	CCO torsion (Schenzel and Fischer 2001)
329	Cellulose	CCC, CO, CCO, ring deformation (Schenzel and Fischer 2001)
331	Cellulose	Heavy atom bending (Wiley and Atalla 1987a, b)
344	Cellulose	Heavy atom stretching (Wiley and Atalla 1987a, b), CCC, CO, CCO, ring deformation (Schenzel and Fischer 2001)
352	Cellulose II	CCC, CO, CCO, ring deformation (Schenzel and Fischer 2001)
373	Cellulose II	CCC, CO, CCO, ring deformation (Schenzel and Fischer 2001)
376	Holocellulose	Holocellulose (Kenton and Rubinovitz 1990)
380	Cellulose	Heavy atom stretching (Wiley and Atalla 1987a, b), CCC, CO, CCO, ring deformation (Schenzel and Fischer 2001)
418	Cellulose II	CCC, CCO, ring deformation (Schenzel and Fischer 2001)
437	Cellulose	Heavy atom stretching (Wiley and Atalla 1987a, b), CCC, CCO, ring deformation (Schenzel and Fischer 2001)
458	Celluloses I and II	Heavy atom stretching (Wiley and Atalla 1987a, b), CCC, CCO, ring deformation (Schenzel and Fischer 2001)
503	Xylan	CCO (Kacurikova et al. 1998)
519	Cellulose	Heavy atom stretching (Wiley and Atalla 1987a, b)
521	Xylan	CCO (Kacurikova et al. 1998)
531	Xylan	CCO (Kacurikova et al. 1998)
553	Xylan	CCO (Kacurikova et al. 1998)
609	Cellulose	CCH torsion (Schenzel and Fischer 2001)
897	Celluloses I and II, holocellulose, xylan	Holocellulose (Kenton and Rubinovitz 1990), CH, ring (Kacurikova et al. 1998), C1-H (Schenzel and Fischer 2001)
904	Cellulose, xylan	CH, ring (Kacurikova et al. 1998; Schenzel and Fischer 2001)
910	Cellulose	HCC and HCO bending (Wiley and Atalla 1987a, b)
969	Celluloses I and II	CC and CO stretching (Wiley and Atalla 1987a, b), CH_2 (Schenzel and Fischer 2001)
977	Xylan	CO (Kacurikova et al. 1998)
995	Cellulose	CC and CO stretching (Wiley and Atalla 1987a, b)
997	Cellulose	CH_2 (Schenzel and Fischer 2001)
1,000–1,010	Cellulose, xylan	CC and COH (Kacurikova et al. 1998)
1,026	Xylan	CC and COH (Kacurikova et al. 1998)
1,037	Cellulose	CC and CO stretching (Wiley and Atalla 1987a, b)
1,057	Cellulose	CC and CO stretching (Wiley and Atalla 1987a, b)

(*continued*)

Table 2 (continued)

Approximate peak location	Polysaccharide	Primary assignment
1,089	Xylan	COC, C-C, ring vibration (Kacurikova et al. 1998)
1,095	Celluloses I and II	CC and CO stretching (Wiley and Atalla 1987a, b), COC, glycosidic, ring breathing, symmetric (Schenzel and Fischer 2001)
1,101	Xylan	COC, C-C, ring vibration (Kacurikova et al. 1998)
1,106	Xylan	COC, C-C, ring vibration (Kacurikova et al. 1998)
1,115	Cellulose II	COC, glycosidic, ring breathing, symmetric (Schenzel and Fischer 2001)
1,117	Cellulose, holocellulose	CC and CO stretching (Wiley and Atalla 1987a, b), holocellulose (Kenton and Rubinovitz 1990)
1,121	Cellulose	CC and CO stretching (Wiley and Atalla 1987a, b), COC, glycosidic, ring breathing, symmetric (Schenzel and Fischer 2001)
1,125	Cellulose, xylan	COC and C-C (Kacurikova et al. 1998)
1,142	Cellulose II	CC, CO ring breathing, asymmetric (Schenzel and Fischer 2001)
1,151	Cellulose	Stretching and HCC and HCO bending (Wiley and Atalla 1987a, b), CC, CO ring breathing, asymmetric (Schenzel and Fischer 2001)
1,202	Celluloses I and II	CH_2, HCC, HOC, COH (Schenzel and Fischer 2001)
1,262	Cellulose II	CH_2, HCC, HOC, COH (Schenzel and Fischer 2001)
1,275	Cellulose	HCC and HCO bending (Wiley and Atalla 1987a, b)
1,291	Cellulose	HCC and HCO bending (Wiley and Atalla 1987a, b), CH_2, HCC, HCO, COH (Schenzel and Fischer 2001)
1,312	Xylan	CH, COH (Kacurikova et al. 1998)
1,331	Cellulose	HCC and HCO bending (Wiley and Atalla 1987a, b)
1,333	Holocellulose	Holocellulose (Kenton and Rubinovitz 1990)
1,337	Celluloses I and II	HCC, HCO, and HOC bending (Wiley and Atalla 1987a, b), CH_2, HCC, HCO, COH (Schenzel and Fischer 2001)
1,365	Xylan	CH, OH stretch (Kacurikova et al. 1998)
1,373	Cellulose II	CH_2, HCC, HCO, COH (Schenzel and Fischer 2001)
1,376	Xylan	CH, OH stretch (Kacurikova et al. 1998)
1,378	Cellulose, holocellulose	HCC, HCO, and HOC bending (Wiley and Atalla 1987a, b; Schenzel and Fischer 2001), holocellulose (Kenton and Rubinovitz 1990), CH_2 (Schenzel and Fischer 2001)
1,407	Cellulose	HCC, HCO, and HOC bending (Wiley and Atalla 1987a, b; Schenzel and Fischer 2001), CH_2 (Schenzel and Fischer 2001)
1,456	Cellulose	HCH and HOC bending (Wiley and Atalla 1987a, b; Schenzel and Fischer 2001), CH_2 scissoring (Schenzel and Fischer 2001)
1,461	Cellulose II	CH_2 scissoring (Schenzel and Fischer 2001)

(*continued*)

Table 2 (continued)

Approximate peak location	Polysaccharide	Primary assignment
1,475	Cellulose	HCH and HOC bending (Wiley and Atalla 1987a, b), CH_2 scissoring (Schenzel and Fischer 2001)
1,736	Holocellulose	Holocellulose (Kenton and Rubinovitz 1990)
2,853	Cellulose	CH_2 symmetric stretch (Liang and Marchessault 1959a)
2,866	Cellulose	CH and CH_2 stretching (Wiley and Atalla 1987a, b)
2,889	Cellulose	CH and CH_2 stretching (Wiley and Atalla 1987a, b)
2,917	Xylan	CH stretch (Kacurikova et al. 1998)
2,933	Xylan	CH stretch (Kacurikova et al. 1998)
2,943	Cellulose	CH and CH_2 stretching (Wiley and Atalla 1987a, b)
2,963	Cellulose	CH and CH_2 stretching (Wiley and Atalla 1987a, b)
3,286	Cellulose	OH stretch (Wiley and Atalla 1987a, b)
3,335	Cellulose	OH stretch (Wiley and Atalla 1987a, b)
3,363	Cellulose	OH stretch (Wiley and Atalla 1987a, b)
3,402	Cellulose	OH stretch (Wiley and Atalla 1987a, b)

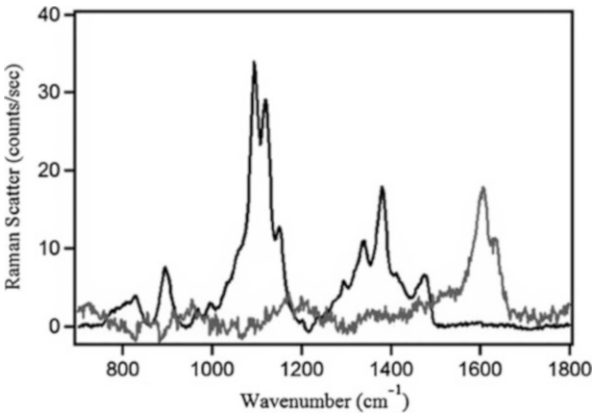

Fig. 1 NIR, dispersive Raman spectra of cellulose (*black*) and lignin (*gray*). The lignin spectrum has been multiplied by a factor of 10 to allow a better visual spectral comparison (Reprinted with permission from Lupoi and Smith 2012)

cell wall sections high in cellulose content were exposed (Agarwal 2006; Lupoi et al. 2013). These images and the corresponding Raman spectra (Fig. 5) reveal higher cellulose content in the secondary S2 region of the cell wall and a much lower cellulose fraction in the cell corners and compound middle lamella, areas known to contain more lignin. Imaging techniques have been utilized to monitor chemical pretreatments that render the biomass less recalcitrant to enzymatic hydrolysis. Recently, the deconstruction of feedstocks using ionic liquids (ILs) has been juxtaposed to acid and ammonia pretreatment strategies in corn stover and *Eucalyptus globulus* (Sun et al. 2010, 2013). Figures 6 and 7 illustrate the employment of Raman imaging for localizing cellulose content in tracheids (Fig. 6) and sclerenchyma cells (Fig. 7) before and during an IL pretreatment. This study demonstrated that while

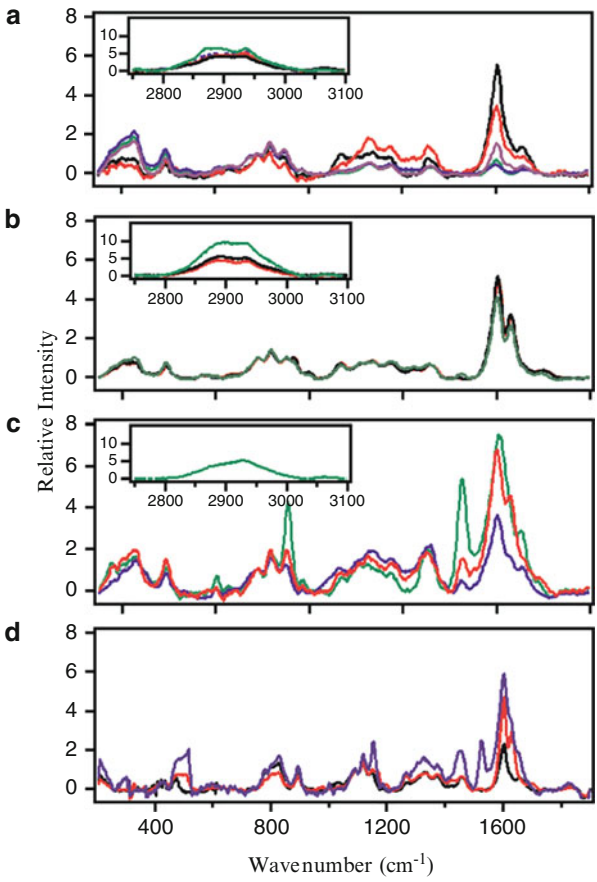

Fig. 2 NIR, dispersive Raman spectra of solid biomass. **a** Spectra of kenaf bast (*blue*), kenaf core (*purple*), oak (*red*), pine (*black*), and poplar (*green*). **b** Spectra of miscanthus (*red*), pampas grass (*black*), and switchgrass (*green*). (**c**) Spectra of alfalfa (*blue*), orchard grass (*red*), and red clover (*green*). (**d**) Averaged spectra from panels (**a**) (*black*), (**b**) (*red*), and (**c**) (*blue*) (Reprinted with permission from Lupoi and Smith 2012)

lignin dissolution preferentially occurred in specific cell wall regions, there was no particular trend in cellulose dissolution. In general, Raman imaging techniques provide real-time evaluations of how plant cell walls are constructed and, more importantly, how efficient a projected pretreatment approach may be in diminishing the complex interlacing of cellulose, hemicellulose, and lignin. This live visualization is paramount for optimizing biomass-to-biofuel conversion efficiency. One of the major drawbacks in generating the images, however, can be long acquisition times due to the weak spontaneous Raman scattering phenomenon.

Coherent anti-Stokes Raman spectroscopy (CARS) and microscopy and stimulated Raman spectroscopy (SRS) have been employed to enhance spectral intensities, thereby alleviating a fundamental limitation of using spontaneous Raman scattering (Saar et al. 2010; Foston and Ragauskas 2012; Zeng et al. 2012; Pohling et al. 2014). Both techniques utilize two lasers that are overlapped spatially and temporally. When the frequency difference is resonated with a Raman-active frequency, the signal intensifies. The more intense spectra have facilitated more rapidly acquired images. Both techniques have the added benefit of reducing

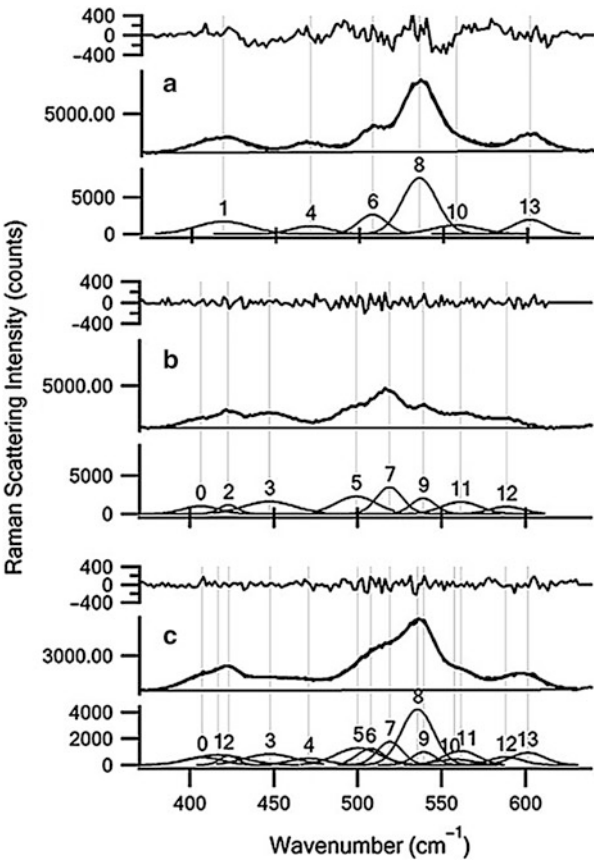

Fig. 3 NIR, dispersive Raman analysis of xylose (**a**) and glucose (**b**) standards in a simulated, complex saccharification matrix. Panel (**c**) represents a mixture of glucose and xylose. From the evaluation of the individual standards, and the mixture, vibrational modes at 519 and 536 cm^{-1} were selected for glucose and xylose analysis, respectively. In each panel, the top plot represents the amount of residuals left from the peak-fitting deconvolution, the middle panel depicts the raw spectra, and the bottom panel shows the peak deconvolution (Reprinted with permission from Shih et al. 2011)

intrinsic biomass fluorescence detrimental to spectra generated via spontaneous Raman scattering. SRS has been more successful for imaging cellulose as CARS suffers from photomultiplier limitations at the optimal acquisition parameters for cellulose detection (Zeng et al. 2012). Another benefit of SRS is the linear dependency of the signal on chemical concentration, like the signal generated using spontaneous Raman scattering. This means that the vibrational modes in a SRS spectrum can be defined using standard Raman peak assignments.

MIR spectroscopy, another nondestructive, noninvasive tool, provides complementary structural information to Raman spectroscopy due to the difference in the selection rules governing the criteria for obtaining MIR and Raman-active vibrational modes, as described above. A significant dissimilarity, however, is that in MIR spectroscopy, molecules are actually excited to higher energy levels, whereas in Raman spectroscopy, molecules are promoted to a short-lived "virtual" state, then quickly relax. Conjunctive use of both techniques can supply a more thorough qualitative structural analysis. Table 3 lists the main polysaccharide vibrational modes measured using MIR spectroscopy. The ratio of the vibrational modes at

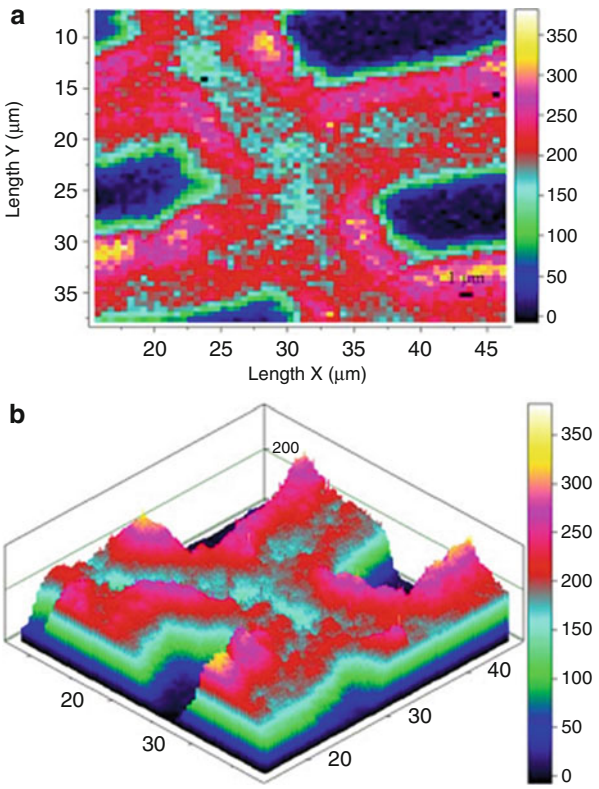

Fig. 4 False color two-dimensional (**a**) and three-dimensional (**b**) Raman images of cellulose distribution in the cell wall of *black* spruce. *Bright white* and/or *yellow* colors reveal cell wall areas high in cellulose, while *dark blue* and/or *black* regions show area very low in cellulose (Reprinted with permission from Agarwal 2006)

1,370 and 670 cm^{-1} enabled the estimation of cellulose I_α and I_β allomorphs in various biomass pulps (Akerholm et al. 2004). Specific bonds like hydrogen bonds in native cellulose or glycosidic linkages in polysaccharides have also been evaluated using MIR spectroscopy (Liang and Marchessault 1959; Nikonenko et al. 2000). For example, polysaccharides possessing 1→4 glycosidic bonds contain distinctive vibrational modes between 1,140 and 1,175 cm^{-1} (Nikonenko et al. 2000). In this same study, the authors presented a method for deconvoluting the spectra between 970 and 1,000 cm^{-1}, revealing multiple bands in the polysaccharides compared to one band when a correlative monomer was measured.

MIR spectroscopy has been extensively employed for evaluating polysaccharide structure and conformation, often subsequent to the chemical and/or physical pretreatment of the biomass (Liang and Marchessault 1959a, b; Mathlouthi and Koenig 1986; Kacurakova et al. 2000; Kacurakova and Wilson 2001; Moore and Owen 2001; Workman 2001; Cui 2005; Coimbra et al. 2010; Persson et al. 2011; Deutschmann and Dekker 2012; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013). As mentioned above, ILs have been increasingly explored as environmentally friendly, deconstruction solvents capable of efficiently solubilizing cell wall constituents. The cellulose can be regenerated using an anti-solvent, such as water. Evaluations of cellulose before and after IL pretreatment of eucalyptus (Cetinkol et al. 2010)

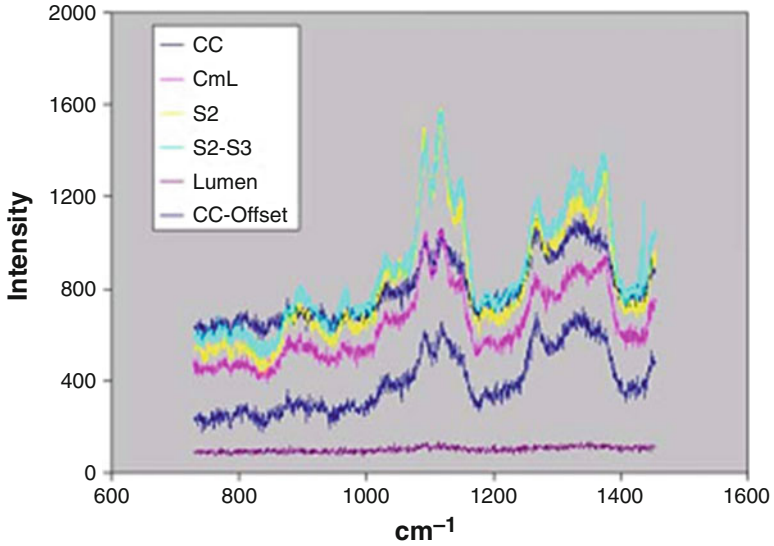

Fig. 5 Raman spectra acquired from various cell wall regions of *black* spruce. Spectrum CC offset (identical to CC) has been spatially offset to facilitate the spectral analysis. A higher cellulose concentration is indicated by more intense peaks at 1,098, 1,123, and 1,150 cm^{-1} (Reprinted with permission from Agarwal 2006)

and the ammonia fiber expansion pretreatment of corn stover (Li et al. 2011) using MIR spectroscopy have enabled researchers to determine if the native structure of cellulose is retained or is significantly altered after pretreatment. Figure 8 illustrates how IR spectroscopy enabled a comparison of *E. globulus* biomass before and after treatment with the IL 1-ethyl-3-methylimidazolium acetate [Cmim][OAc].

Specific bonds like hydrogen bonds in native cellulose or glycosidic linkages in polysaccharides have also been evaluated using MIR spectroscopy (Liang and Marchessault 1959; Nikonenko et al. 2000). For example, polysaccharides possessing 1→4 glycosidic bonds contain distinctive vibrational modes between 1,140 and 1,175 cm^{-1} (Nikonenko et al. 2000). In this same study, the authors presented a method for deconvoluting the spectra between 970 and 1,000 cm^{-1}, revealing multiple bands in the polysaccharides, juxtaposed to one band when a correlative monomer was measured.

In addition to the studies on cellulose, the structural evaluation of xylan has been explored using MIR spectroscopy (Kacurikova et al. 1998; Kacurakova et al. 2000; Kacurakova and Wilson 2001). Xylans reveal significant structural heterogeneity due to the diverse branching from the main polymeric chain. The spectra of β-(1→3)- and β-(1→4)-linked xylose groups show distinguishable signatures dependent on the degree of branching of the main chain and the type of substitutions. The analysis of complex mixtures of polysaccharides, including arabinoglucuronoxylan and galactoglucomannan or arabinogalactan and mannan, displayed at least one unique vibrational mode exclusive to each constituent (Kacurakova et al. 2000).

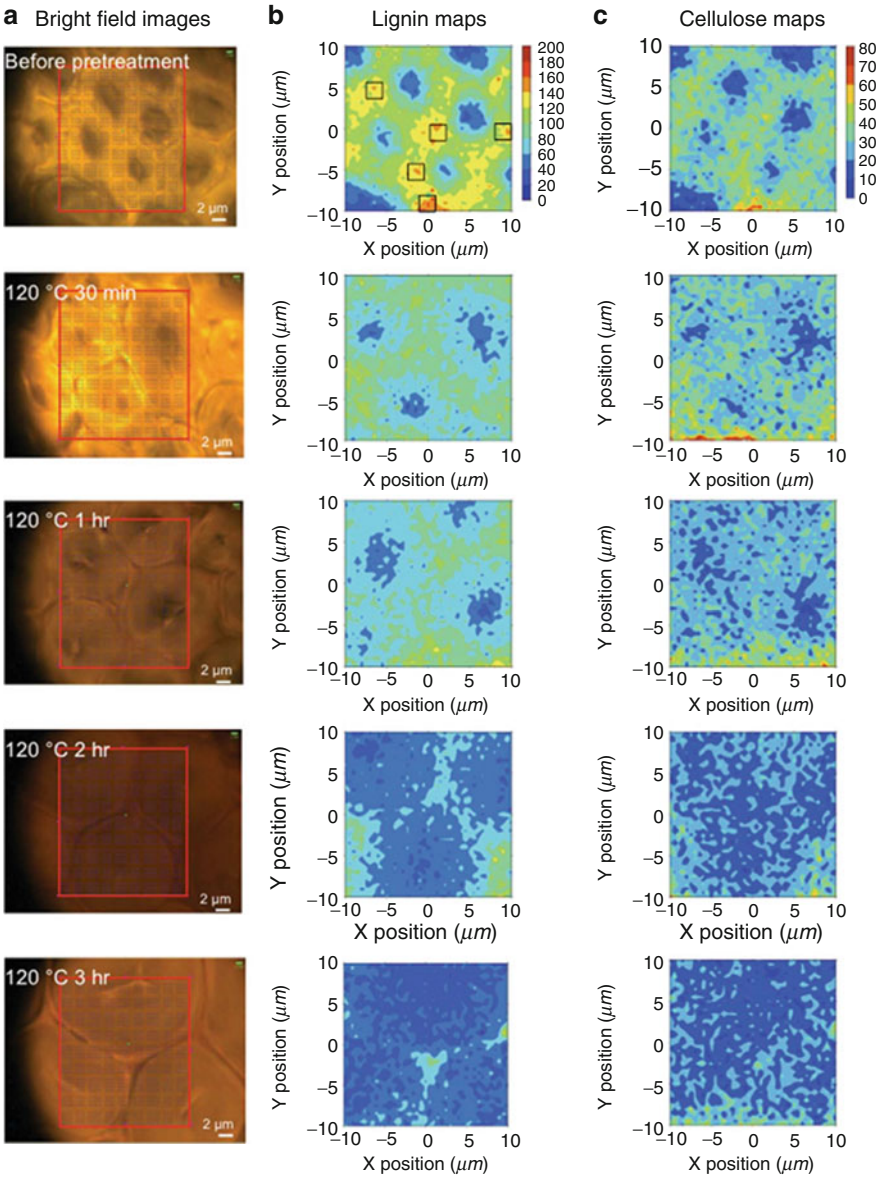

Fig. 6 Raman bright-field images of corn stover tracheids (**a**) and corresponding lignin (**b**) and cellulose (**c**) maps before and during ionic liquid pretreatment (Reprinted with permission from Sun et al. 2013)

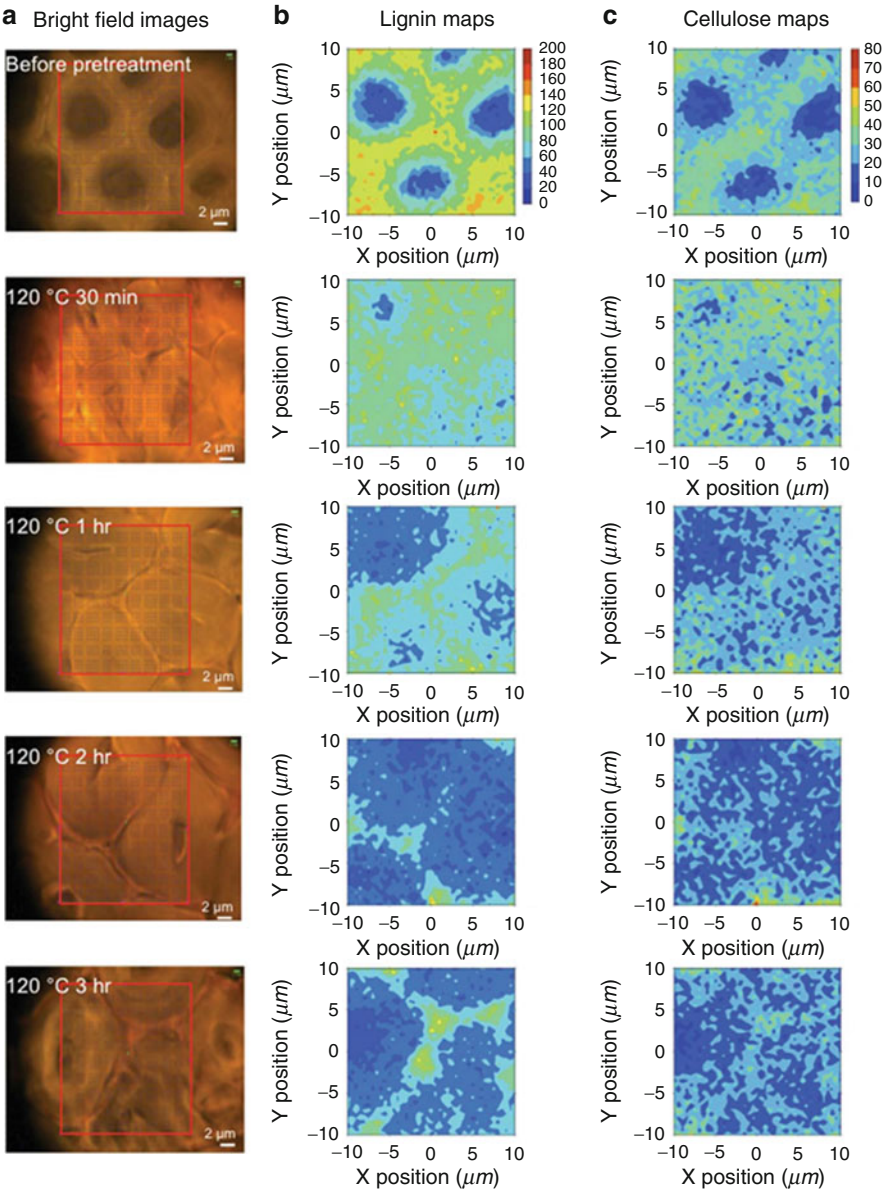

Fig. 7 Raman bright-field images of corn stover sclerenchyma cells (**a**) and corresponding lignin (**b**) and cellulose (**c**) maps before and during ionic liquid pretreatment (Reprinted with permission from Sun et al. 2013)

Table 3 Characteristic MIR vibrational modes for polysaccharides

Approximate peak location	Polysaccharide	Primary assignment
531	Xylan	CCO (Kacurikova et al. 1998)
590	Xylan	CCO (Kacurikova et al. 1998)
604	Xylan	CCO (Kacurikova et al. 1998)
656–667	Cellulose, xylan	CCO, OH (out of plane) (Liang and Marchessault 1959b; Kacurikova et al. 1998)
700	Cellulose	OH out-of-plane bending (Liang and Marchessault 1959b)
740	Cellulose	CH_2 rocking (Liang and Marchessault 1959b)
800	Cellulose	Ring breathing (Liang and Marchessault 1959b)
807	Arabinan, arabinogalactan	CH, ring (Kacurakova et al. 2000)
811	Xylan	Ring (Kacurikova et al. 1998)
814	Galactoglucomannan, glucomannan	CH, ring (Kacurakova et al. 2000)
835	Pectin	CH, ring (Kacurakova et al. 2000)
840	Glucan	CH, ring (Kacurakova et al. 2000)
856	Pectin, xylan	Furanoid ring (Kacurikova et al. 1998), CH, ring (Kacurakova et al. 2000)
868	Arabinogalactan	CH, ring (Kacurakova et al. 2000)
872	Galactoglucomannan, glucomannan	CH, ring (Kacurakova et al. 2000)
875	Hemicellulose	Glycosidic linkage (Sills and Gossett 2012)
883	Galactan	CH, ring (Kacurakova et al. 2000)
893	Galactan	CH, ring (Kacurakova et al. 2000)
897	Arabinan, arabinogalactan, xyloglucan, cellulose, pectin, galactoglucomannan, glucomannan, xylan	CH, ring (Kacurikova et al. 1998; Kacurakova et al. 2000), antisymmetrical out-of-phase stretching (Liang and Marchessault 1959b)
904	Xylan	CH, ring (Kacurikova et al. 1998)
916	Arabinan, glucan	CH, ring (Kacurakova et al. 2000)
930	Cellulose, hemicellulose	CH, ring (Kacurakova et al. 2000), glycosidic linkage (Sills and Gossett 2012)
934	Galactoglucomannan	CH, ring (Kacurakova et al. 2000)
941	Glucomannan	CH, ring (Kacurakova et al. 2000)
945	Xyloglucan	CH, ring (Kacurakova et al. 2000)
953	Pectin	CH, ring (Kacurakova et al. 2000)
960	Galactoglucomannan	CH, ring (Kacurakova et al. 2000)

(*continued*)

Table 3 (continued)

Approximate peak location	Polysaccharide	Primary assignment
985	Cellulose, xylan	CO stretch, OH, ring (Liang and Marchessault 1959b; Kacurikova et al. 1998)
990	Cellulose	CO stretching (Liang and Marchessault 1959b)
1,000	Cellulose	CO stretching (Liang and Marchessault 1959b)
1,015	Cellulose	CO stretching (Liang and Marchessault 1959b)
1,017	Pectin	COH, COC, CC, ring (Kacurakova et al. 2000)
1,026	Glucan, xylan	CC, COH (Kacurikova et al. 1998; Kacurakova et al. 2000), COC, ring (Kacurakova et al. 2000)
1,034	Cellulose, galactoglucomannan, glucomannan, hemicellulose	COH, COC, CC, ring (Kacurakova et al. 2000), CO, CCO stretching (Sills and Gossett 2012)
1,038	Galactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,041	Arabinan, xyloglucan, glucan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,045	Arabinogalactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,047	Pectin, xylan	CC, CO (Kacurikova et al. 1998), COH, COC, ring (Kacurakova et al. 2000)
1,059	Cellulose	COH, COC, CC, ring (Kacurakova et al. 2000)
1,064	Galactoglucomannan, glucomannan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,068	Arabinan, xylan	CO, CC, ring (Kacurikova et al. 1998; Kacurakova et al. 2000), COH, COC (Kacurakova et al. 2000)
1,072	Galactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,074	Arabinogalactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,078	Glucan, xyloglucan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,089	Xylan	CO, CC, ring (Kacurikova et al. 1998)
1,092	Glucomannan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,097	Arabinan	COH, COC, CC, ring (Kacurakova et al. 2000)

(*continued*)

Table 3 (continued)

Approximate peak location	Polysaccharide	Primary assignment
1,101	Pectin, xylan	CO, CC, ring (Kacurikova et al. 1998; Kacurakova et al. 2000), COH, COC (Kacurakova et al. 2000)
1,104	Glucan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,110	Cellulose	Antisymmetrical in-phase ring stretching (Liang and Marchessault 1959b)
1,118	Cellulose, xyloglucan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,121–1,131	Xylan	COC, CC (Kacurikova et al. 1998)
1,134	Galactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,141	Arabinan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,144	Pectin	COH, COC, CC, ring (Kacurakova et al. 2000)
1,149	Galactoglucomannan	COC, CC (Kacurikova et al. 1998; Kacurakova et al. 2000), COH, ring (Kacurakova et al. 2000)
1,151	Glucan, glucomannan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,153	Xyloglucan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,155	Galactan	COH, COC, CC, ring (Kacurakova et al. 2000)
1,162	Cellulose	COH, COC, CC, ring (Kacurakova et al. 2000; Sills and Gossett 2012), antisymmetrical bridge oxygen stretching (Liang and Marchessault 1959b)
1,169	Xylan	COC, CC (Kacurikova et al. 1998)
1,171	Xylan	COC, CC (Kacurikova et al. 1998)
1,200	Cellulose, hemicellulose	OH bending (Sills and Gossett 2012)
1,205	Cellulose	OH in-plane bending (Liang and Marchessault 1959b)
1,235	Cellulose	(Liang and Marchessault 1959b)
1,250	Cellulose	(Liang and Marchessault 1959b)
1,282	Cellulose	CH bending (Liang and Marchessault 1959b; Sills and Gossett 2012)
1,310	Cellulose, hemicellulose	CH_2 wagging (Sills and Gossett 2012)
1,312	Xylan	CH, COH (Kacurikova et al. 1998)

(*continued*)

Table 3 (continued)

Approximate peak location	Polysaccharide	Primary assignment
1,317	Cellulose	CH_2 wagging (Liang and Marchessault 1959b)
1,336	Cellulose, hemicellulose	OH in-plane bending (Liang and Marchessault 1959b; Sills and Gossett 2012), CH vibration (Sills and Gossett 2012)
1,348	Xylan	CH (Kacurikova et al. 1998)
1,358	Cellulose	(Liang and Marchessault 1959b)
1,365	Xylan	CH, OH (Kacurikova et al. 1998)
1,374	Cellulose	CH bending (Liang and Marchessault 1959b)
1,381	Cellulose, hemicellulose, xylan	CH bending, OH (Kacurikova et al. 1998; Sills and Gossett 2012)
1,430	Cellulose	CH_2 bending (Liang and Marchessault 1959b)
1,440	Cellulose, hemicellulose	OH in-plane bending (Sills and Gossett 2012)
1,455	Cellulose	OH in-plane bending (Liang and Marchessault 1959b)
1,730	Cellulose, hemicellulose	Ketone/aldehyde C=O stretch (Sills and Gossett 2012)
1,750	Cellulose, hemicellulose	Free ester (Sills and Gossett 2012)
2,853	Cellulose	CH_2 symmetric stretch (Liang and Marchessault 1959a)
2,870	Cellulose	CH stretching (Liang and Marchessault 1959a)
2,910	Cellulose	CH stretching (Liang and Marchessault 1959a)
2,945	Cellulose	CH_2 antisymmetrical stretching (Liang and Marchessault 1959a)
2,970	Cellulose	CH stretching (Liang and Marchessault 1959a)
3,248	Xylan	OH (Kacurikova et al. 1998)
3,275	Cellulose	(Liang and Marchessault 1959a)
3,305	Cellulose	OH stretching (Liang and Marchessault 1959a)
3,350	Cellulose	OH stretching (Liang and Marchessault 1959a)
3,375	Cellulose	(Liang and Marchessault 1959a)
3,400	Xylan	OH stretching (Kacurikova et al. 1998)
3,405	Cellulose	OH stretching (Liang and Marchessault 1959a)

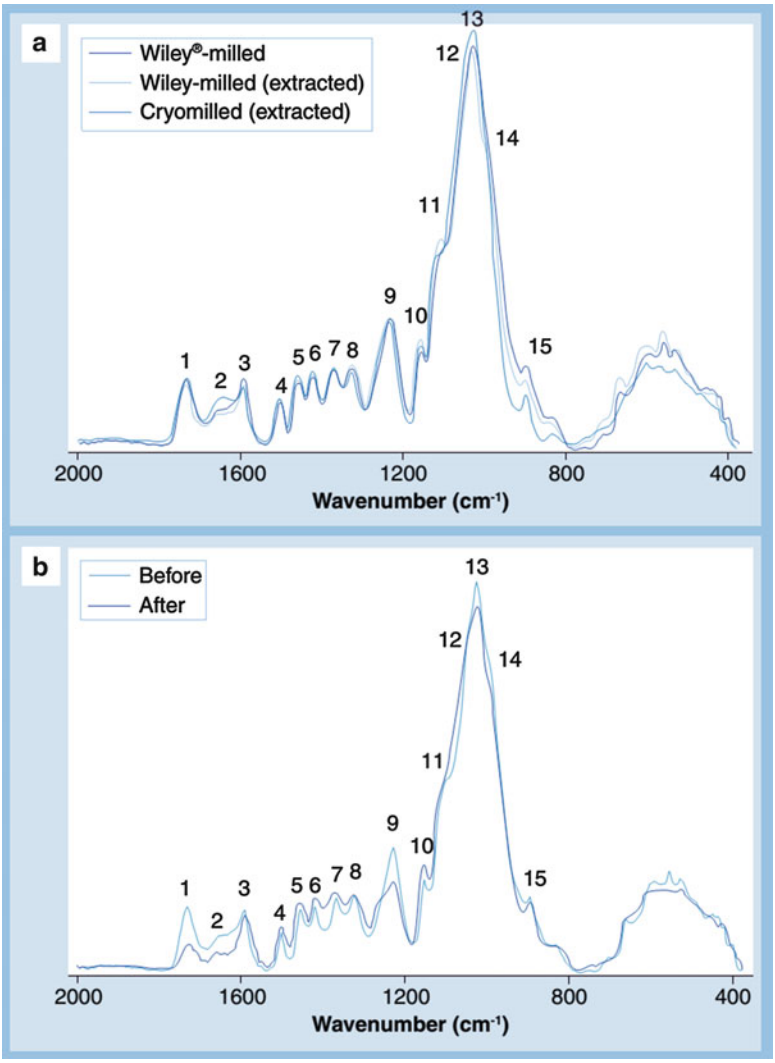

Fig. 8 (**a**) FTIR spectra of Wiley-milled *Eucalyptus globulus* with and without a water/ethanol Soxhlet extraction and cryomilled, water/ethanol Soxhlet extracted *E. globulus*. (**b**) FTIR spectra before and after treatment with an ionic liquid, 1-ethyl-3-methylimidazolium acetate (Reprinted from Centinkol et al. 2010)

1.1.2 Fluorescence Imaging and Spectroscopy

When a molecule absorbs electromagnetic radiation from a light source, such as a lamp or laser, it can be excited to higher energy levels. The molecule will then relax back to a more stable energy state, often with a concomitant emission of radiation in the form of heat or light. When light is emitted, the phenomenon is termed fluorescence. Often, molecules that absorb light in the UV or higher-energy visible

region of the spectrum will emit strong, measurable fluorescence. Fluorescence instrumentation can provide 2–3 orders of magnitude more sensitive quantitation compared to UV/Vis spectrophotometry (Schmidt 2010; Lupoi et al. 2013). Fluorescence imaging and spectroscopy methods have been employed to study the interactions of cellulase enzymes with cellulose (Liu et al. 2009; Wang et al. 2010, 2012). Single molecule and fluorescence resonance energy transfer spectroscopy (SMS and FRET, respectively) are two such techniques that have provided a more fundamental understanding of how enzymes, or constituents of an enzyme, such as the carbohydrate-binding module, catalyze the hydrolysis of cellulose. FRET has often aided researchers in developing an understanding of the distance and interactions between two species, often an enzyme and its substrate (Wang et al. 2010). The FRET process occurs when a molecule labeled with a donor fluorophore transfers energy to a molecule labeled with an acceptor fluorophore, provided the two species are 10 nm or less apart. FRET can provide an in situ glimpse of the processivity of cellulase in real time (Wang et al. 2010). In this experiment, carboxymethylcellulose was labeled with the donor, while cellulase was labeled with the acceptor. In this fashion, real-time analyses of the enzyme kinetics can be studied, as well as the mechanisms of cellulase-cellulose interactions. Confocal fluorescence imaging has also been employed to localize cellulose in switchgrass and monitor changes in the structure of the cell wall during IL pretreatment (Singh et al. 2009b). Hydrogen bond disruption between cellulose fibrils was observed, followed by the subsequent dissolution of the entire cell wall. Figure 9 illustrates the use of confocal fluorescence imaging to monitor changes in corn stover stems and cell wall constituents before and during an IL pretreatment (Sun et al. 2013). The distortion and swelling of tracheids, parenchyma, and sclerenchyma fractions, with concomitant reductions in recalcitrance, can be visualized, illustrating the efficacy of ILs to deconstruct plant cell walls. Although cellulose itself does not fluoresce, fluorescence spectroscopy has been employed to identify molecules potentially causing fluorescence emission (Castellan et al. 2007). This phenomenon has been measured following the delignification of paper via chemical pulping and is hypothesized to result from the introduction of various fluorophores during the chemical processing of the biomass.

1.2 Nuclear Magnetic Resonance Spectroscopy

NMR has provided researchers with unparalleled structural information concerning lignocellulosic biomass (Atalla et al. 1980; Atalla and VanderHart 1999; Ferrier et al. 2002; Maunu 2002; Atalla and Isogai 2005; Cui 2005; Balakshin et al. 2008; Yelle et al. 2008; Persson et al. 2011; Yuan et al. 2011; Deutschmann and Dekker 2012; Foston and Ragauskas 2012; Foston et al. 2012; Li et al. 2012; Mazumder et al. 2012; Du et al. 2013; Lupoi et al. 2013; Miyagawa et al. 2013; Nieto and Jiménez-Barbero 2013; Du et al. 2014). In NMR, nuclei absorb radiation in the radio-wave region of the electromagnetic spectrum. Molecules are introduced to strong magnetic fields to split the electronic states, an essential occurrence, for the

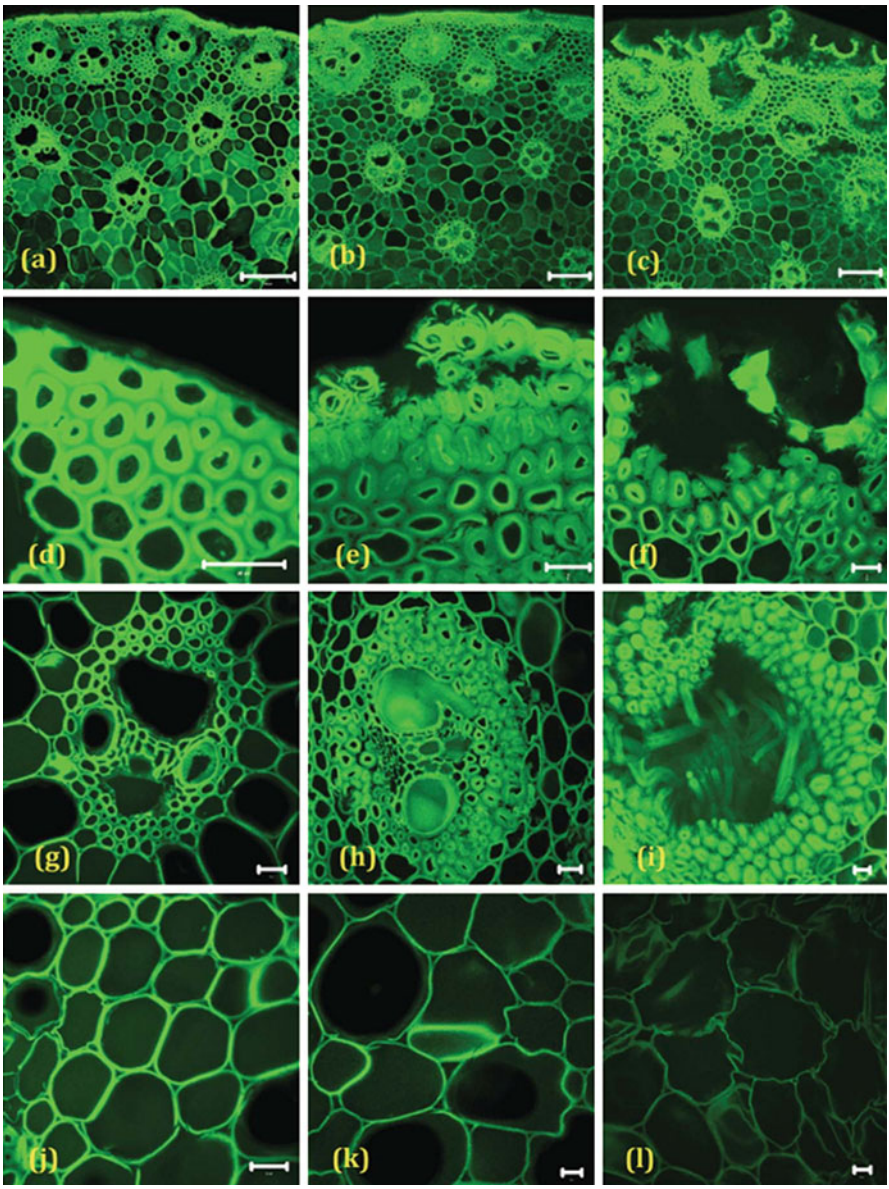

Fig. 9 Confocal fluorescence imaging of corn stover cell structures before and during pretreatment with an ionic liquid. Vertical columns indicate 0, 1, and 3 h of pretreatment, *left* to *right*. (**a**–**c**) Stem overview (100 μm scale bar), (**d**–**f**) sclerenchyma cells (20 μm scale bar), (**g**–**i**) tracheids (20 μm scale bar), and (**j**–**l**) parenchyma cells (20 μm scale bar) (Reprinted with permission from Sun et al. 2013)

absorption process to take place. NMR is a nondestructive, noninvasive technique, since low frequency of the excitation energy is not powerful enough to cause physical or chemical changes in the analyte. The dominant forms of NMR used for polysaccharide analysis include homonuclear techniques, such as proton (^{1}H), carbon-13 (^{13}C), and correlated spectroscopy (COSY), and heteronuclear methods like heteronuclear single quantum correlation (HSQC) 2D NMR. Homonuclear methods provide distinctive spectra for each polysaccharide studied but can often suffer from significant spectral overlap, especially when solely proton NMR is employed. For example, ^{1}H NMR provides a much more sensitive analysis, due to ubiquitous, naturally occurring proton abundance. The signals generated from the protons, however, are detected between 3 and 5 parts per million (ppm), resulting in overlapping peaks and necessitating a transition to 2D methods to resolve the signals (Cui 2005). Figures 10 and 11 show the assignments of key chemical shifts measured using ^{1}H and ^{13}C NMR, respectively. Although ^{13}C NMR does not have the strong signals present in proton NMR, the peaks correspondent to polysaccharides occur over a wider ppm range. Recent developments in both homonuclear (^{1}H-^{1}H) and heteronuclear (^{13}C-^{1}H) two-dimensional NMR spectroscopy have enhanced spectral sensitivity and resolution.

NMR studies can be performed on solubilized or solid biomass. Whole plant cell walls are predominantly insoluble in most customary NMR solvents, necessitating laborious extraction, isolation, and purification strategies. These techniques can potentially transform the chemical composition of the biomass, making their usage undesirable. Recent advances in sample preparation, involving the ball milling and subsequent swelling of the sample in perdeuterated dimethyl sulfoxide (DMSO-d_6), have provided an alternative method to preparing intact plant cell walls (Foston and Ragauskas 2012; Foston et al. 2012). Methods employing these preparatory steps have significantly improved the throughput of NMR experiments. ILs have also been employed as solvents, owing to the complete dissolution of biomass (Foston et al. 2012).

Solid-state NMR alleviates the need to develop efficient dissolution schemes by probing whole biomass. The analysis of cell wall pore size, ultrastructure, and the spatial orientation of the amorphous and crystalline cellulose, hemicellulose, and lignin fractions represent the diversity of solid-state NMR applications. Solid-state NMR has also been used for studying cellulose polymorphs. In fact, it was ^{13}C NMR that made the spectral resolution of cellulose I_α and I_β crystalline forms possible, as can be seen in Fig. 12 (Atalla et al. 1980; Harris et al. 2010). Figure 13 illustrates key spectral differences between crystalline and amorphous celluloses, using ^{13}C cross-polarization magic angle spinning (CP/MAS) NMR (Atalla et al. 1980). In Fig. 14, ^{13}C CP/MAS NMR spectra of pine, *Miscanthus*, and red clover are shown, along with the relative carbon abundance for each chemical shift. This technique can enable the semi-quantitation of specific analytes in biomass when compared against other species; in this example, however, the NMR spectra were used in conjunction with Raman spectroscopy to probe the sources of

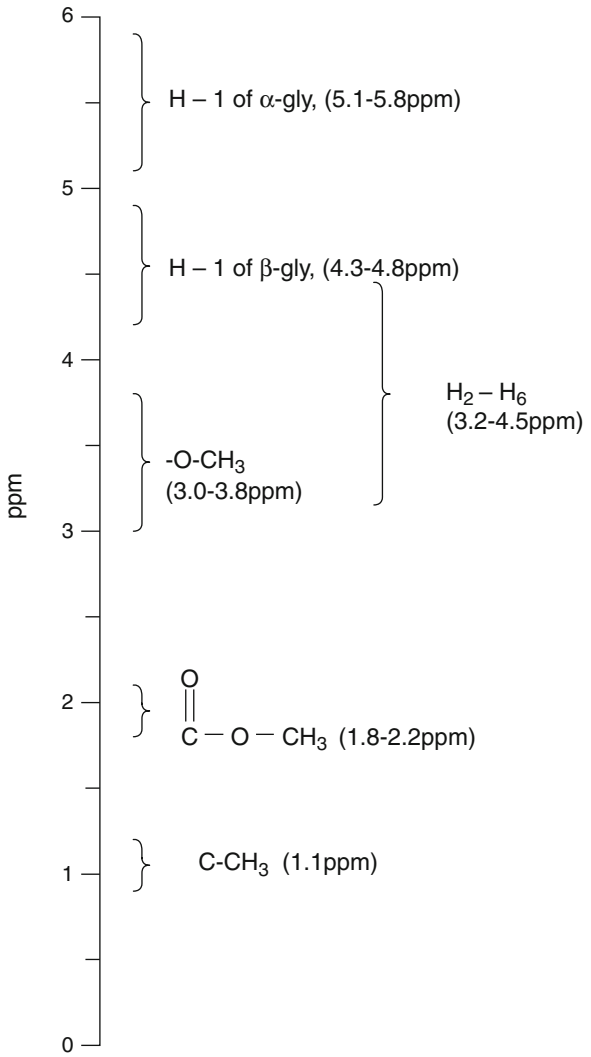

Fig. 10 Key chemical shifts measured using ^{1}H NMR (Reprinted with permission from Springer, from Cui 2005)

anomalous vibrational modes and provide an illustration of the employment of multiple analytical tools to elucidate structural information. Extractives in the herbaceous feedstocks were found to produce the inconsistent spectral peaks, thereby necessitating their removal via a Soxhlet extraction.

HSQC 2D NMR has been one of the most prevalently used techniques to evaluate the structure of lignocellulosic biomass. While many of the studies have focused on the evaluation of lignin, various applications have probed polysaccharides. The linkages responsible for the formation of lignin-carbohydrate complexes (LCCs) have been elucidated using a combination of ^{13}C NMR and HSQC 2D NMR (Balakshin et al. 2008; Yuan et al. 2011; Du et al. 2013). The cognition of

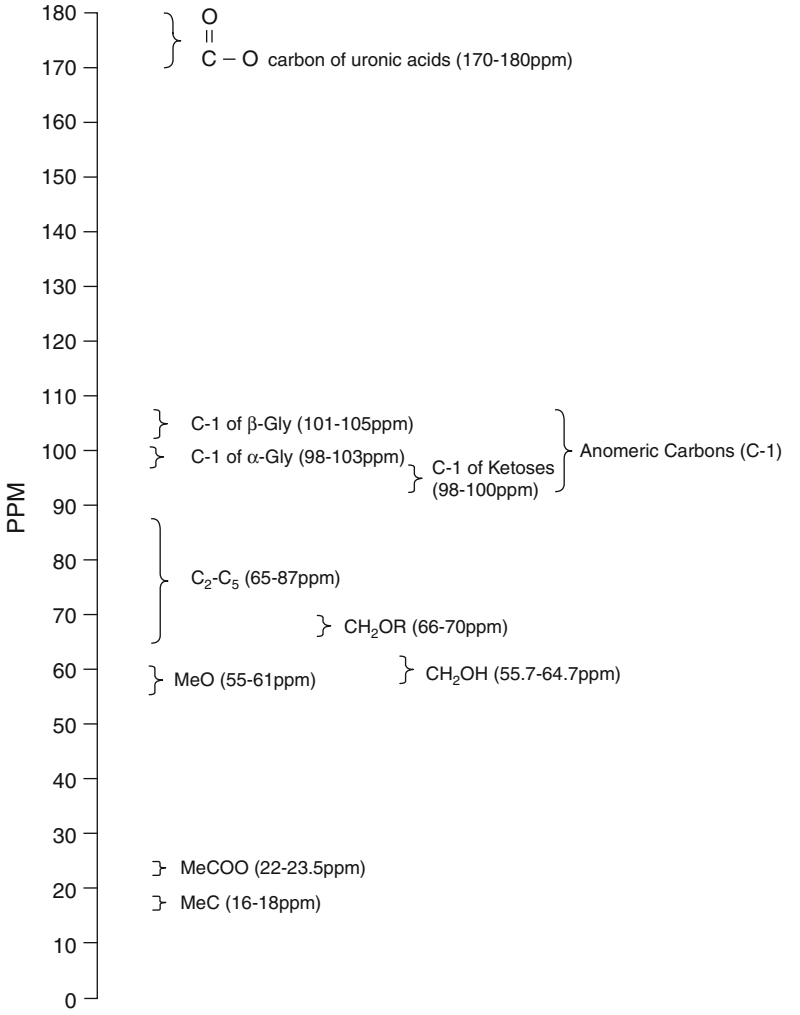

Fig. 11 Key chemical shifts measured using ^{13}C NMR (Reprinted with permission from Springer, from Cui 2005)

how the cell wall constituents are chemically interlaced is paramount for the development of key fractionation strategies for the isolation of each respective portion. For example, acetylated 4-*O*-methylgluconoxylan has been found to be intimately associated with lignin in ball-milled poplar wood (Yuan et al. 2011). β-*O*-4′ aryl ether, resinol, phenylcoumaran, phenyl glycoside, and benzyl ether linkages have all been quantified using ^{13}C NMR. Various studies have developed fractionation protocols to isolate glucan-lignin, glucomannan lignin, and xylan-lignin complexes to evaluate the specific linkages responsible for developing the intricate cell wall LCC networks (Miyagawa et al. 2013; Du et al. 2014).

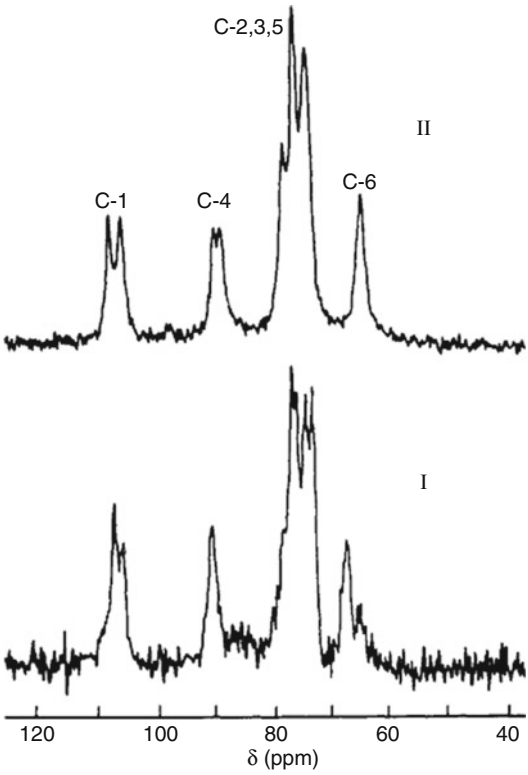

Fig. 12 CP/MAS ^{13}C NMR spectra of crystalline cellulose forms I (*bottom*) and II (*top*) (Reprinted with permission from Atalla et al. 1980)

HSQC 2D NMR has also been used to analyze biomass swelled using DMSO-d_6, permitting the analysis of intact cell walls (Hedenstroem et al. 2009; Rencoret et al. 2009). A combined IL/DMSO-d_6 solvent system coupled with HSQC 2D NMR has been found to appreciably decrease analysis time while simultaneously providing higher spectral resolution (Yelle et al. 2008). The spectral resolving power of HSQC 2D NMR has been conjoined with multivariate analysis (MVA) providing a formidable method for assessing changes in polysaccharide structure and composition between different feedstocks (Figs. 15, 16, and 17) (Hedenstroem et al. 2009). The evaluation of 2D spectral loadings plots in principal component analysis (PCA) can facilitate the nomination of important spectral regions without requiring researchers to rummage spectral regions containing significant overlapping peaks. Figure 15 depicts a typical 2D ^{13}C-^{1}H NMR HSQC spectrum illustrating the main polysaccharide constituents in aspen trees. Figure 16 demonstrates the PCA technique employed by the authors for reducing noise in the 2D NMR spectra and concomitantly elucidating key spectral features in the loadings plot. This method was applied to assess chemical differences between normal and tension aspen wood samples, as shown in Fig. 17. The cell wall constituents in lower or higher concentration in the two wood types could be determined using this

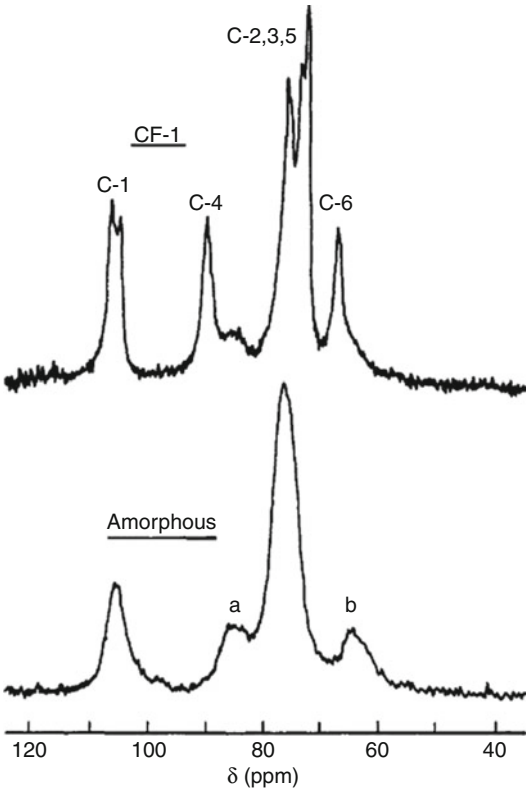

Fig. 13 CP/MAS ^{13}C NMR spectra of native (*top*) and amorphous cellulose (*bottom*) (Reprinted with permission from the American Chemical Society, from Atalla et al. 1980)

MVA technique, whereas traditional spectral analysis required the rummaging of overlapping peaks to expose key compositional differences.

2 Cellulose Crystallinity

Crystalline cellulose has been hypothesized as exhibiting greater recalcitrance to the enzymatic deconstruction of biomass when compared to the amorphous regions, although some studies have been inconclusive (Zhang and Lynd 2004; Hall et al. 2010). Therefore, knowledge of the cellulose crystallinity index (CrI) may assist researchers in developing more efficient degradative strategies. CrI measurements, however, have led to widely varying results. Although XRD remains one of the more prevalently employed analytical techniques for probing cellulose crystallinity (Segal et al. 1959; Thygesen et al. 2005; Beecher et al. 2009; Agarwal et al. 2010; Harris et al. 2010; Park et al. 2010; Foston and Ragauskas 2012; Agarwal et al. 2013; Kim et al. 2013), recent advances in vibrational spectroscopy and NMR have provided supplemental tools for measuring CrIs (Schenzel et al. 2005; Beecher et al. 2009; Agarwal et al. 2010, 2013; Harris et al. 2010; Park et al. 2010; Foston and Ragauskas 2012; Kim et al. 2013; Lupoi et al. 2013; Xu et al. 2013).

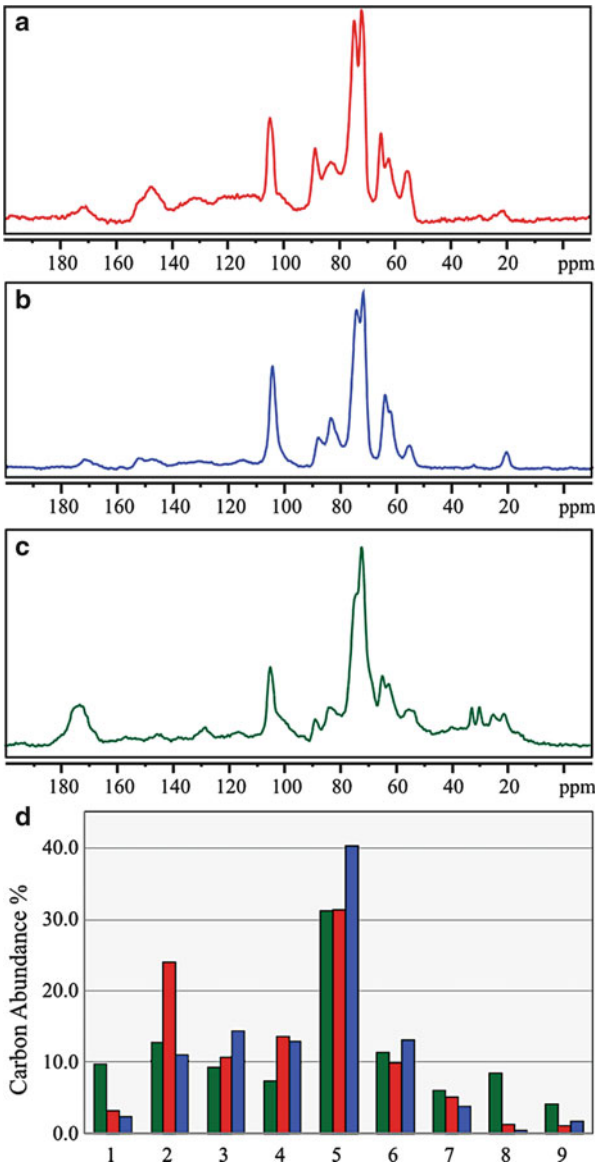

Fig. 14 ^{13}C NMR CP/MAS spectra of pine (**a**), *miscanthus* (**b**), and *red* clover (**c**). (**d**) The relative carbon abundance calculated from the integration ranges listed below. Relative intensities were found by dividing the individual integrated peak height by the total carbon intensity (sum of individual intensities). The integration ranges used and assignments were as follows (in ppm): *1* 180–163, carboxylate carbon in hemicellulose; *2* 158–110, phenyl carbon in lignin; *3* 109.8–100, carbon 1 in cellulose; *4* 92–80.5, carbon 4 in cellulose; *5* 80–68.4, carbons 2, 3, and 5 in cellulose; *6* 68–60.1, carbon 6 in cellulose; *7* 59–52, methoxy carbon in lignin, hemicellulose; *8* 42–27.5, aliphatic C–H; and *9* 24.1–18, methyl in hemicellulose (Reprinted with permission from Lupoi and Smith 2012)

2.1 Vibrational Spectroscopy

Techniques for measuring the CrI of cellulose using Raman spectroscopy have recently been developed (Schenzel et al. 2005; Agarwal et al. 2010, 2013; Kim et al. 2013). The ratio of the 380 and 1,096 cm^{-1} vibrational modes has been nominated for gauging the degree of cellulose crystallinity (Agarwal et al. 2010, 2013). Univariate and multivariate methods have been developed, both revealing

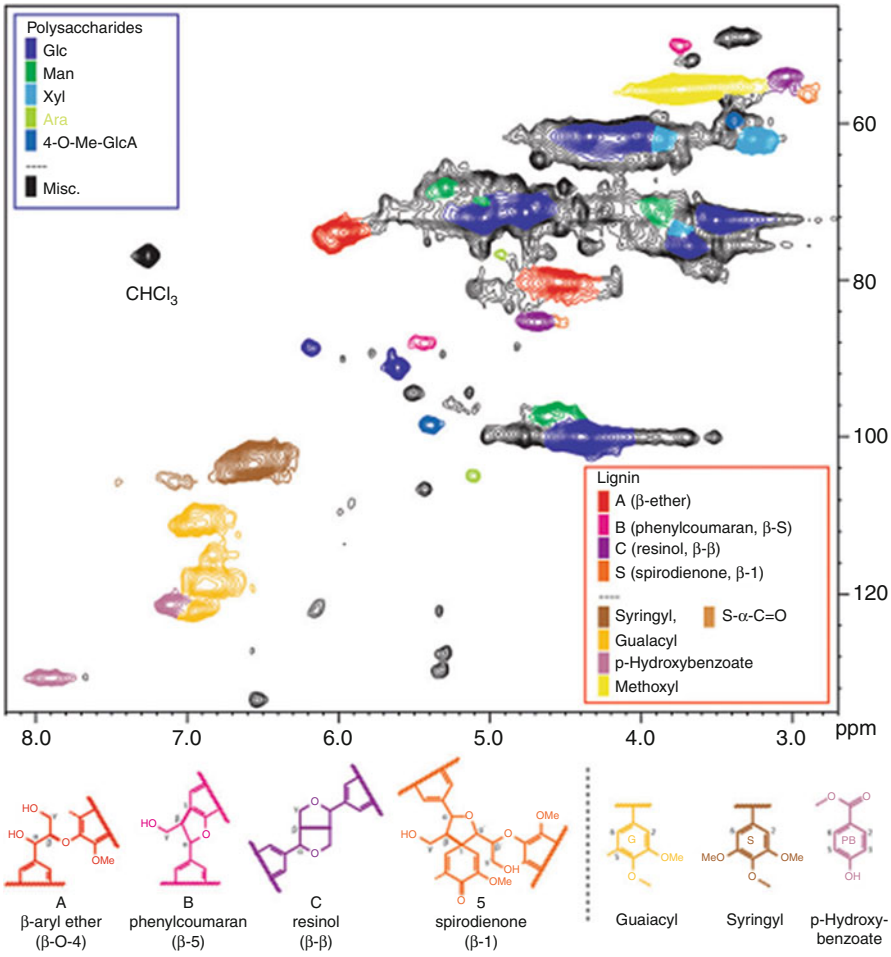

Fig. 15 2D ^{13}C-^{1}H HSQC spectrum of dissolved acetylated cell wall from poplar. Proton NMR chemical shifts are on the x-axis, while carbon NMR is on the y-axis. Spectral assignments are based upon the analysis of standard compounds and reported assignments from the literature. The polysaccharide abbreviations correspond to the following analytes: Glc, glucosyl; Man, mannosyl; Xyl, xylosyl; Ara, arabinosyl; and 4-OMe-GlcA, 4-*O*-methyl glucuronic acid (Reprinted with permission from Hedenström et al. 2009)

strong regression linearity. Another study determined the 1,481 and 1,462 cm^{-1} vibrational modes, assigned to crystalline and amorphous celluloses, respectively, providing a good measure of the CrI of cellulose I (Schenzel et al. 2005). These Raman techniques were optimized using pure cellulose samples, such as filter paper and Avicel. The measurement of the CrI of cellulose in biomass cell walls using vibrational spectroscopy can be complicated by overlapping peaks from hemicellulose, lignin, or other constituents. Thus, it becomes crucial to identify regions of the spectra that can be solely attributable to cellulosic vibrational modes.

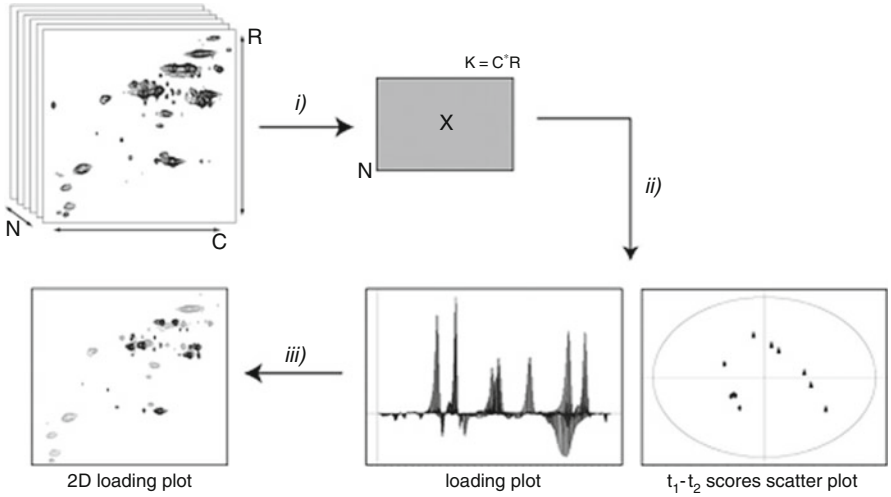

Fig. 16 This figure demonstrates how PCA can be used in conjunction with 2D NMR spectra. (**a**) 2D NMR spectra are converted to row vectors and inserted into data matrix X. Noisy spectral features were eliminated. (**b**) Scores and loadings plots are generated from the MVA analysis of the spectral data. This allows the elucidation of key latent structures with the spectra. (**c**) Loadings plots are unfolded back into 2D NMR spectra by reversing the order outlined in (**a**) (Reprinted with permission from Hedenström et al. 2009)

MIR spectroscopy has also been used to assess cellulose crystallinity (O'Connor et al. 1958; Akerholm et al. 2004; Siroky et al. 2010; Kljun et al. 2011; Xu et al. 2013). As with the Raman CrI measurements, MIR methods have entailed calculating the ratio of specific vibrational modes, such as 1,430 and 893 cm^{-1} to evaluate the lateral order index, which is proportional to crystallinity (O'Connor et al. 1958; Akerholm et al. 2004; Siroky et al. 2010; Kljun et al. 2011). The hydrogen bond index has also been used to indirectly measure cellulose crystallinity using the ratio of the 3,336 and 1,336 cm^{-1} modes (Siroky et al. 2010; Kljun et al. 2011). When juxtaposed to standard methods such as NMR, the MIR results have revealed good correlation.

2.2 Nuclear Magnetic Resonance

NMR has been a standard spectroscopic tool used for measuring the degree of cellulose crystallinity, as amorphous and crystalline celluloses produce different chemical shifts (Beecher et al. 2009; Foston and Ragauskas 2010, 2012; Park et al. 2010; Cao et al. 2012; Kim et al. 2013; Lupoi et al. 2013). The analysis of the C4 carbon has provided a direct way to evaluate crystallinity, as amorphous regions produce a broad peak between 80 and 85 ppm, while crystalline celluloses exhibit sharper peaks between 85 and 92 ppm (Beecher et al. 2009; Park et al. 2010; Foston and Ragauskas 2012). Figure 18 illustrates the use of ^{13}C CP/MAS NMR to quantify

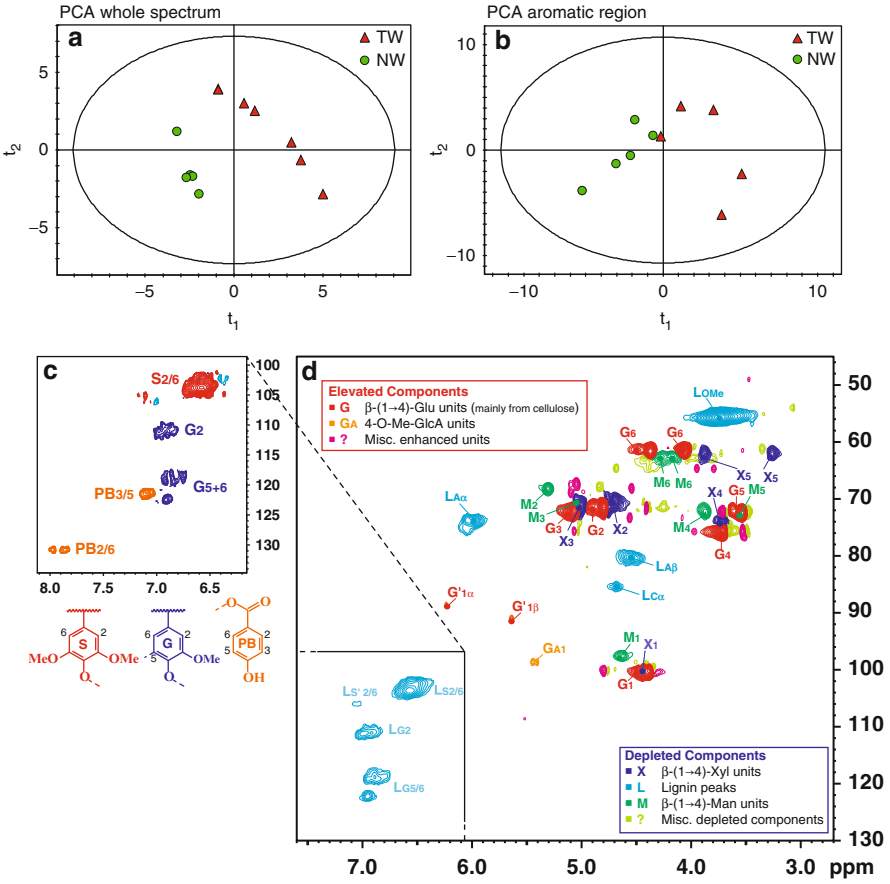

Fig. 17 PCA comparison of normal wood to tension wood. (**a**) PCA scores plot evaluating normal and tension wood using whole NMR spectra. (**b**) PCA scores plot evaluating normal and tension wood using only the aromatic region of the NMR spectra. (**c**) 2D NMR loadings plot explaining the variance along the first principal component in (**b**). S and PB lignin units are increased, while G lignin units are relatively decreased. (**d**) 2D NMR loadings plot derived from the PCA model in (**a**). *Red* and *orange* resonances depict the cell wall constituents in higher concentration in tension wood compared to normal wood. Those colored blue and green correspond to cellular components in higher concentration in normal wood. G′1α and G′1β refer to α- and β-conformations of the glucosyl anomeric units at the cellulose reducing end (Reprinted with permission from Hedenström et al. 2009)

the CrI of Avicel PH-101 (Park et al. 2010). The CrI can be calculated by deconvoluting the C4 signal into its two representative peaks. In Fig. 18, the crystalline cellulose region is labeled as x, while the amorphous fraction is labeled as y. The CrI can be found using the equation x/(x+y). The use of NMR has enabled researchers to evaluate cellulose structural changes resultant from various pretreatment strategies. For example, the changes in the degree of cellulose crystallinity following the dilute

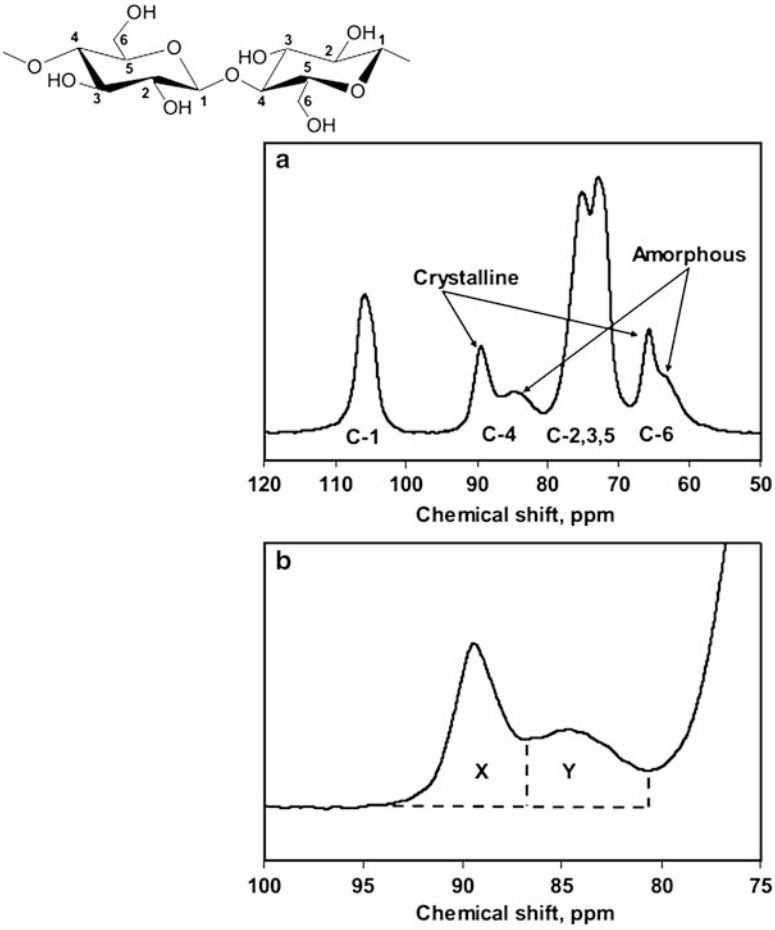

Fig. 18 ^{13}C NMR CP/MAS spectra of Avicel PH-101 used for calculating the cellulose crystallinity index (*CrI*). (**a**) Whole spectrum with the respective carbon peaks assigned using the glucopyranose unit. (**b**) C4 cellulose peak deconvolution elucidating the two peaks used for calculating the CrI (CrI = x/(x + y)) (Reprinted with permission from Park et al. 2010)

sulfuric acid pretreatment of poplar using shorter pretreatment durations proved to be insignificant, while at longer pretreatment times, a slow crystallization of amorphous cellulose was discovered (Foston and Ragauskas 2010; Cao et al. 2012).

3 Total Carbohydrates

Customary techniques for quantifying the total amount of carbohydrates in plant cell walls utilize wet chemical reactions such as the anthrone-sulfuric acid, phenol-sulfuric acid, trifluoroacetic acid (TFA), and the Van Soest methods (Van Soest 1963;

Van Soest and Wine 1968; Fengel and Wegener 1979; Brummer and Cui 2005; Masuko et al. 2005). Total carbohydrate measurements do not distinguish between the various types of sugars in the matrix, but rather provide an estimated quantity based upon the calibration curve of a reference analyte using UV/Vis spectrophotometry (Masuko et al. 2005; Foster et al. 2010). Fluorescence spectroscopy, although not prevalently used for the quantitation of biomass chemical composition, has been recently applied to red oak and yellow poplar woods exhibiting limited success when juxtaposed to NIR methodology (Nkansah and Dawson-Andoh 2010a, b).

Vibrational spectroscopy assessments of total carbohydrates have enabled the prediction of total carbohydrates using two approaches: total holocellulose (cellulose + hemicellulose) content or by quantifying all of the individual sugar producing polymers including glucan, xylan, galactan, mannan, and arabinan (see also Sect. 3.1). FT-Raman spectroscopy coupled with partial least squares (PLS) facilitated the prediction of raw and extractive-free holocellulose content in various eucalypts (Ona et al. 1997, 2003). MIR spectroscopy has also been evaluated for forecasting the total carbohydrate content in *Pinus radiata* using PLS and LC reference data (Meder et al. 1999). These two methods, however, have not been employed as frequently as NIR spectroscopy (Hames et al. 2003; Kelley et al. 2004; Ye et al. 2008; Templeton et al. 2009; Wolfrum and Sluiter 2009; Liu et al. 2010; Nkansah and Dawson-Andoh 2010a, b; Hou and Li 2011). While the analysis of the total carbohydrates using holocellulose may provide a fast way to screen through plant materials for the fermentable sugar content, partitioning the total carbohydrate content into its representative constituents can provide a better survey of potential biofuel feedstocks, as the different polysaccharides require specific enzymes for conversion to monomeric sugars and distinctive microorganisms for subsequent fermentation.

3.1 Cellulose and Noncellulosic Polysaccharide Content

Wet chemical and chromatographic protocols used for the determination of cellulose and noncellulosic polysaccharide content include the Updegraff (1969) (Van Soest and Wine 1968; Van Soest et al. 1991) methods, the two-step acid hydrolysis coupled with HPLC (Cui 2005; Hames 2009; Sluiter Justin et al. 2010), HP anion exchange liquid chromatography (Hausalo 1995; Rohrer 2003; Willfoer et al. 2009; Deutschmann and Dekker 2012), and quantitative GC analysis (Collings and Yokoyama 1979; Willfoer et al. 2009; Deutschmann and Dekker 2012; Sarossy et al. 2012). Although these standard techniques have been ubiquitously employed, they can require stringent reactants and time-consuming sample preparation steps, may destroy the sample, and can entail complex data processing. These limitations necessitate higher-throughput, nondestructive analytical methods, especially for screening large arrays of potential feedstocks to isolate those possessing the ideal phenotypic traits for biofuel and bio-based chemical production. Advances in vibrational spectroscopy have provided researchers with the tools to rapidly assess biomass for polysaccharide content (Lupoi et al. 2013). The coupling of vibrational

spectroscopy with MVA is one approach enabling researchers to diminish the experimental time and expense associated with the traditional protocols. To achieve this, a reference method is used to quantify analytes for a subset of samples, while the spectral data is obtained for all of the samples. The two data sets are correlated in MVA software, enabling a variety of chemometric assessments such as PCA for the classification of the analytes based on sample similarity or PLS regression for quantifying the variables of interest. PLS calibration models are constructed from the reference and spectral data. After the aggressive evaluation of models using validation data sets, where predicted and reference values are compared, the parameter(s) quantified using the reference method can be predicted in the unknown samples. This procedure has facilitated the screening of large pools of plant species for traits such as cellulose and hemicellulose content.

3.1.1 Vibrational Spectroscopy

The combination of vibrational spectroscopy and reference analytical techniques has been used to predict cellulose and hemicellulose content in a diverse assortment of biomass (Ona et al. 1997, 1998, 2003; Hames et al. 2003; Kelley et al. 2004; Poke and Raymond 2006; Ye et al. 2008; Hames 2009; Templeton et al. 2009; Wolfrum and Sluiter 2009; Liu et al. 2010; Tyson et al. 2010; Deutschmann and Dekker 2012; Reeves 2012; Lupoi et al. 2013; Xu et al. 2013). By far, the instrumental workhouse of these tools has been NIR spectroscopy. The spectra in NIR spectroscopy result from the measurement of C-H, N-H, O-H, and S-H bonds that stem from combination and overtone modes of the fundamental vibrations probed in MIR spectroscopy (Workman 1996; Workman et al. 2007). Table 4 lists the main NIR vibrational modes correlative to polysaccharides. Figure 19a depicts a typical NIR spectrum obtained for the measurement of biomass. The effect of differences in particle sizes is illustrated. Although NIR spectra reveal considerable similarity, with relatively broad peaks due to multiple bond vibrations, the coupling of NIR with MVA has led to a diverse assortment of applications regarding the HTP prediction of biomass phenotypes, like cellulose and hemicellulose content. NIR instrumentation provides a cost-efficient, robust tool that has field-portable capabilities. Many of the NIR methods for predicting cellulose and hemicellulose content combine the two-step sulfuric acid hydrolysis with processed NIR spectral data. The spectral processing usually involves the transformation of the spectra into their respective first or second derivative (Fig. 19b, c, respectively) and the application of methods such as standard normal variate (SNV) or multiplicative scatter correction to remove physical disparities, like particle size or path length variation, from the data, thereby honing in on chemical differences. Figure 20 demonstrates the spectral processing of NIR spectral data to diminish spectral contributions from nonchemical features. The raw spectra of *Acacia microbotrya*, a *Corymbia* hybrid, and *E. globulus* are depicted in the top panel. In this example, a second derivative, followed by an SNV transformation, was employed to isolate chemical differences (middle and bottom panels, respectively). Models constructed from NIR spectra have provided robust predictions of polysaccharide content in a diverse array of lignocellulosic feedstocks including corn stover, switchgrass, and eucalypts.

Table 4 Characteristic NIR vibrational modes of polysaccharides

Approximate peak location	Polysaccharide	Primary assignment
3,970–3,990	Cellulose	CO, CH, and CH_2 stretch (Bassett et al. 1963)
4,019	Cellulose	CH and CC stretch (Shenk et al. 2008)
4,063	Cellulose	CH and CC stretch (Osborne et al. 1993)
4,235	Cellulose	OH or CH deformation, CH or CH_2 stretch (Bassett et al. 1963)
4,252	Cellulose	CH stretch and deformation, 2nd overtone CH_2 bend (Shenk et al. 2008)
4,268	Cellulose	CH stretch and deformation and/or 2nd overtone CH deformation (Ali et al. 2001)
4,277	Cellulose	CH stretch and CH_2 deformation (Shenk et al. 2008)
4,281–4,283	Cellulose, hemicellulose	CH stretch and deformation (Tsuchikawa et al. 2005; Shenk et al. 2008)
4,288–4,296	Hemicellulose	CH stretch and deformation (Yonenobu and Tsuchikawa 2003)
4,365	Cellulose	CO and OH stretch, CH_2 bend and stretch (Bassett et al. 1963)
4,392	Cellulose	OH and CC stretch, CH stretch and deformation (Ali et al. 2001)
4,401	Hemicellulose	CH stretch and deformation (Osborne and Fearn 1986)
4,404	Cellulose, hemicellulose	CH stretch and deformation (Tsuchikawa et al. 2005)
4,405	Cellulose	CO and OH stretch (Shenk et al. 2008)
4,591, 4,608	Cellulose, hemicellulose	Not assigned (Schwanninger et al. 2011)
4,686	Hemicellulose	CH and C=O stretch (Schwanninger et al. 2011)
4,739	Cellulose	OH deformation and stretch (Ellis and Bath 1940)
4,760–4,780	Cellulose	OH deformation and stretch, CH deformation (Bassett et al. 1963)
4,795	Cellulose, hemicellulose	OH deformation and stretch, CH deformation (Bassett et al. 1963)
4,808	Cellulose	OH stretch, CH deformation (Osborne and Fearn 1986)
5,236, 5,245	Hemicellulose	2nd overtone CH stretch (Workman et al. 2007)
5,464	Cellulose	OH stretch and 2nd overtone CO stretch (Osborne and Fearn 1986; Fujimoto et al. 2007)
5,495	Cellulose	OH stretch and 2nd overtone CO stretch (Osborne et al. 1993; Workman et al. 2007)
5,577	Cellulose	1st overtone CH stretch (Ali et al. 2001)
5,587, 5,593	Cellulose	1st overtone CH stretch (Osborne and Fearn 1986)
5,618	Cellulose	1st overtone CH_2 stretch (Workman et al. 2007; Shenk et al. 2008)
5,776	Cellulose	1st overtone CH stretch (Barton and Himmelsbach 1993)

(*continued*)

Table 4 (continued)

Approximate peak location	Polysaccharide	Primary assignment
5,800	Hemicellulose	1st overtone CH stretch (Osborne and Fearn 1986; Fujimoto et al. 2007)
5,814, 5,816	Cellulose, hemicellulose, lignin	1st overtone CH stretch (Michell and Schimleck 1996; Schwanninger et al. 2011)
5,848	Hemicellulose	1st overtone CH stretch (Michell and Schimleck 1996; Yonenobu and Tsuchikawa 2003)
5,865	Hemicellulose	1st overtone CH stretch (Wust and Rudzik 1996)
5,872	Cellulose	1st overtone CH stretch (Ali et al. 2001)
5,950	Hemicellulose	1st overtone CH stretch (Wust and Rudzik 1996)
6,003	Hemicellulose	1st overtone CH stretch (Wust and Rudzik 1996)
6,126	Cellulose	1st overtone OH stretch (Watanabe et al. 2006)
6,257	Cellulose	1st overtone OH stretch (Fackler and Schwanninger 2010; Schwanninger et al. 2011)
6,281	Cellulose	1st overtone OH stretch (Tsuchikawa and Siesler 2003; Fujimoto et al. 2007)
6,267–6,307	Cellulose	1st overtone OH stretch (Fackler and Schwanninger 2010)
6,329	Cellulose	1st overtone OH stretch (Tsuchikawa and Siesler 2003)
6,334	Cellulose	1st overtone OH stretch (Tsuchikawa and Siesler 2003)
6,450	Cellulose	1st overtone OH stretch (Fackler and Schwanninger 2010)
6,460	Cellulose	1st overtone OH stretch (Tsuchikawa and Siesler 2003; Fujimoto et al. 2007)
6,472	Cellulose	1st overtone OH stretch (Ali et al. 2001; Schwanninger et al. 2011)
6,495	Cellulose	1st overtone OH stretch (Schwanninger et al. 2011)
6,520	Cellulose	1st overtone OH stretch (Fackler and Schwanninger 2010; Schwanninger et al. 2011)
6,622	Cellulose	1st overtone OH stretch (Watanabe et al. 2006; Schwanninger et al. 2011)
6,660	Cellulose	1st overtone OH stretch (Fackler and Schwanninger 2010; Schwanninger et al. 2011)
6,700	Hemicellulose	1st overtone OH stretch (Fackler and Schwanninger 2010; Schwanninger et al. 2011)
6,715	Cellulose	1st overtone OH stretch (Shenk et al. 2008)
6,740–6,770	Cellulose	1st overtone OH stretch (Osborne et al. 1993; Schwanninger et al. 2011)
6,775	Cellulose	1st overtone OH stretch (Fujimoto et al. 2007)
6,790	Cellulose	1st overtone OH stretch (Fujimoto et al. 2007; Schwanninger et al. 2011)

(*continued*)

Table 4 (continued)

Approximate peak location	Polysaccharide	Primary assignment
6,800	Hemicellulose	1st overtone OH stretch (Fackler and Schwanninger 2010; Schwanninger et al. 2011)
6,993–7,003	Cellulose/water	1st overtone OH stretch (Tsuchikawa and Siesler 2003; Fujimoto et al. 2007)
7,300	Hemicellulose	1st overtone CH stretch, CH deformation (Schwanninger et al. 2011)
7,315–7,321	Cellulose	1st overtone CH stretch, CH deformation (Ali et al. 2001)
7,400, 7,410	Hemicellulose, lignin	1st overtone CH stretch, CH deformation (Schwanninger et al. 2011)
8,160–8,250	Cellulose	2nd overtone CH stretch (Michell and Schimleck 1996)
8,450, 8,525, 8,540, 8,654	Hemicellulose	2nd overtone CH stretch (Wust and Rudzik 1996; Shenk et al. 2008)

Raman spectroscopy, although not utilized to the extent of NIR spectroscopy, has enabled the development of MVA predictive models for assessing polysaccharide content (Ona et al. 1997, 1998, 2003; Lupoi et al. 2013). The lack of abundant studies employing Raman spectroscopy could be due to higher instrumentation costs compared to NIR spectroscopy. One of the main spectroscopic instrumental challenges is obtaining high signal-to-noise (S/N) spectra. In NIR and MIR spectroscopy, this can be achievable by increasing the number of scans the instrument acquires, which reduces random noise in the spectra. Raman spectroscopy can have the added advantage of tunable excitation lasers. By increasing the laser power, spectral signals intensify, further elucidating subtle spectral differences between samples. The noise can be simultaneously diminished by acquiring more scans, thereby providing a two-pronged approach to increase S/N. Figure 21 illustrates the spectral processing of FT-Raman spectra for the development of predictive PLS models. Juxtaposed to the NIR spectra in Fig. 20, the Raman spectral data of the same feedstocks contains significantly more spectral information and a greater amount of spectral variance. MVA models flourish from the variance remaining once the average spectrum has been calculated and subtracted from all spectra, a process called mean centering. More diverse spectra should exhibit better partitioning between the samples, resulting in more accurate predictive models. Ona et al. have been one of the leading teams in applying FT-Raman spectroscopy and PLS to evaluate *Eucalyptus* for cellulose and hemicellulose content, as well as the monomeric sugars comprising hemicellulosic fractions (Ona et al. 1997, 1998, 2003). Although there have been recent advances in NIR, dispersive Raman instrumentation (Meyer et al. 2011), this arena remains relatively untapped for the development of MVA models.

Although typically used for biomass structural analysis, MIR spectroscopy has also been employed for the development of MVA predictive models for assessing

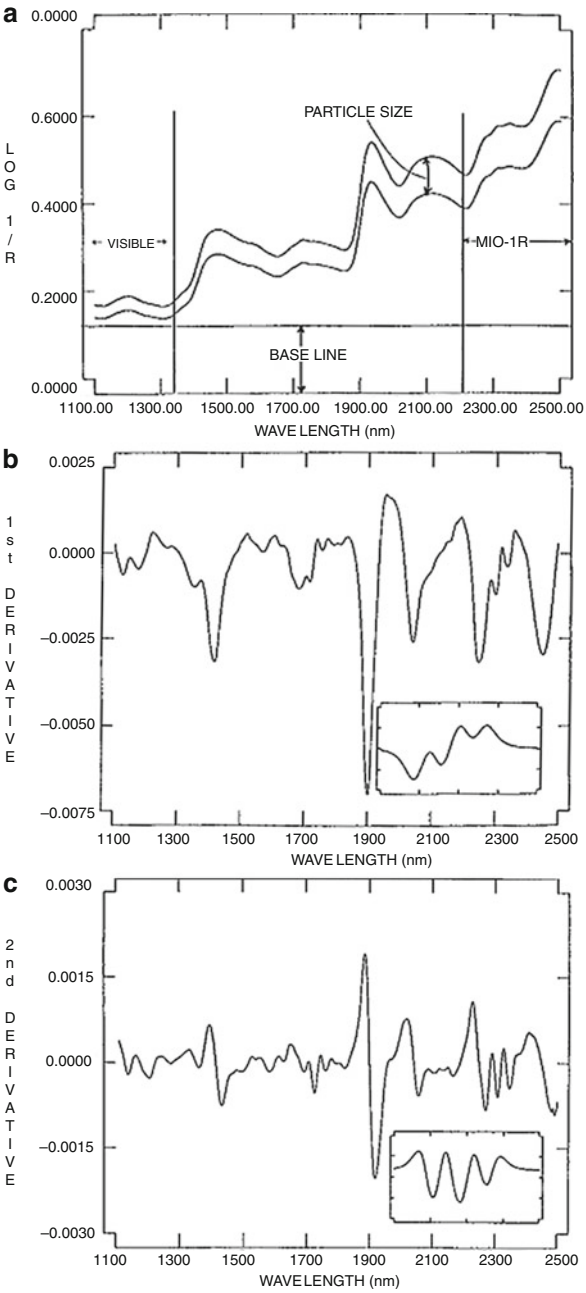

Fig. 19 (**a**) Demonstrative NIR spectral features from lignocellulosic biomass. (**b**) First-derivative spectrum of a forage product. (**c**) Second-derivative spectrum of a forage product (Reprinted with permission from Shenk et al. 2008)

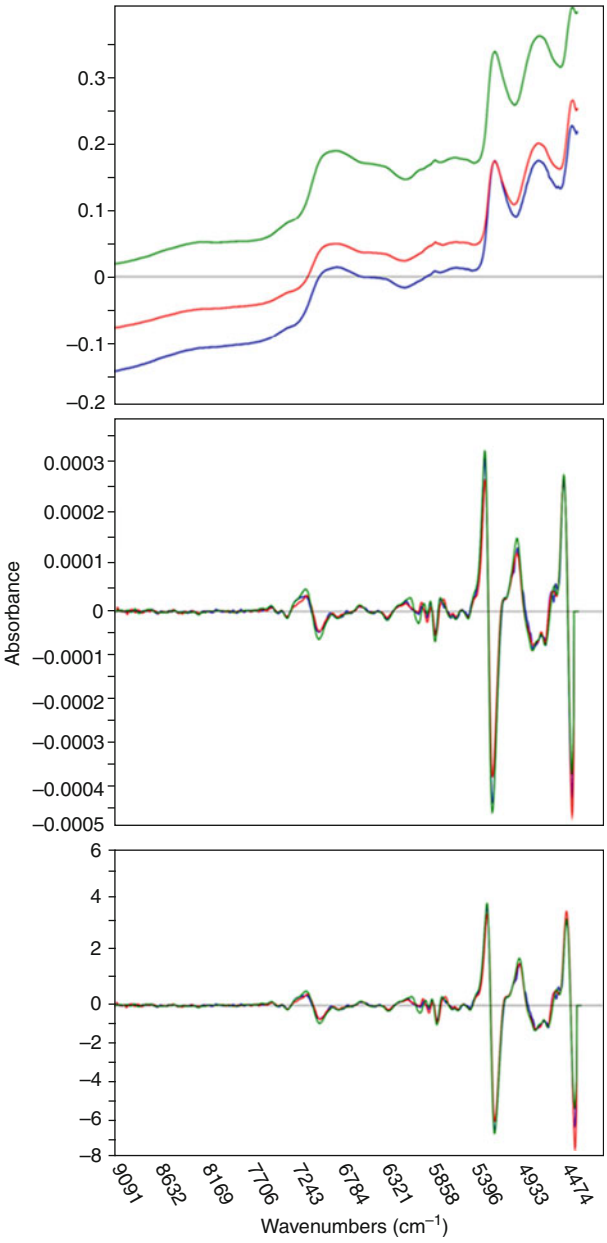

Fig. 20 Near-infrared spectra of *Acacia microbotrya* (*green*), *Corymbia* hybrid (*blue*), and *Eucalyptus globulus* subspecies *maidenii* (*red*). The *upper panel* (**a**) shows the untreated spectral data, while the *middle* (**b**) and *bottom* (**c**) *panels* show the second-derivative and second-derivative + standard normal variate (SNV) spectral transformations, respectively. The x-axis is in wavenumbers, while the y-axis is the absorbance (Reprinted with permission from Lupoi et al. 2014)

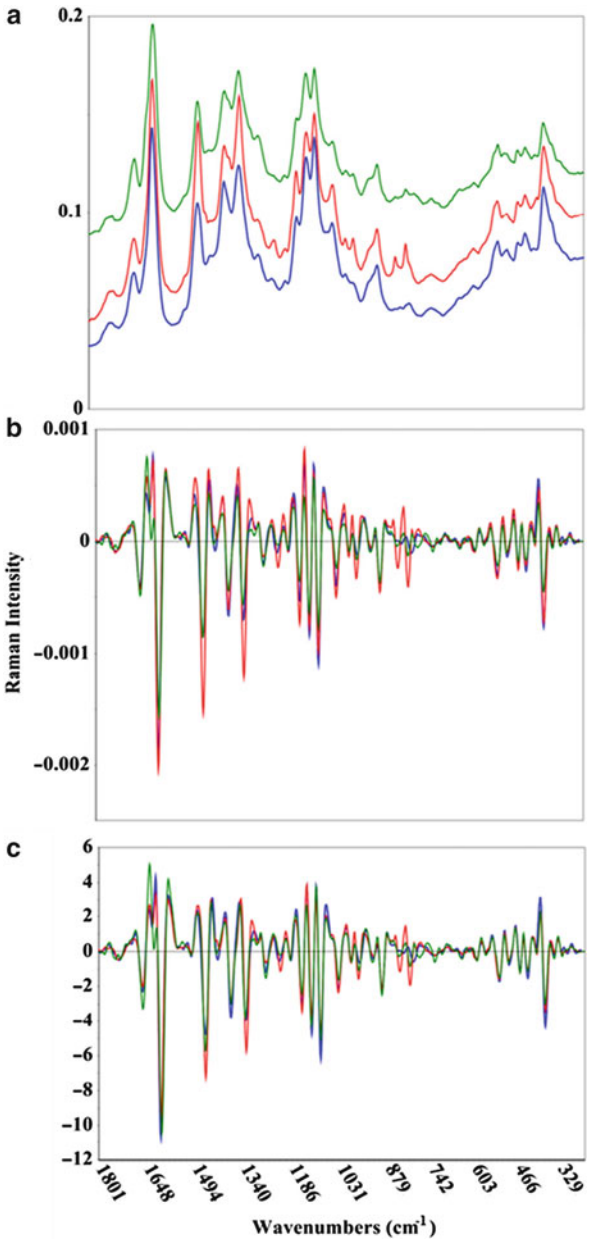

Fig 21 Raman spectra of *Acacia microbotrya* (*green*), *Corymbia* hybrid (*blue*), and *Eucalyptus globulus* subspecies *maidenii* (*red*). The *upper panel* (**a**) shows the untreated spectral data, while the *middle* (**b**) and *bottom* (**c**) *panels* show the second-derivative and second-derivative + standard normal variate (SNV) spectral transformations, respectively. The x-axis is in wavenumbers, while the y-axis shows the Raman intensity (Reprinted with permission from Lupoi et al. 2014)

polysaccharide content (Bjarnestad and Dahlman 2002; Jones et al. 2002; Reeves 2012; Lupoi et al. 2013). Since fundamental vibrational modes are being excited to higher energy levels in MIR spectroscopy, the spectral peaks are much more intense compared to those obtained in NIR spectroscopy. As described above for Raman

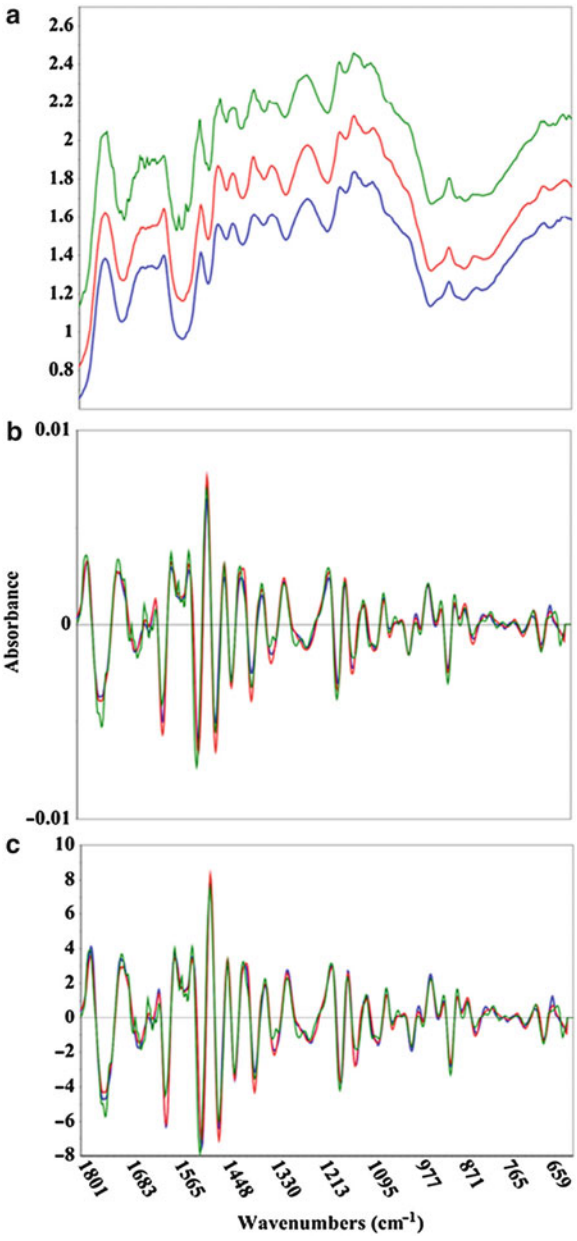

Fig. 22 Mid-infrared spectra of *Acacia microbotrya* (*green*), *Corymbia* hybrid (*blue*), and *Eucalyptus globulus* subspecies *maidenii* (*red*). The *upper panel* (**a**) shows the untreated spectral data, while the *middle* (**b**) and *bottom* (**c**) *panels* show the second-derivative and second-derivative + standard normal variate (SNV) spectral transformations, respectively. The x-axis is in wavenumbers, while the y-axis is the absorbance (Reprinted with permission from Lupoi et al. 2014)

spectroscopy, MIR spectra are more diverse when contrasting different samples, presenting another option for the development of robust MVA models (Fig. 22). While MIR spectroscopy has been occasionally used for predicting the chemical composition of different feedstocks in a static environment, Jones et al. illustrated the viability of online thermal emission MIR spectroscopy to predict glucan and

hemicellulose in a moving process stream containing samples of 96 hard- and softwood chips (Jones et al. 2002). The ratio of the vibrational modes at 1,510 (lignin) and 900 cm^{-1} (cellulose) has been used to estimate the relative lignin-to-cellulose content of plant cell walls following different pretreatment strategies, such as dilute sulfuric acid and IL (Li et al. 2010, 2011). Variations of MIR spectroscopy, such as diffuse reflectance infrared Fourier transform (DRIFT) or photoacoustic FTIR spectroscopy, have also exhibited viability for the analysis of biomass (Meder et al. 1999; Bjarnestad and Dahlman 2002). These techniques, used for solid analyte analysis, require little to no sample preparation, can probe larger sample areas, and are not sensitive to sample morphology, significantly increasing the analytical throughput.

When endeavoring to construct robust MVA models based on vibrational spectroscopy, the methodical analysis of the regression coefficients used to build the model is essential. Regression coefficient plots illuminate which wavenumber locations have been critical in designing the model. These vibrational modes should correspond to the representative peaks established for the analyte of interest. Analyzing these plots is more straightforward when employing MIR or Raman spectra, since there are more discrete vibrational modes representative of the plant cell wall constituents. Although cellulose, hemicellulose, and lignin, for example, have common vibrational modes, leading to peak overlap in that portion of the spectrum, there are often specific peaks indicative of a sole analyte. This can be illustrated when comparing the spectra of microcrystalline cellulose to that of isolated lignin (see Fig. 1). The main peaks from lignin occur near 1,600 cm^{-1}, whereas cellulose does not contain vibrational modes in this region. The overlapping modes and broad peaks in NIR spectra complicate the determination of what is actually being predicted since correlating the developed models to spectral peaks is challenging.

3.1.2 Ultraviolet/Visible Spectrophotometry

UV/Vis spectrophotometry probes a molecule's electronic transition to higher energy levels when light is absorbed. Quantitative analysis can be achieved using Beer's law, in which the absorbance intensity is linearly proportional to the analyte concentration. By reading the absorbance value at a specific wavelength or wavenumber location for standard solutions with differing, known concentrations, a calibration curve can be developed. The equation of the trend line produced from the calibration curve can then be used to calculate the concentration of an unknown.

UV/Vis spectrophotometry has typically been employed in conjunction with wet chemical, often colorimetric, methods such as the phenol-sulfuric acid assay for carbohydrate quantitation (Masuko et al. 2005) or following the enzymatic transformation of key analytes such as glucose or xylose to derivative products with a concomitant side reaction, such as the reduction of NAD^+ to NADH, which can then be monitored at 340 nm (Bondar and Mead 1974; Selig et al. 2011). Crystalline cellulose content has also been measured spectrophotometrically by employing the Updegraff method (Updegraff 1969; Foster et al. 2010). Recent high-throughput techniques have enabled the rapid, nondestructive quantitation of glucan and xylan in a wide array of potential biofuel feedstocks, substantially diminishing analysis time (Selig et al. 2011).

4 Monosaccharide Content

The quantitation of monosaccharide content has typically involved wet chemical or chromatographic methodology such as methylation (Cui 2005), sulfuric acid or TFA hydrolysis (Collings and Yokoyama 1979; Fengel and Wegener 1979; Brummer and Cui 2005; Foyle et al. 2007; TAPPI 2009; Sluiter Justin et al. 2010; Lygin et al. 2011), derivatization followed by reductive cleavage (DRFC) coupled with GC (Lu and Ralph 1997; Brummer and Cui 2005), high-performance anion exchange chromatography (HPAEC) (Brummer and Cui 2005), HPLC (Sluiter Justin et al. 2010; Dvorackova et al. 2014), or CE (Goubet et al. 2002; Persson et al. 2011). Often these techniques are used to decompose the polysaccharides inside plant cell walls to their monomeric constituents. Then, the polymeric content can be calculated using stoichiometric conversion factors. The measurement of glucose and xylose release from the enzymatic saccharification of biomass is another way in which monosaccharide content is evaluated. This is an important step in the screening of potential biofuel feedstocks, as plants possessing greater cellulose content should translate into higher glucose yields for subsequent fermentation to bioethanol. One of the more customary detection techniques for monomeric sugar release is HPLC. One of the major impediments with HPLC, however, is the data collection run time associated with each sample. For the screening of large sample sets, higher-throughput methods are essential.

4.1 Vibrational Spectroscopy

Although vibrational spectroscopy has not been frequently exploited for the quantitation of monosaccharide content, a few recent developments are worth mentioning (Ona et al. 1998, 2003; Rodrigues et al. 2001; Smith-Moritz et al. 2011). FT-Raman spectroscopy has been used to predict which monomeric sugars comprised the hemicellulose fraction of *E. camaldulensis* and *E. globulus* (Ona et al. 1998, 2003). FTIR spectroscopy has been utilized for the characterization of monosaccharide content following the sulfuric acid hydrolysis of *E. globulus* (Rodrigues et al. 2001). In this study, both univariate and multivariate analyses were considered. Univariate analyses require the detection of a single vibrational mode that corresponds to the desired analyte(s). Due to the complexity of lignocellulosic biomass, and the similarity in the vibrational modes measured for cell wall polysaccharides, the experimental spectral resolution can substantially limit the use of univariate methods, as many bands will overlap. Univariate techniques, when viable, however, can significantly facilitate quantitative analysis. The integrated peak area or peak height of the distinctive vibrational mode can be used in conjunction with calibration curves from measured standards. NIR spectroscopy has enabled the screening of cell wall monosaccharide changes in wild-type and mutated *Arabidopsis* and rice (Smith-Moritz et al. 2011). As researchers attempt to develop biofuel feedstocks possessing more ideal phenotypic traits, high-throughput screens are paramount to the

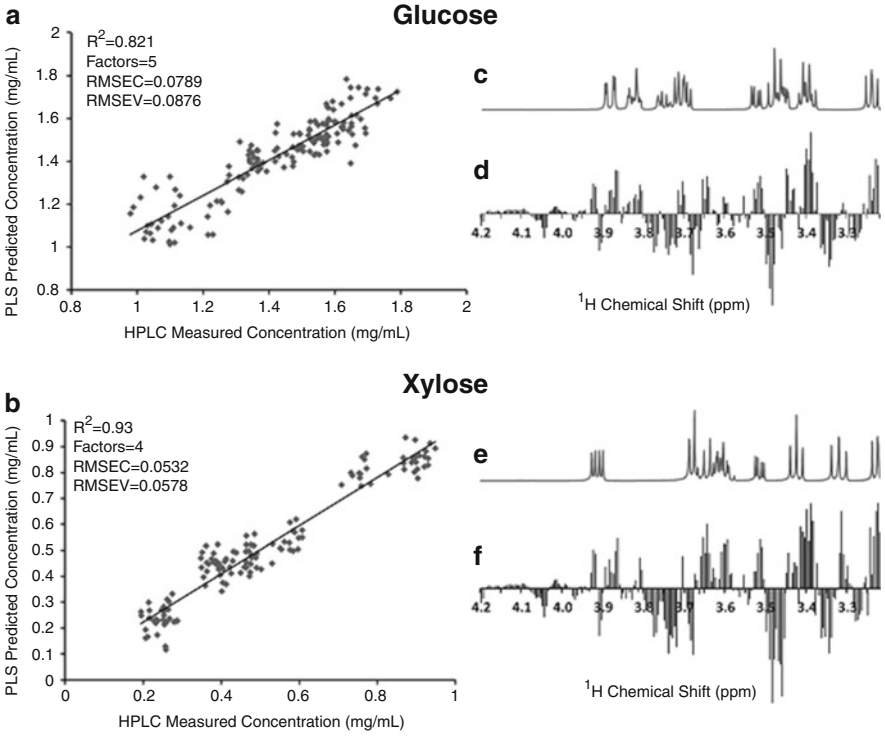

Fig. 23 PLS models constructed with ^{1}H NMR spectra and the HPLC analysis of (**a**) glucose and (**b**) xylose from multiple feedstocks. The R^2 for the validation set shows the linearity of the models. (**c**, **e**) show the NMR spectra and (**d**, **f**) show the regression coefficients for the glucose and xylose models, respectively (Reprinted with permission from Gjersing et al. 2013)

identification of successful mutations. In this study, the authors evaluated monosaccharide content variance in a rice mutant population containing thousands of unknown samples.

4.2 Nuclear Magnetic Resonance

A recent ^{1}H NMR analysis showcased the instrument's capability of quantifying monosaccharides in bamboo, eucalyptus, and sugarcane bagasse (Alves et al. 2010). The results showed a high correlation with those obtained using HPLC, especially for glucose and xylose. Another novel technique for assessing monosaccharide content coupled NMR spectral data with glucose, xylose, galactose, arabinose, and mannose concentrations measured with HPLC (Gjersing et al. 2013). PLS models were generated to predict multiple sugars simultaneously. Figures 23 and 24 illustrate the predictive capacity of the models. Although NMR

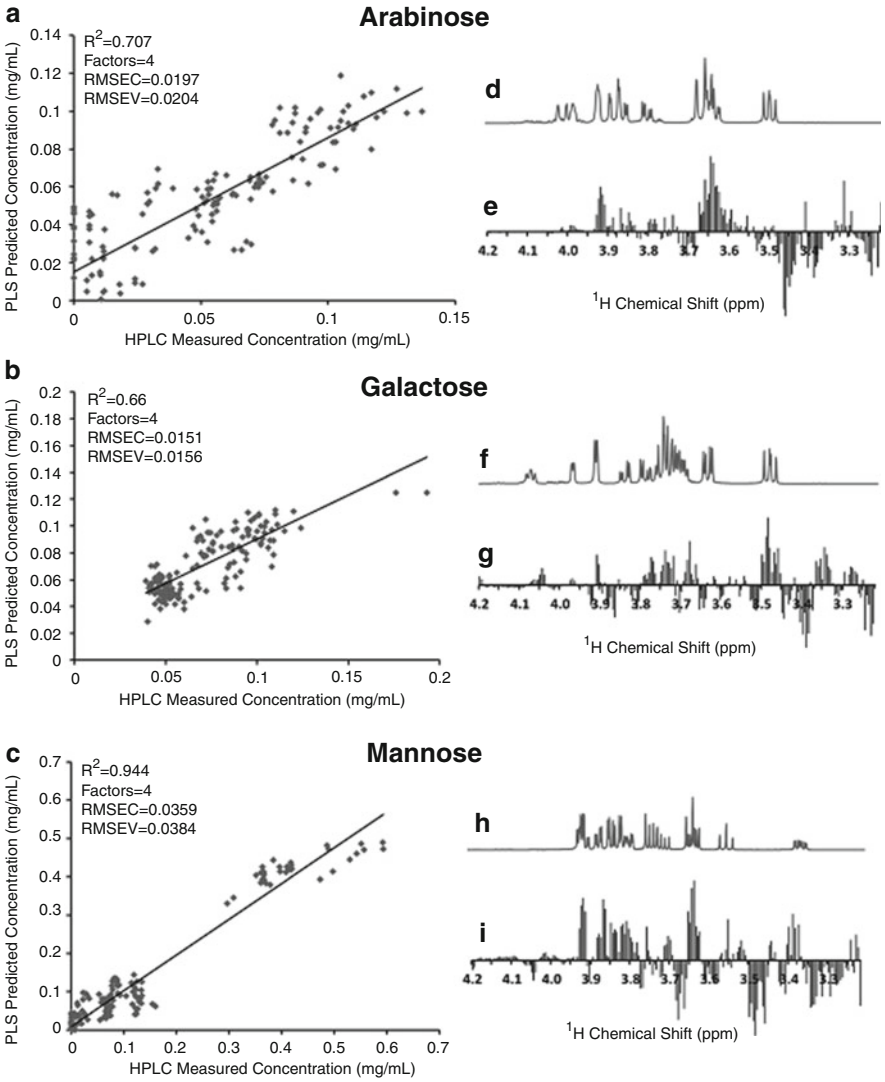

Fig. 24 PLS models constructed with ^{1}H NMR spectra and the HPLC analysis of (**a**) arabinose, (**b**) galactose, and (**c**) mannose. The R^2 for the validation set shows the linearity of the models. (**d**, **f**, and **h**) show the NMR spectra and (**e**, **g**, and **i**) show the regression coefficients for the arabinose, galactose, and mannose models, respectively (Reprinted with permission from Gjersing et al. 2013)

spectral data has not often been employed for the development of MVA models, perhaps due to the time-consuming nature of the technique, this study reduced the experimental time to one-third that required for the respective HPLC analysis, thereby significantly increasing the analytical throughput.

5 Conclusions

This chapter has provided a brief survey of how analytical spectroscopy has facilitated the qualitative and quantitative study of structural polysaccharides in lignocellulosic biomass. Techniques employing 2D NMR, such as COSY and HSQC, have allowed researchers to elucidate the labyrinthine entanglement between lignin and cell wall polysaccharides by the identification of key lignin-carbohydrate linkages. Developments in Raman and MIR spectroscopy have provided new methods for rapidly assessing cellulose crystallinity. Some of the most significant advances in the use of spectroscopy for polysaccharide analysis are the contemporary developments in Raman, MIR, and NIR spectroscopy that have enabled the progress of high-throughput methods for quantifying carbohydrates inside plant cell walls. This is paramount for the rapid screening of diverse arrays of naturally occurring or genetically designed plants to gauge their suitability for deconstruction to biofuels and bio-based chemicals. As the potential applications of analytical spectroscopy continue to be established, a more exhaustive comprehension of cell wall structural polysaccharides will be achieved, significantly facilitating the isolation of plants possessing high cellulose content, as well as the improvement of the degradative strategies paramount for efficient enzymatic hydrolyses.

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Carbohydrate Secondary and Tertiary Structure Using Raman Spectroscopy

40

Shaun Thomas Mutter and Ewan William Blanch

Contents

Abstract

Raman spectroscopy is a long-established analytical technique that has now proliferated into a variety of research tools that are able to identify and characterize almost any type of molecule under most conditions. As such, Raman spectroscopies are well suited to the study of carbohydrates, from simple monosaccharides to the largest glycosaminoglycans and from industrial bioreactors to *in situ* measurements on living cells. This review covers a range of examples of how Raman techniques are addressing the questions of glycobiologists working on diverse aspects of this fascinating but poorly understood class of biomolecules.

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Focus is placed on the application of Raman, surface-enhanced Raman, Raman optical activity, and related spectroscopies to characterizing carbohydrates of all types, with only a general introduction to the theory of the techniques themselves. Particular attention is also paid to the computational tools now regularly used by spectroscopists to analyze complex data. Although this review is aimed at the glycobiology community, the examples discussed also demonstrate to the expert spectroscopist how their techniques can impact on the exciting opportunities presented by working with carbohydrates.

1 Introduction

While the importance of carbohydrates to both the natural world and human society is clear, it is surprising how limited is our current understanding of the structural parameters that control the function and behavior of complex carbohydrates, particularly in contrast to proteins and DNA which are far better understood. While high-resolution structural techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) have revolutionized biology through their ability to reveal detailed information on proteins and nucleic acids, they are less applicable to carbohydrates. Most glycoproteins and polysaccharides do not form diffractable crystals, while their typical combination of large size and low sequence complexity often prevents the acquisition of well-resolved NMR spectra. The development of our understanding of how sugars, glycans, and polysaccharides behave therefore requires the use of other biophysical techniques.

Raman spectroscopy, which measures the vibrational motions of molecules induced through the inelastic scattering of light, possesses several characteristics that makes it particularly suitable as an analytical technique for carbohydrates. Raman spectra can be obtained from samples in any physical state, which is particularly significant considering that complex carbohydrates are commonly found in solution, solid, and gel states; the technique is noninvasive and nondestructive; there is no size limit to the samples that can be studied, allowing studies on high molecular weight polysaccharides and large glycoproteins; and the spectra contain information about all levels of biomolecular structure. There are also a number of variants of conventional Raman spectroscopy that have been used to provide important new insights into carbohydrate structure. Surface-enhanced Raman scattering (SERS) exhibits a significant boosting of Raman bands due to surface plasmons generated by specific metal surfaces, thereby increasing sensitivity to even trace levels of biomolecules and forming the basis for a new generation of biosensors. Raman optical activity (ROA) is the chirally sensitive form of Raman scattering and displays an exquisite sensitivity to stereochemistry and conformational dynamics. These complementary Raman techniques together form a valuable toolkit for glycobiology, and this review presents an introduction to each of these methods and examples of their application to carbohydrates. We also discuss the capabilities of density functional theory (DFT) modeling and chemometrics for revealing further structural information about carbohydrates from these spectra.

The purpose of this review is not to exhaustively cover all previous research in this field but to illustrate to glycobiologists, through the use of relevant examples, how different Raman techniques can generate new information on carbohydrates and their behavior.

1.1 Raman Spectroscopy

Photons interact with matter in a number of ways including through absorbance and reflection. A proportion of photons will be scattered from molecules, and most of these elastically scatter, with no energy transfer, which is called Rayleigh scattering. However, a small proportion of the scattered photons (around 10^{-7} of the incident photons) will undergo a transfer of energy, either from the photon to the molecule, so raising it to an excited vibrational state (Stokes scattering), or from a molecule in an excited vibrational state to the photon, thus returning it to a lower energetic state (anti-Stokes scattering). In Raman spectroscopy, monochromatic radiation is used to excite Raman scattering, and, most typically, the Stokes-scattered photons are collected and analyzed as this leads to the optimum signal intensity as Boltzmann averaging dictates that there are more molecules in the ground vibrational state than in excited states. The differences in energy between the Raman-scattered and incident photons will correspond to vibrational motions of the molecule, which depend upon the atoms involved in the vibrational mode and their local environments. There are 3n–6 vibrational modes of a nonlinear molecule (where n is the number of atoms), with many, or most, of these potentially giving rise to Raman transitions. Therefore, the Raman spectrum of any molecule is an information-rich fingerprint of the molecule and any structural changes induced by the experimental conditions, e.g., through changes in temperature, pH, and binding interactions, or any other chemical or physical factor. Many different types of Raman spectrometer can be found, with these being optimized for different purposes, with Raman microscopes being particularly popular for studies on solids, including cells and tissues. For biological samples, care may have to be exercised in selecting the most appropriate type of Raman instrument. This is particularly true in terms of the competing factors of scattering efficiency, which is related to the excitation wavelength (λ) by λ^{-4} and so is higher at shorter wavelengths, and fluorescence, which can overwhelm a Raman spectrum and is more intense at shorter visible wavelengths. In the case of pure samples of carbohydrates, fluorescence is not typically a serious problem; however, for biofluids and tissues, this may be an issue.

A typical Raman spectrum of a simple carbohydrate is shown in Fig. 1, in this case for D-ribose in water at ~20 °C. Characteristic band patterns related to the functional groups of the saccharide are evident over the presented range of 300–1,800 cm^{-1}. Although they are not shown in this figure, other spectral regions can also be studied, particularly the C–H stretching region from ~2,800 to 3,100 cm^{-1}, N–H stretching region from ~3,200 to 3,600 cm^{-1}, with O–H stretching modes usually appearing ~3,600–3,700 cm^{-1}, and carboxylic acids

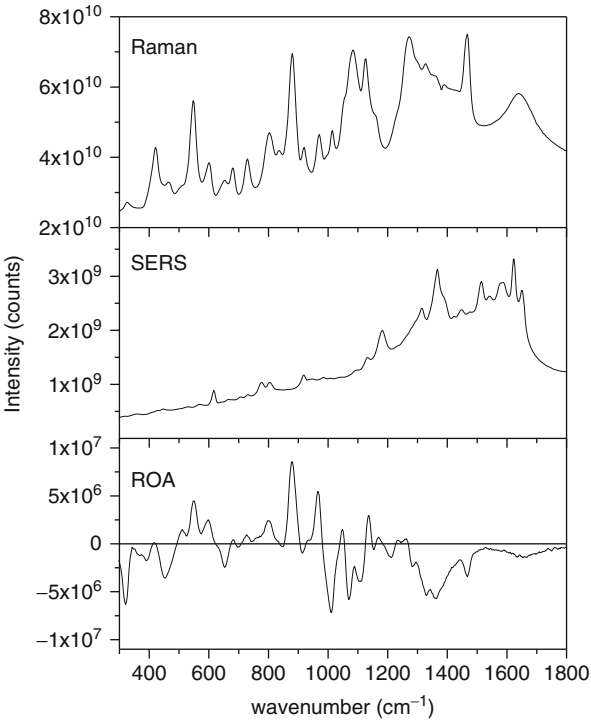

Fig. 1 Raman (*top*), SERS (*middle*), and ROA (*bottom*) spectra of D-ribose in solution. The Raman and ROA spectra were recorded at a concentration of 2.66 M for 237 min and with laser power at the sample of 625 mW. The SERS spectrum of D-ribose was measured with a concentration of 1.67 mM, for 20 min with 100 mW laser power. For the SERS measurement, silver citrate-reduced colloids and 20 mM K_2SO_4 as the aggregating agent were used (Figure was adapted from Ostovar Pour et al. 2011)

generating additional bands between 2,500 and 3,200 cm^{-1}. Detailed analysis of the functional group origins of these Raman bands can be found elsewhere (Ostovar pour et al. 2011), but in general many of the Raman modes of sugars over this range tend to involve complex combinations of molecular vibrations, with many of the Raman bands presented here for D-ribose from 700 to 1,100 cm^{-1} originating from various C–C and C–O stretching motions, sometimes with coupling to O–H deformations. Raman bands of carbohydrates from 1,300 to 1,600 cm^{-1} generally arise from CH deformations, and below 700 cm^{-1} can be seen bands signifying deformation modes of the skeletal ring backbone. The anomeric ratio can also be directly characterized from Raman bands in the region of ~630–700 cm^{-1} (Mathlouthi et al. 1983; Carmona and Molina 1990). The complex band patterns of carbohydrate Raman spectra make them useful fingerprints of identity and functional group composition, as well as the effects on structure induced by the local environment.

1.2 Surface-Enhanced Raman Scattering (SERS)

Although Raman spectroscopy is now widely used in the chemical, biological, and even medical sciences, it still is generally viewed as a relatively weak effect. One solution to this problem is the use of plasmon resonance enhancement mechanisms to greatly reduce detection limits and increase spectral intensities, which leads to

surface-enhanced Raman scattering (SERS). There are many detailed reviews on the origin and potential applications of SERS (Anker et al. 2008; Schlucker 2009, 2014; McNay et al. 2011; Larmour and Graham 2011; Kitahama et al. 2012; Ringe et al. 2013; Yamamoto et al. 2014), and so we present only a brief summary here of the relevant aspects.

While the first observation of SERS, of pyridine on an electrochemically roughened silver surface, was made by Fleischmann et al. (1974), it was in 1977 that two other groups suggested mechanisms for the enhancement process that are today widely accepted as being correct (Jeanmaire and van Duyne 1977; Albrecht and Creighton 1977). Considerable debate has been generated on the relative importance of different contributing factors to the SERS effect, but it is generally recognized that there are two main effects involved. The electromagnetic (EM) mechanism describes how an incident light wave can excite localized surface plasmons in the vicinity of roughened or geometric features on the surface of certain metals, with the most commonly used being silver or gold. The greatly enhanced local electric field then leads to an increase in Raman intensity. Both the Raman incident and emitted fields may be enhanced, generally by different amounts. The nature of the metal surface will affect the plasmon resonance frequency, so changing the SERS response. In general, silver surfaces give strongest enhancements for visible excitation frequencies, while gold surfaces work best at near-infrared excitation frequencies.

Although the EM mechanism is thought to be responsible for much of the observed SERS spectrum, it does not explain the whole process, and an additional contribution comes from the chemical enhancement (CE) mechanism. The CE mechanism involves charge transfer between the chemisorbed analyte molecule and the metal surface. It can be generalized that while the EM mechanism is responsible for most of the signal enhancement observed, the CE mechanism gives rise to the selective band enhancement that makes the SERS spectrum distinctive and different to the parent Raman spectrum. In Fig. 1, the SERS spectrum of D-ribose clearly has a different profile to the corresponding Raman spectrum due to the chemical enhancement of selected Raman bands. Although establishing the molecular origins of SERS bands is a subject of ongoing research for many group, most SERS bands measured for carbohydrates are not yet assigned in the literature. These SERS spectra are therefore commonly used as spectral fingerprints for the detection of specific sugars under conditions of low concentration and laser power. We note that the principal bands shown in the SERS spectrum for D-ribose are between 4 and 5 orders of magnitude stronger than the corresponding Raman features, on a per molecule basis. Such enhancement factors are commonly obtained, verifying the sensitivity of SERS as a bioanalytical tool.

1.3 Raman Optical Activity (ROA)

Raman optical activity (ROA) measures a small difference in the intensity of Raman scattering from chiral molecules using right- and left-circularly polarized

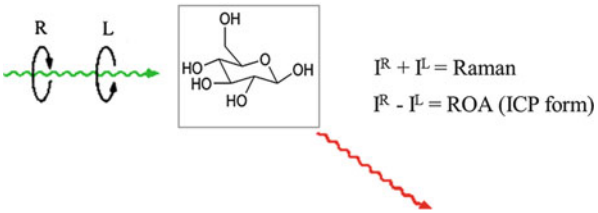

Fig. 2 Schematic of the ROA scattering process, specifically showing the incident circular polarization (*ICP*) form. If the scattered radiation was circularly polarized instead, this would correspond to the SCP form. The SCP form is most widely used, but in the absence of resonance effects, the ICP and SCP forms are equivalent and generate the same spectra (Figure is adapted from Barron et al. 2000)

light; see Fig. 2. ROA was first predicted in 1969 by Atkins and Barron (1969) and then observed experimentally in 1972 by Barron et al. (1973). Until recently, ROA spectroscopy was only used in a handful of laboratories around the world as it is a weak effect which requires careful measurement, with ROA scattering intensities typically being 10^{-3} to 10^{-5} of the corresponding Raman scattering. Although still a niche technique, at the time of writing, ROA was now available in around 30 laboratories globally, in both universities and pharmaceutical companies.

As is shown in Fig. 2, the circular polarization may be in either the incident light, leading to incident circular polarization (ICP) ROA, or the scattered light, leading to scattered circular polarization (SCP) ROA, or even in both, which gives rise to dual circular polarization (DCP) ROA. Most early ROA measurements, until around 2004, were made with the ICP form, but since then SCP ROA is the most common. However, this does not present a problem for comparing spectra measured with the different forms of ROA as in the far-from-resonance limit they give the same spectra. As can be clearly seen in Fig. 1, as ROA is a difference measurement, the spectrum is bisignate, with both positive and negative features. The ROA bands observed originate from the Raman bands, but as ROA spectral features derive from not only the electric polarizability but also the magnetic polarizability and the quadruple moment, ROA features display different intensities and bandshapes to the corresponding Raman features. They can also present both mono- and bisignate features. This is apparent for several of the ROA bands of D-ribose, shown in Fig. 1. Further details of the sensitivity of ROA bands to the local stereochemistry and structure of carbohydrates are discussed in this review, below.

The ROA measurement can be represented quantitatively by the circular intensity difference (CID), defined as:

$$\Delta = \left(I^R - I^L\right)/\left(I^R + I^L\right) \tag{1}$$

where I^R and I^L are the scattered Raman intensities in right- and left-circularly polarized incident light, respectively. If the R and L labels were subscripted, this would denote right- and left-circularly polarized scattered light intensities. As was mentioned above, in the absence of electronic enhancement, which is the typical

case for ROA studies on carbohydrates, the incident and scattered CID expressions are equivalent. For readers interested in the theory of ROA or its general application to other classes of molecules, we recommend any of several comprehensive reviews (Blanch et al. 2003; Barron et al. 2003, 2004; Barron and Blanch 2009).

2 Studies on Simple Carbohydrates

Early Raman studies on mono-, oligo-, and polysaccharides were mainly conducted on crystalline samples; see, e.g., Cael et al. (1974), Mathlouthi and Koenig (1986), Wells and Atalla (1990), and Dauchez et al. (1994a, b). These studies established that many of the vibrational modes of monosaccharides are complex and involve greater mixing of internal coordinates than is generally found for peptides and amino acids. Although this can make detailed analysis of the vibrational modes of sugars more challenging than for other biological molecules, the results from these studies indicated that characteristic spectral fingerprints could still be identified. Thus, the ability of Raman spectroscopy to differentiate between different monosaccharides, and so serve as a sensitive probe of carbohydrate identity, has been known for some time. Kačuráková and Mathlouthi (1996) found that they could use the Raman profiles to distinguish between a selection of simple sugars (D-glucose, D-galactose, lactose, maltose, melibiose, maltotriose, raffinose, and trehalose) in solution. These authors also found that distinctive Raman bands of the monosaccharides could be detected in the spectra of several of the disaccharides as the conformations of the individual sugars were very similar to those of the corresponding subunits. This contrasted with the larger changes observed in the spectra of melibiose and trehalose, as a result of the larger structural differences induced by formation of the glycosidic linkages. Similarly, Arboleda and Loppnow found that their Raman spectra of each of nine different monosaccharides were unique (Arboleda and Loppnow 2000). These monosaccharides were *O*-methylated to block racemization of the anomeric carbon. The authors found that they could even distinguish between the α- and β-anomers of several of these monosaccharides. They also reported that such distinctions could be made on samples of less than 0.1 mg, though typically larger amounts are generally used in most studies. Arboleda and Loppnow were also able to use Raman marker bands to identify the monosaccharide components of unknown disaccharide samples (Fig. 3).

Although carbohydrates do not display as well-defined coordination properties as many other natural ligands, they do bind transition metal ions. The large number of electronegative functional groups and well-defined stereochemistries of sugars makes them attractive synthons for the synthesis of more complex structures. The mode of coordination of metal ions with the different hydroxyl groups of sugars is a subject of importance for guiding new syntheses, but quantitative characterization of the anomeric and conformational species of the carbohydrates involved in these complexes is difficult. Cerchiaro et al. used Raman spectroscopy and electron paramagnetic resonance (EPR) to investigate complexes of Cu(II) ions with D-glucose, D-fructose, and D-galactose (Cerchiaro et al. 2005). They found that

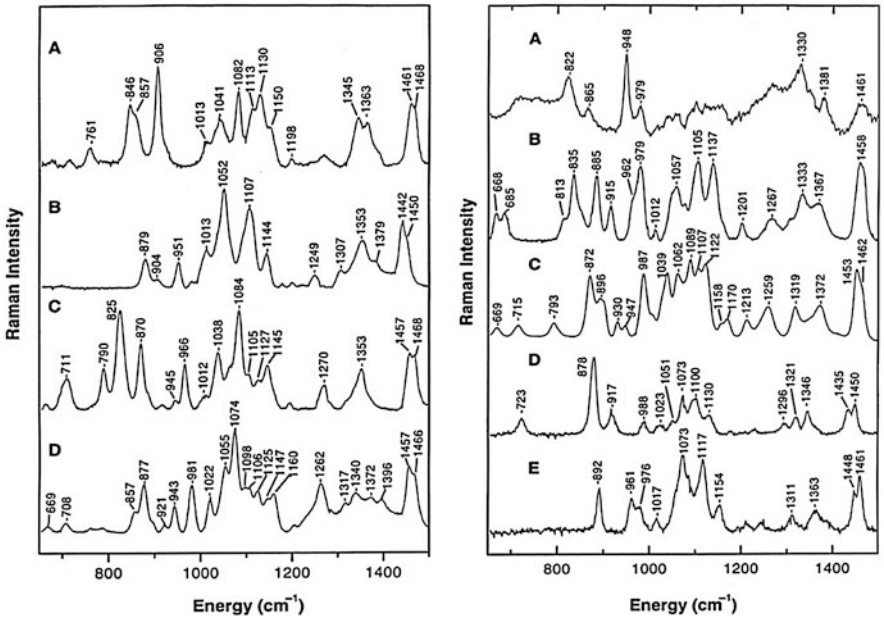

Fig. 3 Raman spectra of 1-*O*-methyl-monosaccharides. *Left-hand panel*: (*A*) 1-*O*-methyl-α-D-glucoside, (*B*) 1-*O*-methyl-β-D-glucoside, (*C*) 1-*O*-methyl-α-D-galactoside, and (*D*) 1-*O*-methyl-β-D-galactoside. *Right-hand panel*: (*A*) 1-*O*-methyl-α-D-*N*-acetylgalactosamine, (*B*) 1-*O*-methyl-α-D-mannoside, (*C*) 1-*O*-methyl-β-D-mannoside, (*D*) 1-*O*-methyl-α-D-xyloside, and (*E*) 1-*O*-methyl-β-D-xyloside. All spectra were measured with 2 W of 514.5 nm light. Note that the label of the ordinate axis used here, "energy," means the same as the more common "wave number" and "Raman shift" labels (Figure is Adapted from Arboleda and Loppnow 2000)

the β-anomer was the dominant form of D-glucose when complexed, but both α- and β-anomers were found in the complexes of D-galactose. For the complexes of D-fructose, both furanose and pyranose forms were detected, with furanose being the major one. Specific hydroxyls and oxygens were also identified as being coordination sites for the Cu(II) ions, from changes in their signature Raman bands upon complexation.

Mixtures of simple sugars are commonly found, and overlap of Raman bands from these sugars can yield complex spectra. However, standard analytical tools can successfully resolve the differences between the Raman spectral signatures and hence the identities and relative contributions of each. A nice illustration of this is a teaching experiment designed by Wang and colleagues (2009), who used principal component analysis and regression models (PCA/PCR) from the Raman spectra to quantify the relative compositions of three sugars (D-(−)-fructose, D-(+)-glucose, and D-(+)-galactose) in a solution mixture. It is particularly noteworthy that in this study undergraduate students were able to use this approach to obtain accurate results.

Previously, Mrozek et al. had used both Raman and SERS spectroscopies to differentiate between two sugars, maltotetraose, and stachyose (Mrozek et al. 2004). Their reported SERS spectra, following a procedure developed by

Mrozek and Weaver to measure SERS of several monosaccharides (Mrozek and Weaver 2002), were very similar to the corresponding Raman spectra, but apparently with enhancement factors of 2–3 orders of magnitude, so significantly reducing detection limits. These authors also considered the question of separating and quantifying the spectral signatures of each of the two sugars in a binary mixture and found this could be achieved accurately using PLS algorithms.

As previously stated, one advantage of SERS as an analytical technique is that it does not require an extrinsic label. However, in cases where even higher signal enhancements are sought, facilitating even down to attomolar detection, a widely used strategy is to chemically link a chromophoric reporter group, typically a dye compound, to either the analyte molecule or the metal surface. This further electronic resonance enhancement gives rise to the technique of surface-enhanced resonance Raman scattering or SERRS. Vangala et al. adopted this approach and coupled rhodamine tags to three different sugars: glucose, lactose, and glucuronic acid (Vangala et al. 2010). These tagged carbohydrates were shown to increase sensitivity for not only Raman detection but also for fluorescence and mass spectrometric analysis as well.

All sugars are chiral, making Raman optical activity (ROA) an obvious technique for investigating their solvated conformations. The Barron group in Glasgow undertook a number of studies on small sugars and found that detailed and informative spectra could be measured (Bell et al. 1994a, b, 1995; Macleod et al. 2006). Most significantly, Wen et al. (1993) verified that ROA can determine the absolute configuration about each chiral center of a carbohydrate and differentiate between α- and β-anomeric linkages; that large ROA spectral differences can be observed between epimers; that ROA spectra can also discriminate between homomorphic sugars, which have the same stereochemistry around each chiral carbon but different substituents, though these spectral differences are weaker than those found for epimers; and finally, that ROA can infer the relative conformations of CH_2OH groups, which are important for intramolecular hydrogen bonding and for stabilizing backbone conformations.

Further studies were conducted on cyclodextrins (Bell et al. 1997; Barron et al. 1990), and these established that ROA can provide detailed information about the conformational dynamics of oligosaccharides. The first ROA spectra of glycoproteins were, again, first reported by the Glasgow group (Bell et al. 1994c; Smyth et al. 2001; Zhu et al. 2005), who then subsequently demonstrated that ROA could simultaneously probe the structural complexity of both the protein and glycan components of a glycoprotein (Johannessen et al. 2011).

3 Conformational Studies on Polysaccharides and Glycoproteins

Raman and ROA spectroscopies have also been used to characterize the conformations and behavior of complex polysaccharides. One notable example of this is the study by Yaffe et al. on hyaluronan (HA), a nonsulfated glycosaminoglycan (GAG)

composed entirely of repeating disaccharides of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) linked by alternating β-1,3 and β-1,4 glycosidic bonds (Yaffe et al. 2010). Hyaluronan is found in all vertebrate tissues as a high molecular mass polysaccharide and performs a wide range of biological functions, leading to its widespread use in medicine, tissue engineering, and cosmetics. However, we understand relatively little about the structural parameters that regulate hyaluronan organization and function. Yaffe et al. were able to use the stereochemical sensitivity of ROA in order to monitor characteristic intersaccharide interactions in a short hyaluronan subunit (the HA_4 tetramer) but then found that there were no signs of extensive interchain interactions being formed in a much longer HA chain (see Fig. 4). In this way, the authors were able to conclude that there was no extensive tertiary structure formation in the HA polymer, in agreement with several NMR studies (Kaufmann et al. 1998; Blundell et al. 2006).

Rudd et al. have also reported the Raman and ROA spectra for several glycosaminoglycans (Rudd et al. 2010), further emphasizing the potential of the technique in glycobiology.

Recently, Ashton et al. have used both Raman and ROA spectroscopies to investigate the interactions between mucin glycoproteins (Ashton et al. 2013). Mucins are highly glycosylated, high molecular weight proteins which perform diverse roles in the formation of mucosal gels in metazoans. Mucins are important for determining the physical properties of gastrointestinal and salivary mucus, and they function as mesh-like, size, and charge exclusion barriers. Most mucins have a block copolymer structure with nonglycosylated domains at both termini that are separated by an extended glycosylated domain which is rich in serine, threonine, and proline residues. O-Linked glycosylation occurs through conjugation of *N*-acetylgalactosamine (GalNAc) sugars to the hydroxyl moieties of the serines and threonine side chains, giving rise to the so-called hinge region that is thought to be important for molecular control of the formation of the mucosal mesh. Using 2D correlation analysis with both Raman and ROA spectra, the authors were able to distinguish the order of conformational changes occurring over concentration ranges relevant to the formation of the gastric mucosal layer. They found that at mucin concentrations from 20 to 40 mg/ml, these GalNAc moieties underwent conformational changes, with other saccharides changing conformation above 40 mg/ml, together with other structural transitions observed in the protein core, specifically the formation of β-structure. In this way, Raman and ROA were able to monitor the formation of transient entanglements formed by what are thought to be brush–brush interactions between the oligosaccharide combs of mucin molecules.

One particular great challenge for glycobiology is to map and understand the expression patterns of component sugars in the glycan components of glycoproteins. With most techniques it is difficult to determine linkage types and between oligosaccharides with similar compositions. Johannessen et al. examined yeast invertase from *S. cerevisiae*, a high-mannose glycoprotein that is widely used in the sugar industry as a biocatalyst, and a number of its component oligosaccharides. Structures of these sugars are shown in Fig. 5 along with the Raman and ROA spectra of the mannose monosaccharide and three disaccharides. Unsurprisingly,

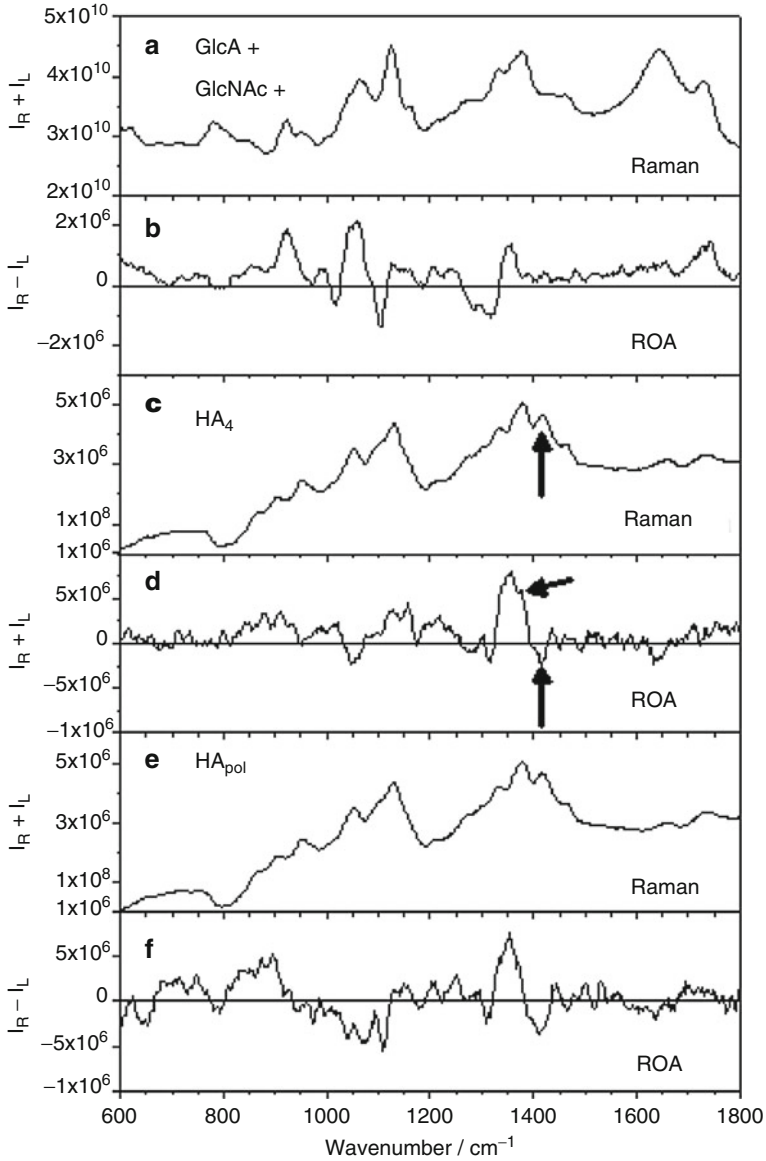

Fig. 4 Raman ($I_R + I_L$) and ROA ($I_R - I_L$) spectra, respectively, of a 1:1 stoichiometric mix of GlcA and GlcNAc (panels *A* and *B*), HA_4 tetramer (panels *C* and *D*), and the HA_{pol} polymer (panels *E* and *F*). Secondary structure marker bands are shown with *arrows*. The large differences between the ROA spectra shown in panels *B* and *D* reflect the formation of secondary structure-type interactions between saccharides, while the strong similarity between the ROA spectra for HA_4 and HA_{pol} (panels *D* and *F*) indicates that no significant tertiary interactions are formed in the polymer (Figure is taken from Yaffe et al. 2010)

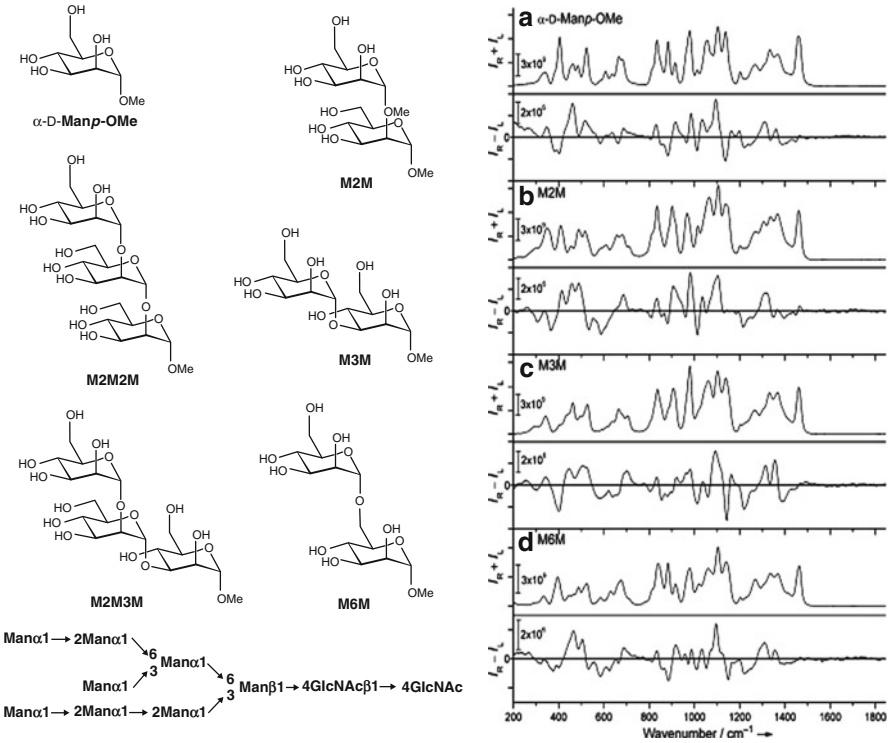

Fig. 5 Shown on the *left* are the structures of mannose oligosaccharides found in yeast invertase, along with the linkage scheme of the $Man_8GlcNAc_2$ unit. On the *right* are the corresponding Raman and ROA spectra for the mannose mono- and disaccharides (Figure is taken and adapted from Johannessen et al. (2011))

the four Raman spectra are all similar, though with some differences in detail. Far more significant differences can be observed between their corresponding ROA spectra. The obvious changes in ROA band profiles and intensities originate from the different glycosidic linkages and the resulting effects on conformation and dynamics of the component sugars.

Further differences between the ROA spectra were observed for the two mannose trisaccharides, labeled as M2M2M and M2M3M, and measured by Johannessen et al., as is shown in Fig. 6. Most strikingly, the complex ROA band structures from ~250 to 700 cm^{-1}, shown by shading, present in the spectra of M2M2M and M2M3M are also clearly apparent in the ROA spectrum of the yeast invertase. The authors concluded that this band pattern originated from the α-(1 → 2)- and α-(1 → 3)-glycosidic linkages, possibly with some minor contributions from α-(1 → 6)-linkages. As the authors stated, this close similarity in ROA band patterns also indicated that the conformations around these glycosidic links in the glycoprotein are very similar to those of the free mannose trisaccharides in aqueous solution. Although the use of Raman spectroscopies for studying protein

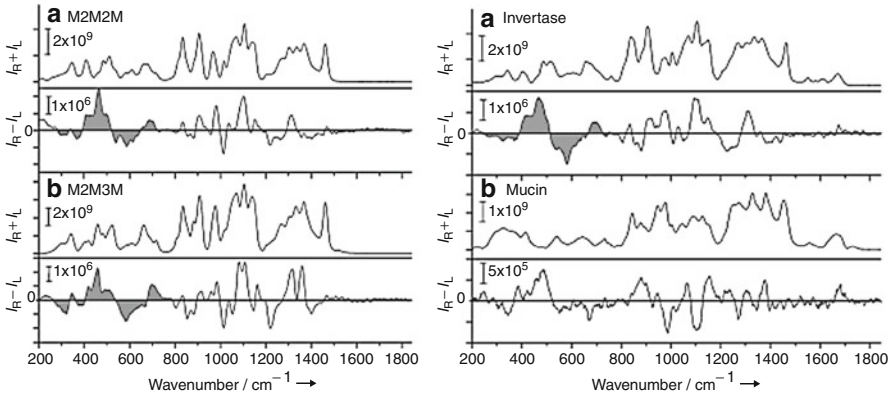

Fig. 6 Shown on the *left* are the Raman and ROA spectra for the mannose trisaccharides α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-OMe (M2M2M) and α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-α-D-Man*p*-OMe (M2M3M). Presented on the *right* are the Raman and ROA spectra of (*a*) yeast external invertase and (*b*) bovine submaxillary mucin in water. The shaded ROA band pattern in the low-wave number region of the invertase spectrum is very similar to the shaded patterns in the ROA spectra of the trisaccharides M2M2M and M2M3M (Figure is taken and adapted from Johannessen et al. 2011)

structure lies outside the purposes of this review, it should be noted that Raman spectroscopies, including ROA, can simultaneously probe both the protein and carbohydrate moieties of a glycoprotein. Johannessen et al. were able to determine from the absence of protein structural ROA marker bands that invertase was completely disordered, presumably due to the glycan content, similarly to a commercial sample of the highly glycosylated bovine submaxillary mucin.

Recently, this group extended their study on glycoproteins to ribonuclease B (RNase B). As already described, RNase B has the same protein sequence as RNase A as well as a single N-linked glycan with two GlcNAcs and a variable number of mannose residues. These researchers were able to reconstruct the Raman and ROA spectral signatures for this glycan component, as shown in Fig. 7, by subtracting the protein spectral signatures measured for the nonglycosylated RNase A (Mensch et al. 2014). Although there is insufficient information yet to analyze the glycan spectra in detail, this work demonstrates the future potential for Raman spectroscopies for investigating the structures of the glycan moieties of glycoproteins in situ.

4 Probing Hydration Interactions of Sugars

The dynamics of water molecules have long been thought to play important roles in the behavior and properties of carbohydrates. Water is a weak Raman scatterer so making Raman measurements on aqueous solutions easier than when using infrared spectroscopy. However, Raman spectra of solutes, including carbohydrates, are

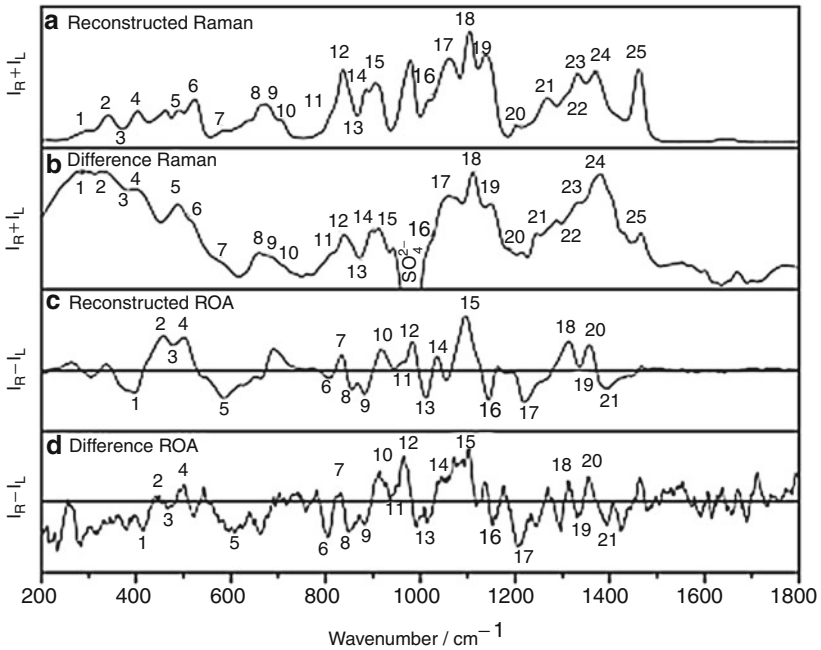

Fig. 7 Reconstructed Raman (*A*) and ROA (*C*) spectra of the glycan moiety from RNase B from the constituent disaccharide spectra and difference Raman (*B*) and ROA (*D*) spectra of RNase B after subtraction of the respective RNase A spectra. Particular bands are numbered (Figure is taken from Mensch et al. 2014)

sensitive to interactions with water molecules. Soderholm et al. investigated the sensitivity of specific Raman bands to hydration for samples of glucose and fructose (Soderholm et al. 1999). In their study of honey samples, de Oliveira et al. found that Raman bands of the component saccharides showed subtle changes in relative intensity as a function of water content (de Oliveira et al. 2002). Paola Sassi and colleagues in Perugia have performed a number of studies on hydration interactions with carbohydrates using different biophysical techniques including Raman spectroscopy (Fioretto et al. 2007; Paolantoni et al. 2007). They have shown that Raman bands from solvent water molecules are sensitive to the disordering of tetrahedral water structures by sugar molecules (Gallina et al. 2006; Perticaroli et al. 2008), with glucose and fructose appearing to have a different effect on bulk water structure than trehalose and dextran.

Irradiation of foods is now a widely used method for controlling microbial and insect contamination and can also extend the shelf life of fruits and vegetables by delaying ripening and inhibiting sprouting. However, ionizing radiation can, of course, cause chemical damage, and in the case of carbohydrates, this can lead to both the direct breaking of chemical bonds and indirect effects from formation of reactive hydroxyl radicals from water molecules within the foodstuffs.

5 Characterizing the Chemical Modification of Sugars

Raman spectroscopies show particular promise for the identification of chemical modifications in carbohydrates, particularly of sulfation. The vibrational modes of the sulfonyl group are typically intense and sharp and so are often easy to distinguish from the many other vibrational modes of carbohydrates, as was shown many years ago (Bansil et al. 1978; Cabassi et al. 1978). More recently, a number of other research groups have further explored the capabilities of Raman techniques for studying the sulfation of glycosaminoglycans. Matsuhiro and colleagues (2012) combined Raman and SERS spectroscopies in addition to FTIR and NMR to investigate the composition of material extracted from a sea cucumber (*Athyonidium chilensis*). They found that the SERS spectra were more informative than the corresponding Raman spectra, and their collective experiments characterized the glycosaminoglycan composition of the extract, with chondroitin 4,6-disulfate substituted at position O-3 of glucuronic acid and partially 2,4-disulfated-fucopyranosyl residues being found.

6 Industrial and Healthcare Applications

One of the greatest challenges currently facing the pharmaceutical sector is the characterization of posttranslational modifications (PTMs) of protein pharmaceuticals, with the most common of these being glycosylation. The stability, immunogenicity, and pharmacokinetics of a protein drug can all be affected by glycosylation, but detection and characterization of the glycan content typically require complex, destructive, or time-consuming methodologies. Raman spectroscopy has the potential to meet the requirement for a fast, noninvasive, and quantitative technique for the characterization of the glycan content of protein pharmaceuticals. Brewster et al. demonstrated that Raman spectroscopy coupled with multivariate data analyses could differentiate between the nonglycosylated ribonuclease A (RNase A) and the glycosylated ribonuclease B (RNase B, which has an identical amino acid sequence but also a single N-linked glycan containing two GlcNAcs and from three to nine mannoses), as well as deglycosylated versions of RNase B (Brewster et al. 2011). The Raman spectra shown in Fig. 8 display significant differences, highlighted in purple, due to glycosylation. Using the first principal component from the resulting PCA analysis performed on these Raman spectra, the authors were able to clearly distinguish between the glycosylated (RNase B) and nonglycosylated (RNase A) proteins.

Raman bands are quantitative, and the authors were also able to use another chemometrics technique, principal least squares regression (PLSR), to show that this methodology could accurately determine the amount of carbohydrate in a glycoprotein. In their results, presented in Fig. 9a, we observe the strong correlation between the predicted and known proportions of RNase B in mixtures of RNase A and B. Although this was a proof-of-principle experiment on a simplified system, this work is a good exemplar of the use of Raman for quantitative glycobiology.

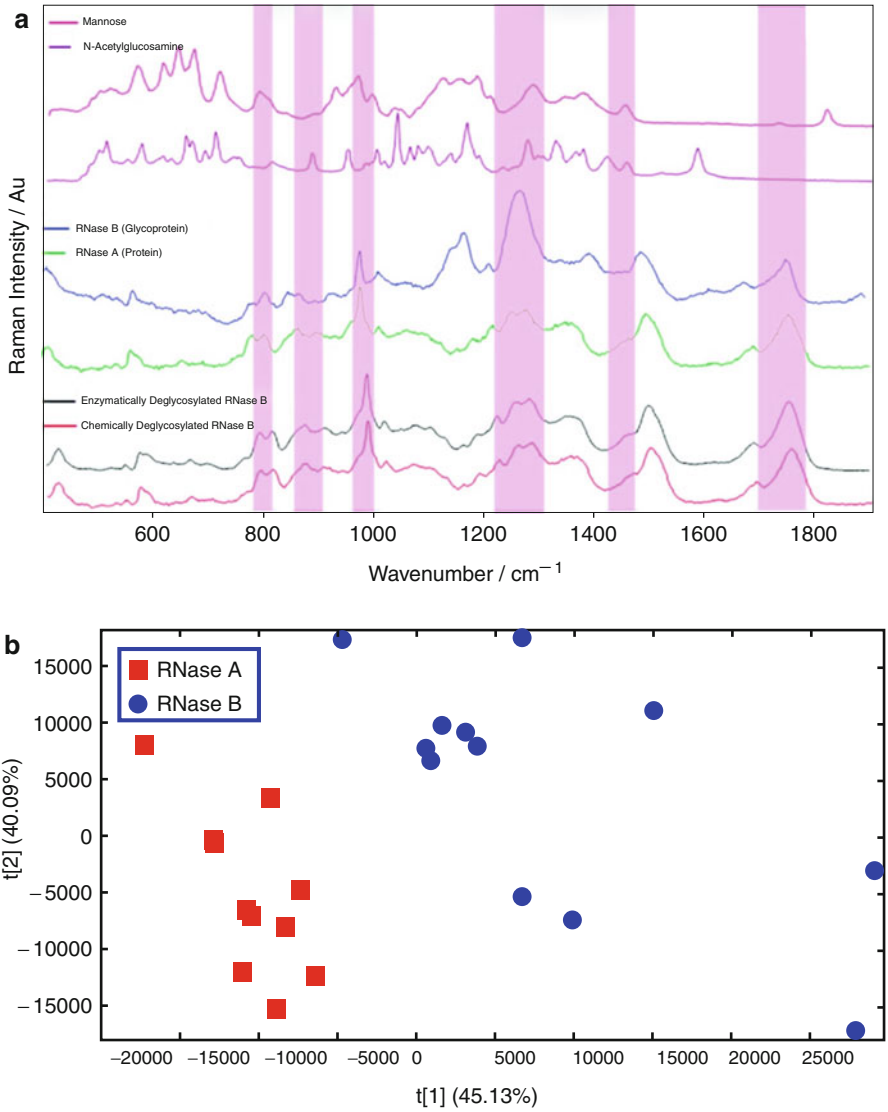

Fig. 8 Panel (**a**) shows averaged Raman spectra of mannose, GlcNAc, ribonuclease A and B, and chemically and enzymatically deglycosylated ribonuclease B, measured at 785 nm. Areas highlighted are regions of significance, as indicated by the PLS loading in panel (**b**), which presents the PCA scores plot (PC1 vs. PC2) of the ribonuclease data, showing ribonuclease A and B spectra resolved into separate clusters allowing separation of the glycosylated and nonglycosylated forms (Figure is taken from Brewster et al. 2011)

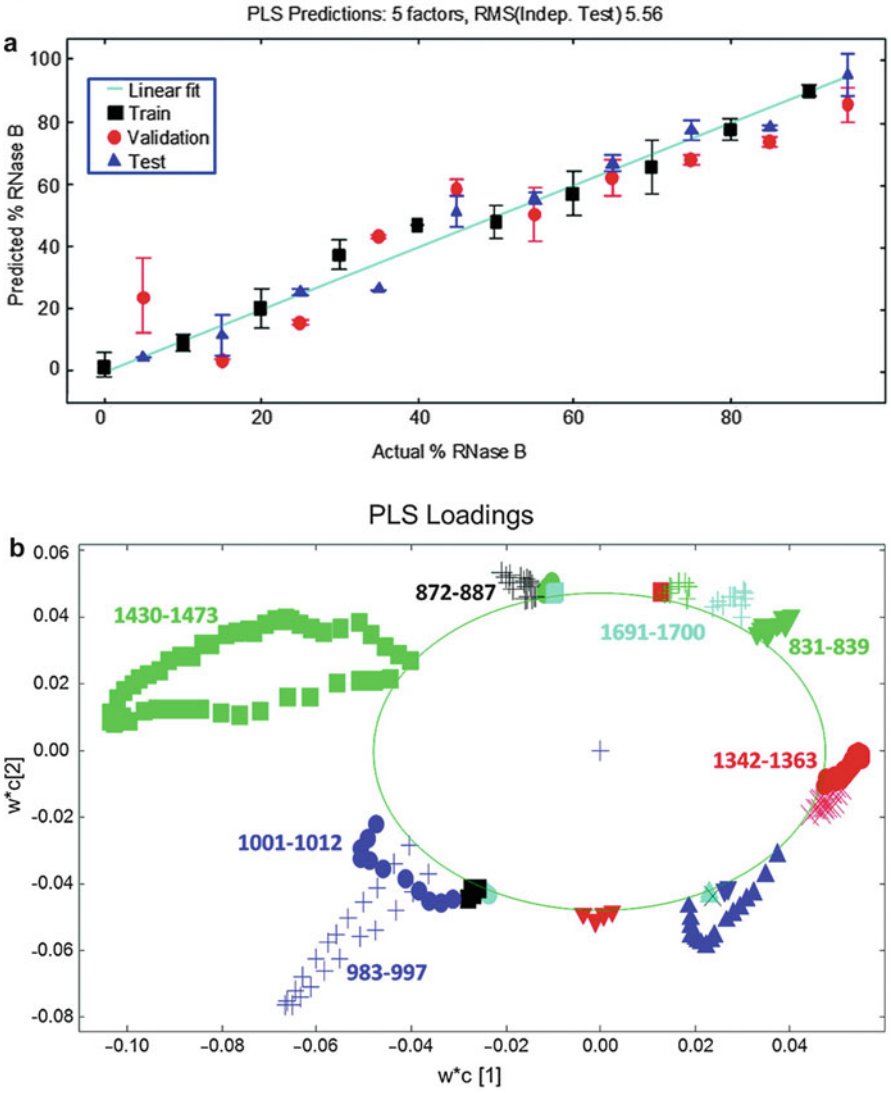

Fig. 9 (**a**) PLS predictions from Raman data of RNase mixtures (mean predictions (from $n = 5$) are plotted with standard error bars). (**b**) PLS loading plot of the first two latent variables (LVs; w_c[1] vs. w_c[2]); the *green circle* indicates the 95 % confidence limit. *Numbers* shown refer to Raman peak positions (Figure is taken from Brewster et al. 2011)

Furthermore, chemometrics analyses can also reveal greater structural detail from complex Raman spectra. Panel (b) of Fig. 9 shows the PLS loading plot from this study, with the first two latent vectors, which were responsible for most of the variance in this data, being sensitive to relative changes in selected marker bands from the regions highlighted in Fig. 8a.

A long-standing bioanalytical challenge is the detection and control of glucose levels in the bloodstream, for the control of diabetes mellitus. Among many other techniques, Raman spectroscopies have been applied to this problem. In particular, the van Duyne group at Northwestern University has developed nanolithographic surfaces as SERS-based sensors for monitoring glucose levels (Schafer-Peltier et al. 2003; Lyandres et al. 2005). More recently, this group has used a specialized spatially offset form of SERS for transcutaneous monitoring of glucose within living rats (Yuen et al. 2010; Ma et al. 2011). An alternative approach to monitoring glucose levels directly has been explored by Barman et al. who propose the detection of glycated hemoglobin (HbA1c), which is known to display a strong correlation with bloodstream glucose levels and is an approved target for screening diabetic and prediabetic conditions (Barman et al. 2012). These researchers coupled Raman excitation at 785 nm with drop coating deposition (DCD), which is a relatively new but popular analytical Raman technique (Ortiz et al. 2006). In brief, DCD uses special hydrophobic surfaces to preconcentrate the analytes in dried solution samples, so greatly increasing signal intensity. Analytes tend to coalesce into a coffee-ring pattern, and this phenomenon is still a subject of investigation, particularly with reference to the homogeneity of material deposited within the ring. Through their experiments on both HbA1c and nonglycated hemoglobin (Hb), Barman et al. found that DCD Raman combined with PCA and PLS informatics could distinguish spectra signatures of HbA1c that provided a limit of detection (LOD) of as low as 3.8 μM. This compares very favorably with clinically determined concentrations of HbA1c (60 μM and higher). Figure 10 shows how the PCA scores plot generated from their DCD Raman data could directly differentiate between the glycated HbA1c and nonglycated Hb. Although this paper by Barman et al. was another proof-of-principle experiment, they also consider in some detail the potential of this approach for clinical application, and we direct interested readers to their paper as well as a similar study on glycated albumin (Dingari et al. 2012).

The long-term and widespread prevalence of carbohydrates in foods and beverages makes the food and drink industry an obvious sector of interest for Raman spectroscopies. The rapid, noninvasive, and label-free nature of Raman scattering makes it well suited to many situations in food science and agriculture, though the complexity of spectra from food samples often requires the use of informatics during data analysis. Such a strategy was followed by Delfino et al. in their use of a standard Raman microscope to quantify glucose content in different commercial sports drinks (Delfino et al. 2011). They collected Raman spectra with a simple He–Ne laser and 50× optical objective on droplets of sample, with example spectra being shown in Fig. 11. Interval partial least squares (iPLS) was then employed to identify key marker bands and construct calibration models that allowed accurate determination of glucose concentration, within experimental error of the results from enzymatic assays.

Although the result from each Raman analysis had a higher experimental uncertainty than for the corresponding enzymatic (biochemical) assay, as is apparent in Fig. 12, this work shows that accurate quantitative analysis of sugars can be

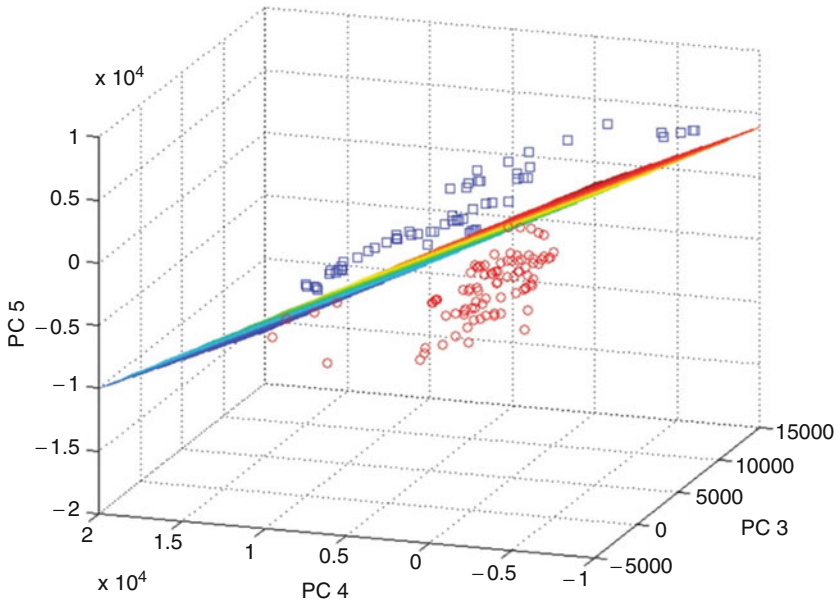

Fig. 10 Three-dimensional scores plot corresponding to principal components (*PCs*) 3, 4, and 5 for the Raman dataset acquired from single-protein Hb and HbA1c drop-coated rings. Hb samples are indicated by *red circles* and HbA1c by *blue squares* (Figure is taken from Barman et al. 2012)

obtained using relatively standard Raman equipment and multivariate analytical tools and a simple calibration model. As such Raman analyses of carbohydrates in food and beverage samples could be made rapidly and without specialist sample preparation, there is great potential for such techniques in on-site quality control and characterization of foodstuffs and beverages.

7 Applications in Virology

Glycoproteins play critical roles in the mechanisms of viral infection, such as membrane fusion, with conformational changes in these glycoproteins being important for driving these mechanisms. As already mentioned, conventional structural techniques are often difficult to apply to this problem. In the requirement for rapid clinical diagnosis of viral infection, the time constraint becomes increasingly important. The fast data acquisitions inherent to Raman technologies, coupled with the wealth of structural information that can be obtained, are leading to their application in structural virology, including the roles of glycoproteins in viral infection and cell–cell fusion. Lu et al. (2013) have used a confocal Raman microscope, along with chemometrics, to discriminate glycoproteins on the surfaces of virions and viruslike particles (VLPs) of Nipah virus (NiV). NiV is an

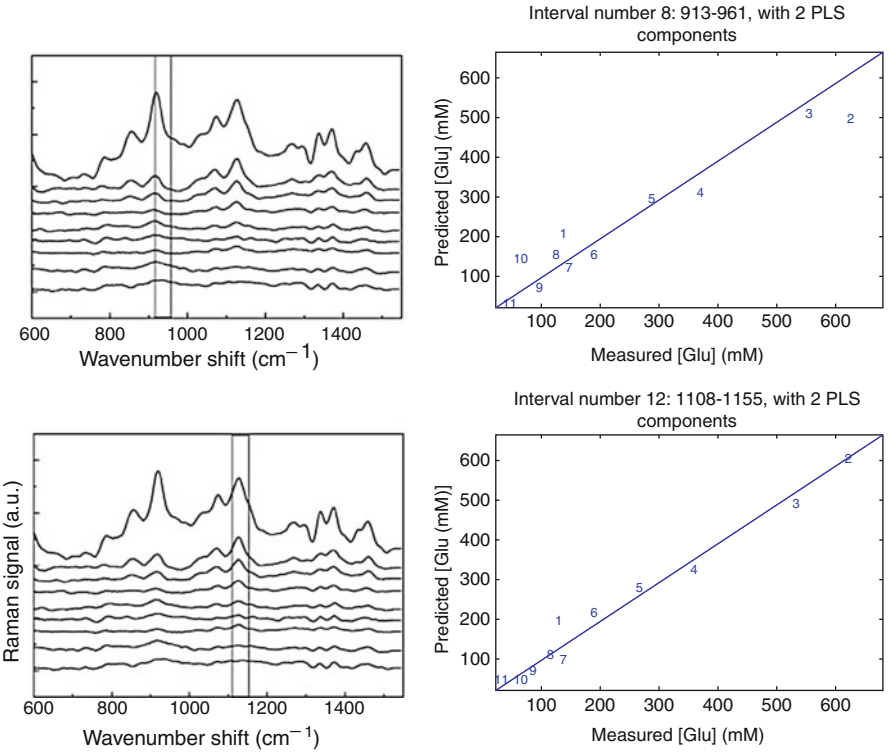

Fig. 11 Selected spectra intervals used in the iPLS model (*left panels*) for Raman spectra of solutions of different glucose concentrations with the corresponding predictions of concentration obtained using single spectral intervals (*right panels*, from 913–961 cm^{-1} (*top*) to 1,108–1,155 cm^{-1} (*bottom*)). Characteristic Raman marker bands for the α- and β-anomers are found at 919 and 1,128 cm^{-1}, respectively. *Numbers* represent the identities of individual measured spectra (Figure is taken from Delfino et al. 2011)

enveloped virus from the *Paramyxoviridae* family and zoonosis that is a disease that crosses species barriers and that causes severe illness in humans (encephalitis and respiratory disease) with a 40–75 % mortality rate and no approved therapy. Its relative aerosol stability and propensity for cross-species infection make NiV a potential bioterrorism agent and a priority pathogen for the National Institutes of Health and other healthcare authorities. In NiV, the F- and G-glycoproteins are required for membrane fusion during cell entry and pathognomonic cell–cell fusion. Figure 13 shows Raman spectra collected by Lu et al. for various NiV VLPs and pseudovirions expressing the F- and G-glycoproteins, as well as the matrix (M) protein.

A commonly used method for differentiating between similar Raman spectra such as those shown in Fig. 14 is to take the second derivative, with these derivatives being shown for NiV-M, NiV-G, and NiV-F in Fig. 14, panels (a) and (b). Second-derivative Raman spectra are very sensitive to differences in identity and

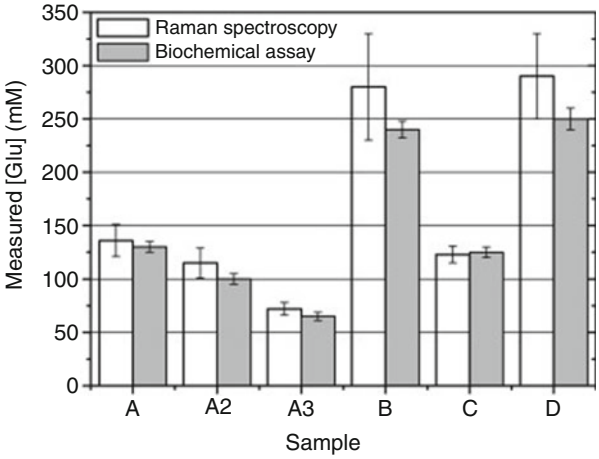

Fig. 12 Measured glucose concentrations for the investigated commercial beverage samples as obtained by iPLS analysis of Raman spectra, benchmarked against the enzymatic (biochemical) assays (Figure is taken from Delfino et al. 2011)

composition, and Lu et al. were able to utilize these for constructing a PCA model, shown in panel (c) of Fig. 14, which could clearly differentiate between the three VLPs based on the glycoproteins expressed. The rapid capability of Raman spectra to characterize viruses and identify structural changes makes it a viable real-time diagnostic technique for virology and vaccine development.

Raman spectra are sensitive to the specific vibrational modes that characterize changes in the structure of biomolecules, so making it an increasingly important biophysical technique. An example of this is presented here from the Lu et al. paper in Fig. 15, where they used the second derivatives of the measured Raman spectra in order to increase spectral sensitivity and remove the effects of baseline variations. Panels (a–c) display characteristic spectral changes induced by receptor binding to the F- and G-glycoproteins attached to the NiV pseudovirions. The authors suggested that they were probably monitoring the formation of a prehairpin intermediate. Note that no conformational change occurs in panel (d), and the two traces overlap perfectly. This work illustrates both the applicability of Raman spectroscopies to studying large biomolecular complexes, such as virions, and the sensitivity of specific marker bands to conformational changes, in this case in glycoproteins.

8 Studies on Carbohydrates Within Living Cells

Raman spectra can also be readily obtained from living single cells, such as bacteria and yeast, using microscope systems. Standard methods used for monitoring the uptake and metabolic flux of nutrients, such as sugars, typically involve isotopic labeling combined with biophysical methods such as chromatography or mass spectrometry. These approaches are complex and time-consuming and require bulk cell populations and experiments performed on cell extracts, so potentially losing information about the effects of cellular variation and dynamics.

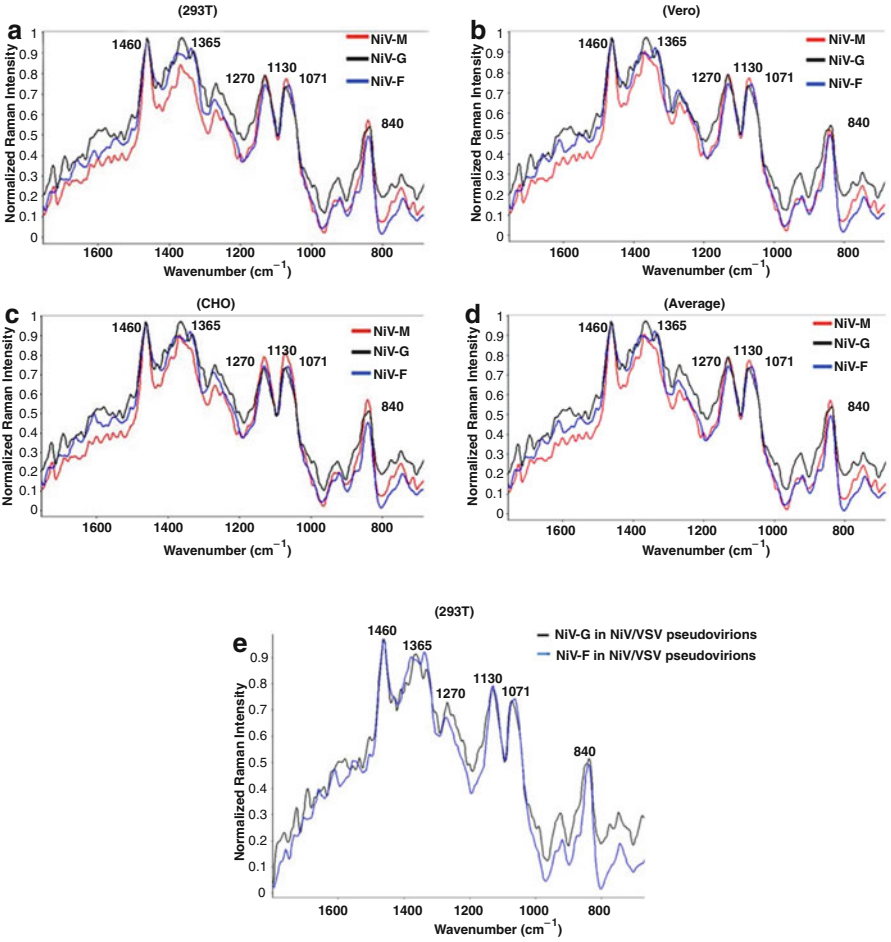

Fig. 13 Raman spectral features of M-, F-, and G-proteins on NiV VLPs cultivated in three different cell lines (panels **a**–**c**), the average of VLP signals produced from three distinct cell cultures (panel **d**), and NiV-G and NiV-F spectra from pseudovirions (panel **e**) (Figure is taken from Lu et al. 2013)

While fluorescent labels and stains can reveal useful information about sugar uptake and metabolism, this approach introduces potential problems due to the size and chemistry of exogenous fluorophores affecting the structure and behavior of the investigated biomolecules, issues of inhomogeneous expression and distribution, as well as photobleaching reducing signal intensity and reproducibility. The ability of Raman microscopy to measure detailed chemical and structural information from intrinsic vibrational motions of carbohydrates, without the requirement of a fluorescent label being added, makes it a more widely applicable and label-free probe of carbohydrate biochemistry in living cells. A recent demonstration of this can be found from the work of Avetisyan et al. (2013). This group used laser

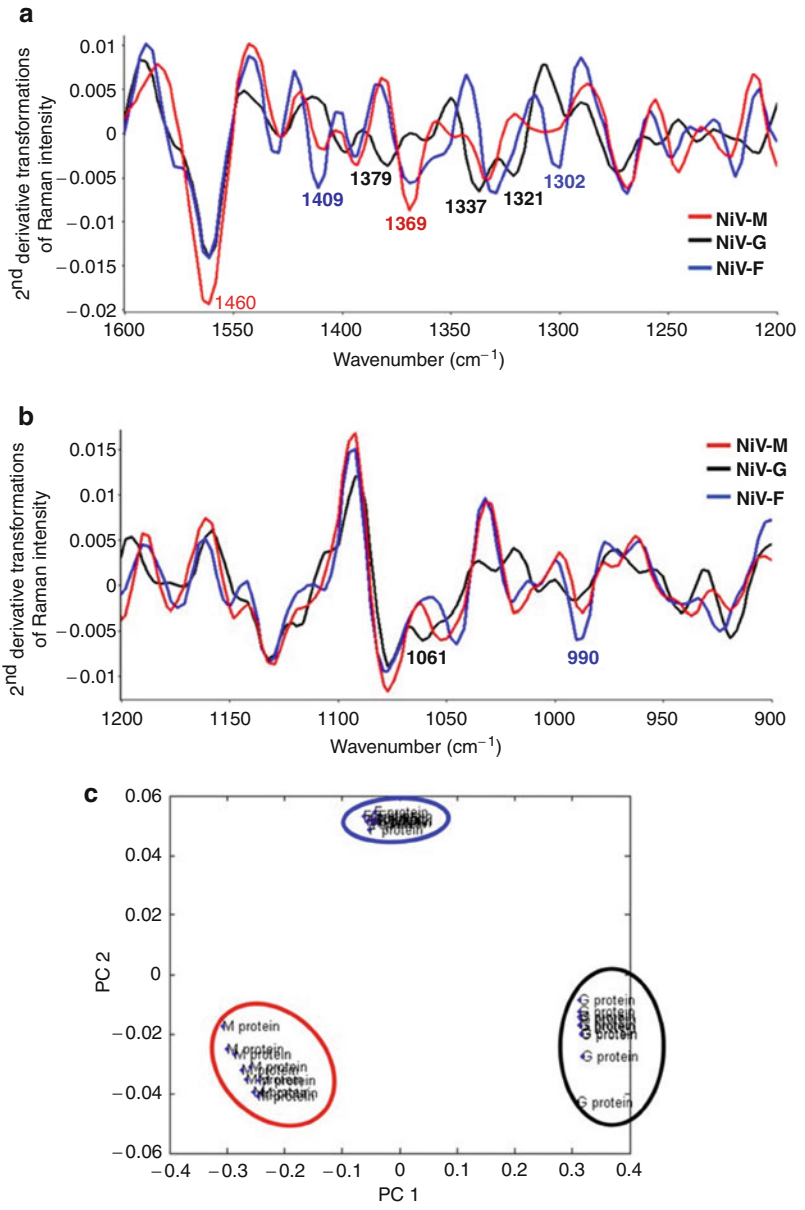

Fig. 14 Second-derivative analyses of Raman spectral features showing specific M, F, and G spectral peaks. Panel (**a**) shows the second-derivative transformation of Raman spectra (1,500–1,200 cm^{-1}) of the M-, F-, and G-proteins on NiV VLPs (from the *Vero cell line*). Panel (**b**) shows the same as for panel (**a**) but for the range of 1,200–900 cm^{-1}. Panel (**c**) shows a representative PCA classification model established and cross-validated to differentiate NiV proteins (*n*_4), with data points being circled as *blue*, F-protein; *black*, G-protein; *red*, M-protein (Figure is taken from Lu et al. 2013)

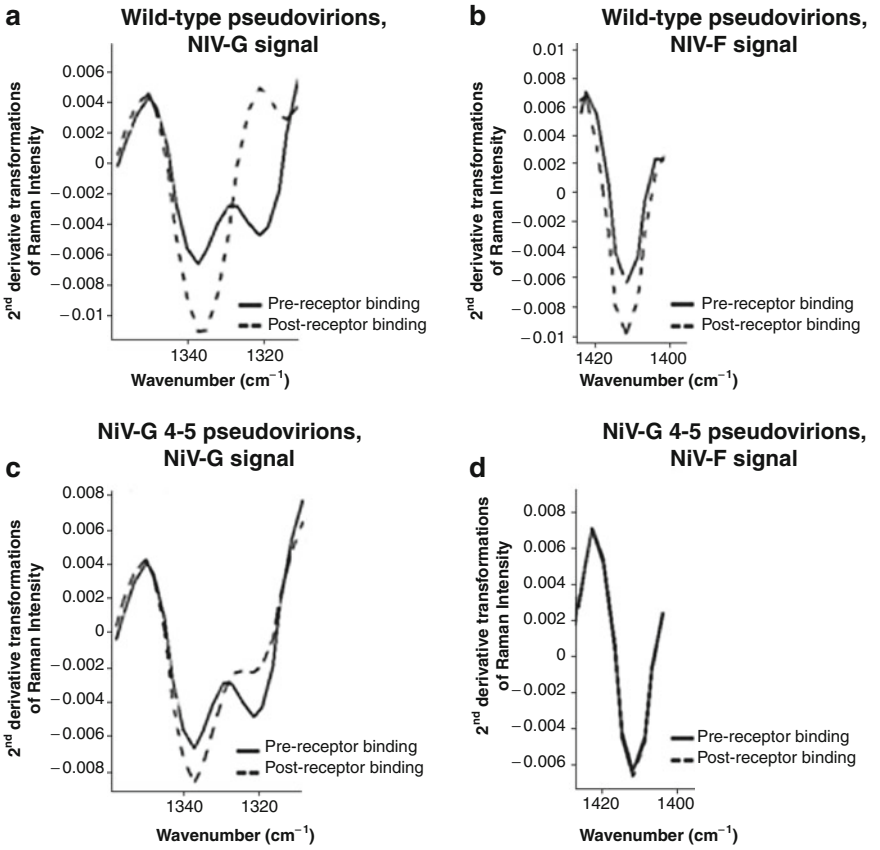

Fig. 15 Examples of Raman spectral changes (from the second derivative) corresponding to receptor-induced conformational changes in the NiV-F and NiV-G-glycoproteins. In panels (**a**–**c**) conformational changes are monitored but not in panel (**d**) (For specific details of these experiments please refer to Lu et al. 2013)

tweezers to isolate and localize single *Sinorhizobium meliloti* cells, a soil bacterium, and then collected Raman spectra at 785 nm to investigate the uptake of trehalose. This approach is also referred to as laser tweezers Raman spectroscopy (LTRS).

Figure 16 shows the sensitivity of Raman spectra to a small chemical difference in the disaccharide trehalose, in this case substitution of a single hydroxyl with a carbonyl moiety. Through following such changes in characteristic marker bands, Avetisyan et al. could monitor uptake and metabolism of trehalose by individual *S. meliloti* cells, as is shown in Fig. 17. Although care must be taken to minimize the risk of photodegradation, the use of longer-wavelength lasers (in this case 785 nm) and short spectral acquisition times (as low as 10 s in this study) can achieve this. The authors were able to quantitatively follow the metabolism of trehalose in situ, determining that intracellular concentrations of the disaccharide were an average of

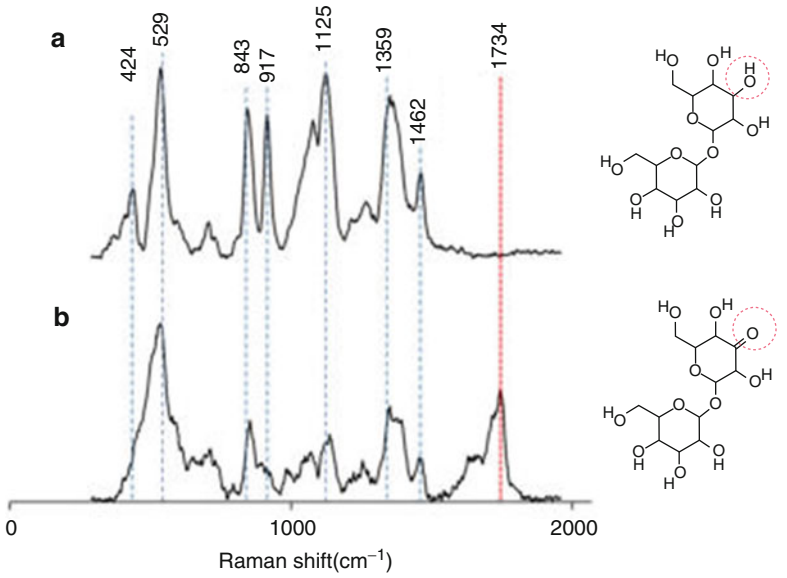

Fig. 16 Chemical structures (*right*) and Raman spectra (*left*) of a 5 % (w/v) aqueous solution of trehalose (*A*) and 3-ketotrehalose (*B*). Significant spectral differences are observed between the two closely related sugars, such as the carbonyl stretching vibration at 1,734 cm^{-1} distinguishing 3-ketotrehalose from trehalose. Key Raman peaks are labeled (Figure is taken from Avetisyan et al. 2013)

22 mM but varied from 18 to 65 mM. As the authors point out, their approach could be readily adapted for studies on carbohydrate metabolism, utilization, or production in a wide range of microorganisms.

Raman imaging is now a widely used technique in biomedical and materials research as it allows the direct visualization of the chemical complexity of surfaces and living material. To date, there have been few developments in the imaging of carbohydrates, but one relevant paper is that of Åkeson et al. who used coherent anti-Stokes Raman (CARS) spectroscopy to image both glucose fluxes in lipid bilayer vesicles and the metabolic responses to a glucose pulse by living yeast cells (Åkeson et al. 2010). Examples of their results on yeast cells are shown in Fig. 18, with a specific Raman vibration at 2,870 cm^{-1} being used to generate the images. For readers new to Raman spectroscopic imaging, please refer to more extensive reviews of this topic, e.g., Dochow et al. (2011), Chan (2013), Diem et al. (2013), Galler et al. (2014), Xu et al. (2014), but the key conceptual difference to fluorescence imaging is that in the various Raman imaging techniques images of a picture or map can be generated using any of the spectral marker bands measured. In this way, the chemistry of the sample, or changes in its chemical profile, is directly observed and spatially resolved, providing a powerful means to investigate the biochemistry of living cells and tissues in situ and in real time. In this case, Åkeson et al. were able to probe metabolic changes throughout all regions of a living cell induced by the glucose pulse.

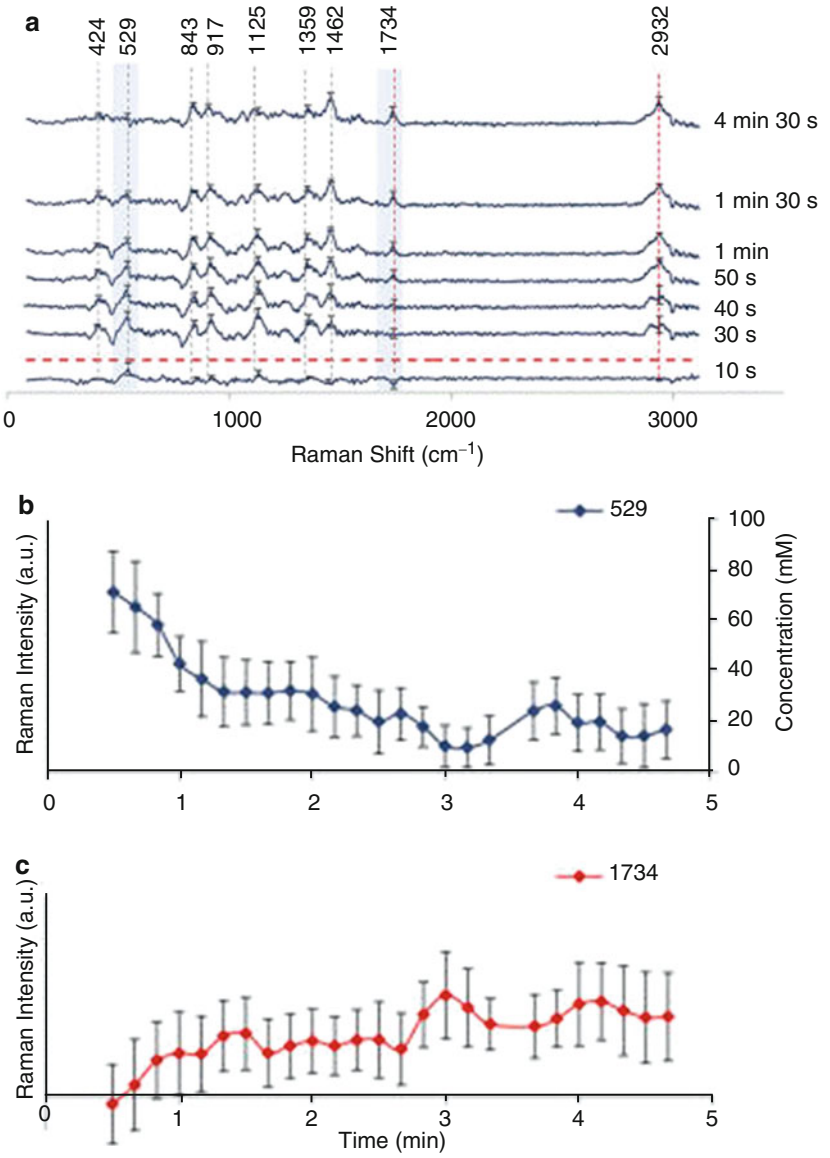

Fig. 17 (**a**) Time dependence of trehalose uptake and metabolism in single *S. meliloti* cells. Spectral average with standard error represents three different cells. Note that the acquisition time for each spectrum was 10 s (excitation power of 30 mW at 785 nm). Data are presented as continuous acquisitions of a single cell for 4 min and 30 s where the initial cell spectrum, i.e., at $t = 0$ s, was subtracted. *Dashed gray* and *red lines* show the peaks present and absent in the trehalose standard, respectively. Decrease of the peak at 529 cm^{-1} and appearance of the band at 1,734 cm^{-1} are due to conversion of trehalose to 3-ketotrehalose. The intensity changes of peaks occurring from 30 s to 4 min and 30 s with standard errors ($n = 3$) are presented in (**b**), for peak at 529 cm^{-1}, and (**c**) for peak at 1,734 cm^{-1}) (Figure is taken from Avetisyan et al. 2013)

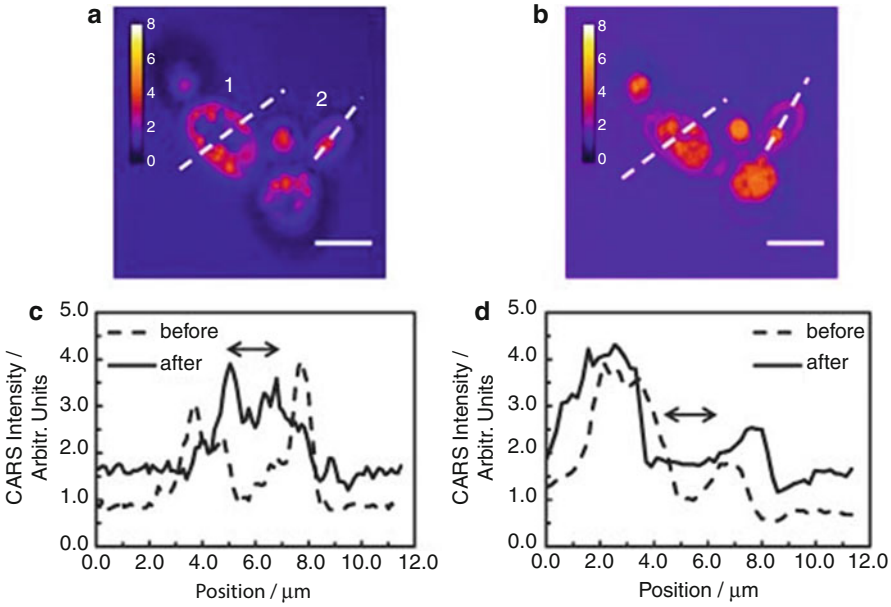

Fig. 18 CARS images (from the band at 2,870 cm^{-1}) of yeast cells before (**a**) and after (**b**) glucose addition. For the cells labeled *1* and *2*, the accompanying intensity profiles corresponding to the *dashed white lines* are shown in (**c**) and (**d**), respectively. After addition of glucose, increased CARS signals are observed both in the surrounding medium and within the cells. In addition, reduced cell size and a redistribution of organelles can be observed. The *horizontal arrows* above the graphs highlight the cytosol regions in the cells, characterized by higher CARS signal after addition of glucose compared to before. Image size is 25 × 25 µm (scale bar 5 µm), integration time 20 s (Figure is taken from Åkeson et al. 2010)

9 Computational Raman and ROA Spectroscopies

As shown above, experimental Raman and ROA spectroscopies can offer a great insight into the structure of carbohydrates. While a large amount of important data can already be elucidated in these cases, an even greater understanding can be exposed from using quantum chemical simulations.

This area of spectroscopy is one where there is a useful complement between the experimentally obtained data and computationally simulated spectra. It is possible to determine structural features, assign absolute configurations, or obtain otherwise unavailable vibrational data and structural dynamics. While early ab initio calculations of Raman intensities date back to the 1970s (Janosche 1973), the first reported ROA calculations came as late as 1989 (Bose et al. 1989), and following several important advancements in theory (Liegeois et al. 2007; Ruud and Thorvaldsen 2009) and computing, it is now possible to routinely simulate spectra of small- to medium-sized molecules.

ROA spectroscopy is a very powerful chiroptical technique and due to its treatment of chiral molecules makes it particularly useful for analyzing carbohydrates. Although while the combination of calculated and experimental data makes this method ideally suited to study these molecules, especially in light of shortcomings of other structural biology techniques, there are very few publications in this area. This is in part due to the difficulty of carrying out accurate calculations. Carbohydrates exhibit strong solvent effects requiring accurate treatment of water as well as exhibiting high conformational flexibility, meaning multiple calculations are needed to simulate the conformational dynamics. With these complications, the calculations can be computationally expensive, with full quantum simulations usually difficult on molecules larger than a few monosaccharides.

The computational treatment of carbohydrates using ROA spectroscopy is still very much an up-and-coming field, and as such much of the work referenced within this section focuses on understanding the structure of monosaccharides, and no work has been reported on larger molecules. As such the higher-order structure of carbohydrates has yet to be explored in this way. However, with refinements to the techniques, it is possible to get very accurate representations of experimental spectra with future aims to increase the size and scope of the research being carried out in this area.

9.1 Calculation of Raman and ROA Spectra

When carrying out the calculation of Raman and ROA intensities, it is important to use a suitable method and basis set. The early reported calculations utilized Hartree–Fock or multi-configurational self-consistent field methods with relatively small basis sets, and due to computational constraints at the time, only very small molecules could be studied (Polavarapu 1990; Barron et al. 1991a, 1992; Helgaker et al. 1994). Advancements in the theory as well as computation mean that DFT methods are now considered the standard in this area, and as such several benchmark studies have been carried out to find the most suitable functional and basis sets.

Early benchmark studies examined the importance of the basis set used for calculation of ROA. Pecul and Rizzo found that diffuse functions are an essential part of the basis set (Pecul and Rizzo 2003), and a more rigorous investigation by Zuber and Hug yielded a highly rarefied basis set called rDPS, constructed from 3-21++G with semi-diffuse p functions on all hydrogens that offered excellent results for ROA intensities when compared to much larger basis sets (Zuber and Hug 2004). In the basis set studies, Reiher et al. (2005) also examined the DFT method needed, finding that generalized gradient approximation and hybrid methods perform to a similar level, and both outperform local density approximation methods. In general several DFT functionals show potential but one of the most suitable choices is that of B3LYP, which exhibits a reasonable compromise between accuracy and cost (Ruud et al. 2002).

The most recent benchmark study published by Cheeseman and Frisch examined not only the basis set requirements for calculation of ROA and Raman intensities but also the requirements for optimization and calculation of the force field (Cheeseman and Frisch 2011). There are two different algorithms that can be used for simulation of the spectra: the one-step algorithm, where the force field and ROA invariants are calculated at the same level of theory, and the two-step algorithm, where the force field and ROA invariants are calculated at different level of theories. The authors offer suggested schemes for calculations of differing size systems; for small systems, the one-step procedure can be used, and the basis sets aug-cc-pVDZ and aug(sp)-cc-pVDZ are ideal. The two-step procedure however is more efficient and as such offers the ability to carry out calculations on larger systems. The recommend basis sets for medium to large systems are a combination of cc-pVTZ or 6-31G* with aug(sp)-cc-pVDZ or rDPS for the calculation of the force field and ROA tensors, respectively.

As mentioned above, the calculation of ROA spectra can be very time-consuming and computationally expensive, and as such calculations are limited to small- to medium-sized systems, meaning it can be difficult to get information about higher orders of structure of carbohydrates. To be able to expand calculations to larger systems, further approximations need to be made, and several have been suggested (Luber and Reiher 2009a; Ghysels et al. 2007) with one of the most popular being the Cartesian coordinate tensor transfer (CCT), proposed by Bour et al. (1997). This approach aims to calculate property tensors for large molecules by using values of overlapping smaller fragments. While some errors have been reported for this approach (Yamamoto and Bour 2011), it offers the scope of getting a greater structural understanding of larger carbohydrate molecules.

Due to the strong solvent effects that carbohydrates undergo, it is important to include an aqueous environment within calculations. There are three approaches that can be used to fulfill this: implicit solvent models, a small number of explicit solvent molecules treated at the quantum level, or a large number of explicit solvent molecules in a hybrid quantum mechanical/molecular mechanical (QM/MM) treated with a force field method. Implicit solvent models are the cheapest computationally but are also the least accurate by only modeling solvent as a dielectric continuum and foregoing any interactions with the solute. A small number of explicit solvent molecules are a more accurate approach and can be used alongside implicit models, but this approach fails to show information about solvation layers as a whole. The hybrid QM/MM approach is likely the most suitable when studying carbohydrates as it allows water molecules numbering in the hundreds to be included in simulations, and therefore multiple solvation shells can be included.

9.2 Structural Investigation of Carbohydrates Using ROA Spectroscopy

The first reported computational ROA study of carbohydrates was carried out by Macleod et al. (2006) who studied hydrated and isolated glucose, galactose, and

lactose in the gas phase and in aqueous solution. In experimental measurements of Raman and ROA spectra in aqueous solution at 298 K, infrared ion-dip spectroscopy was conducted at low temperatures. Gas phase calculations of the low-lying conformational structures, at the B3LYP/6-31+G* level of theory, were used to give sets of computed spectra, which in turn were used to create weighted sums in an attempt to approximate the experimental spectra recorded in solution. These calculations were compared with estimates based upon NMR measurements as well as molecular mechanics and molecular dynamics simulations (MD), giving results deemed surprisingly successful. The singly hydrated complexes of the two monosaccharides studied showed altered conformational preferences in the gas phase experiments, which were sustained in aqueous solution. These results supported the view that explicit hydration has a strong influence on aqueous phase conformations.

Luber and Reiher calculated the Raman and ROA spectra of the carbohydrate molecule 1,6-anhydro-β-D-glucopyranose (Luber and Reiher 2009b), to offer a deeper insight into an earlier published experimental ROA spectrum (Barron et al. 1991b). Boat and chair conformations of the monosaccharide were studied as well as rotamers arising from the three hydroxyl groups, giving rise to 54 conformers. These calculations exhibited the great sensitivity of ROA by showing large variations of peak intensity between rotamers. The need for solvent in calculations was treated in two ways, the implicit solvation model COSMO was used initially, and then the molecule was explicitly hydrated using several water molecules. The implicit model gave rise to minor alterations in ROA bands, but the inclusion of explicit solvent resulted in a markedly different spectrum, showing the large effect hydrogen bonding can have on the results. Final spectra were constructed by weighting individual conformers using three approaches: electronic energies, Gibbs free energies, and no weighting. The agreement with experiment was good for all types of weighting; however, it was noted that on the route to better experimental agreement, combined MD and QM/MM approaches would offer better prospects at modeling solvent effects and conformational dynamics.

Kaminsky et al. (2009) used Raman and ROA calculations coupled with MD simulations as well as the CCT transfer technique to interpret the experimental spectra of a sugar derivative, the gluconic acid anion. This linear carbohydrate exhibits large conformational flexibility due to ten dihedral angles that if carrying out mapping of the conformational space at 120° intervals would result in close to 60,000 conformers, an unfeasible number to be able to calculate a spectrum for each. To circumvent this, the authors carried out MD simulations to get a 1,000 structures and took a small sample of geometries from these simulations and calculated force fields, polarizability, and optical activity tensors at the B3LYP/6-31 + G**/CPCM level of theory. The CCT transfer technique was used with the DFT-calculated results to generate a spectrum from the MD library that reproduced most of the experimental features. It is also noted that despite a high level of spectral averaging, it is still possible to identify characteristic vibrational normal modes such as C–H and O–H bending.

One of the more recent publications in this field by Cheeseman et al. (2011) shows the future potential of the Raman and ROA calculations of carbohydrates,

by incorporating MD simulations with explicit solvation alongside QM/MM calculations. Studying the monosaccharide methyl-β-D-glucose, a selection of MD snapshots was used as the starting point for ONIOM calculations, where optimizations and force field calculations were carried out at the B3LYP/6-31G* level of theory for the monosaccharide and AMBER for the water. Calculation of Raman and ROA tensors was carried out at the HSEH1PBE/rDPS level of theory for the monosaccharide and AMBER for the water, using the two-step procedure. By incorporating the conformational dynamics and explicit solvation from the MD simulations with the quantum calculation of the ROA spectrum, excellent agreement with experiment is achieved, especially when compared to gas phase or implicit solvent calculations of the same molecule. The authors also note that this combined approach solves problems associated with the inability to model the sensitivity of ROA spectra, and it surpasses all previous approaches to include hydration in these calculations.

9.3 Structural Investigation of Carbohydrates Using Raman Spectroscopy

Due to the chiroptical nature of ROA, it makes it particularly useful in the study of carbohydrates particularly when combined with calculations, much more so then the non-chirally sensitive analogous technique Raman spectroscopy. As such there is also a lack or reported research using only Raman spectroscopy, with the only publications occurring only in the last few years.

Brizuela et al. (2012) reported one of the first examples of using computational modeling of Raman spectra to study carbohydrates in 2012, by offering a complete characterization of the Raman and FTIR spectra of the disaccharide sucrose in the solid state. They had a particular interest in structural properties including bond order, charge transfer, and topological properties of the monosaccharide rings studied using DFT calculations, natural bond orbital (NBO) analysis as well as atoms in molecules (AIM) analysis. Calculations at the B3LYP/6-31G* and B3LYP/6-311++G** levels of theory gave results that were in good agreement with experimental data and resulted in a complete assignment of the normal vibrational modes of this molecule.

A similar study to the one presented above was also carried out by Brizuela et al. (2014) on sucrose but in an aqueous medium instead of the solid phase. Self-consistent reaction field calculations were used to model the presence of solvent, and full assignment of the normal vibrational modes was achieved as well as confirming the presence of pentahydrate and dihydrate sucrose species in aqueous solution.

Quesada-Moreno et al. (2013) studied the conformational preference of the carbohydrates D-ribose and 2-deoxy-D-ribose in the aqueous and solid phase. Experimentally IR and Raman measurements were taken as well as another chiroptical technique, vibrational circular dichroism (VCD). Calculations at several levels of theory were used in this study, including MP2, B3LYP, and M06-2X, with the PCM implicit solvation model and in some calculations

a single explicit water molecule. The combination of the two approaches allowed the dominant configurations of the carbohydrates in question to be elucidated as well as discovered that for 2-deoxy-D-ribose, there are different conformer populations between solution and the solid phase.

9.4 Guidelines for Raman and ROA Calculations of Carbohydrates

In calculation of Raman and ROA spectra, several things need to be taken into account. Choice of method and basis set is important, as already shown above, but it is also important to account for the presence of all important conformers, especially when carrying out calculations on carbohydrates that exhibit high conformational flexibility.

Firstly, the structure of the molecule of choice must be constructed, and then a search of this molecule's conformational space must be undertaken. This can be carried out in several ways, such as manually exploring the angular degrees of freedom of the molecule. Although this approach is more suitable for small rigid molecules than carbohydrates, as such a better approach would be to use MD simulations, which also offers the advantage of being able to include explicit solvent molecules. The MD trajectories can then be used to obtain snapshots as starting points for Raman and ROA calculations as well as giving conformational population analysis.

Selected snapshots are used as starting points for optimization and these optimized structures are used for calculation of the force field and Raman and ROA intensities. Spectra can be plotted from these intensities using Lorentzian or Gaussian curves with ranging half-peak widths, although for most applications 10 cm^{-1} is recommended. The spectra of the individual snapshots can then be used to create a final spectrum be carrying out weighted averages, either based on Boltzmann distributions or using the population analysis from MD simulations. These computationally obtained spectra can then be compared to experimentally obtained data (Fig. 19).

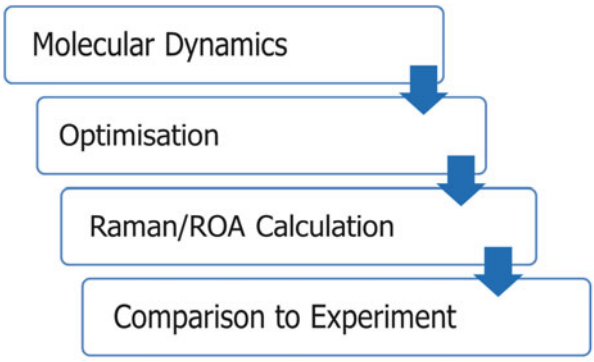

Fig. 19 Scheme showing the approach for the calculation of Raman and ROA spectra of carbohydrates

10 Conclusion

Carbohydrates present, arguably, the greatest challenge for structural biology today, not least because of their flexible natures and their heterogeneous compositions (in the case of glycoproteins) or limited complexity sequences (a significant issue for NMR studies on glycosaminoglycans). Raman spectroscopies now comprise a highly adaptable toolkit of analytical techniques that are able to provide detailed information about carbohydrates, their structures and behavior. The ability to collect such information noninvasively from environments as disparate as living cells, bioreactors, and foodstuffs can allow Raman spectroscopies to make a significant impact in glycobiology, not only in research but also in the industrial and biomedical sectors. Although this review has presented examples from a number of Raman techniques (Raman microscopy, SERS, ROA, CARS), this is not an exhaustive list, and other Raman methods are likely to prove valuable in the future. For example, the capability of tip-enhanced Raman (TERS) (Bailo and Deckert 2008) to combine the chemical sensitivity of Raman with the site specificity of atomic force microscopy has already created great interest for studies on proteins and nucleic acids (Kurouski et al. 2012; Rasmussen and Deckert 2006). The potential of TERS to provide detailed site- or region-specific information about the glycosylation patterns of individual glycoproteins would open new opportunities for researchers of these important but poorly understood systems. As we have discussed, Raman spectroscopy can be readily combined with other technologies, and hyphenated variations in the form of a single instrument package, such as Raman-mass spectrometry, are likely to prove useful in this field in the future. In parallel with technological developments, it is also likely that chemometrics will become a routine data processing step due to the already demonstrated ability of both clustering and regression algorithms to extract information about carbohydrates of any form. The many modeling tools being developed for systems biology are well suited to mining the rich detail of carbohydrate Raman spectra. Finally, the exquisite sensitivity of quantum and molecular mechanics to the vibrational modes of molecules can provide the atomic resolution level of understanding that we currently lack for most carbohydrates.

We hope that this review provides our glycobiology colleagues with an insight into how the family of Raman spectroscopies can help them understand this fascinating class of biomolecules. We encourage them to contact their local Raman experts to explore the many opportunities available for doing some new and exciting science. And to our fellow Raman spectroscopists, the future is indeed looking sweet.

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Production, Upgrading and Analysis of Bio-oils Derived from Lignocellulosic Biomass

41

Pankaj K. Kanaujia

Contents

Abstract

In view of the increase in global energy requirements, pyrolysis of lignocellulosic biomass gains significant impetus as a promising source of renewable energy and valuable chemicals. This pyrolysis oil is a complex mixture of simple organic, inorganic, and macromolecular compounds formed as a result of thermochemical breakdown of lignocellulosic biomass. It has high oxygen content and consequently a very low calorific value which renders it useless for fuel applications. As a result, appropriate upgrading is essential to make it a viable alternative to petroleum fuels. Analytical chemistry plays a key role in revealing compositional important information and helps in developing molecular-level understanding. Bio-oil production and upgrading research aimed at building up of commercial production and refining units concerns monitoring of quality and stability of initial and final products which rely strongly on analytical approaches. In the ensuing discussion, fundamental and practical aspects of

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research in this area have been presented. Chemical analysis with different state-of-the-art analytical techniques has been discussed in addition to the sample preparation methods.

Keywords

Lignocellulosic biomass • Bio-oil • Pyrolysis • Gas chromatography • Thermogravimetry • Infrared spectroscopy • Mass spectrometry • Upgrading • Sample preparation

Abbreviations

AAS	Atomic absorption spectroscopy
AES	Atomic emission spectroscopy
APCI	Atmospheric pressure chemical ionization
ASTM	American Society for Testing and Materials
CI	Chemical ionization
DART	Direct analysis in real time
DEPT	Distortionless Enhancement by Polarization Transfer
DESI	Desorption electrospray ionization
DTG	Differential thermogravimetry
EI	Electron ionization
ESI	Electrospray ionization
FAB	Fast atom bombardment
FI	Field ionization
FID	Flame ionization detector
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC × GC	Two dimensional gas chromatography
GC–MS	Gas chromatography–mass spectrometry
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
HRMS	High-resolution mass spectrometry
HS-SPME	Headspace solid-phase microextraction
ICP	Inductively coupled plasma
LC–MS	Liquid chromatography–mass spectrometry
LDI	Laser desorption ionization
LLE	Liquid–liquid extraction
MALDI	Matrix-assisted laser desorption ionization
NMR	Nuclear magnetic resonance
PAH	Polycyclic aromatic hydrocarbon
Py-FI–MS	Py-FI–MS
Py–GC–MS	Pyrolysis–gas chromatography–mass spectrometry
SDME	Single-drop microextraction
SEM	Scanning electron microscopy

SFE	Supercritical fluid extraction
SLE	Solid-supported liquid–liquid extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TCD	Thermal conductivity detector
TGA	Thermogravimetric analysis
TOF	Time of flight
UV	Ultraviolet spectroscopy
XRD	X-ray diffraction

1 Introduction

Energy requirements across the globe are incessantly increasing due to fast growths in population and economies especially in China, India, Latin America, the Middle East, and Russia. Extensive use of fossilized organic substances such as coal, oils, and gases for energy generation and production of chemicals has resulted in massive carbon dioxide generation which is responsible for global climate change. Fast-expanding transportation sector demands huge energy which is currently met through fossil fuels. Power generation heavily relies on either coal or natural gases in large parts of the developed as well as developing world. Diminishing geological reserves and grave environmental concerns have necessitated the exploration of new and renewable sources of energy (Sorrell et al. 2010). One such approach is the conversion of biomass to biofuels and chemicals through thermal degradation in an integrated refining process (Ragauskas et al. 2006).

As shown in Fig. 1, biomass is the most abundant and renewable source from which fuels and chemicals may be obtained in reasonable yields, shorter span, and ecological and environmentally friendly manner. First-generation biofuels essentially comprise ethanol which is produced from edible biomass such as food grade agricultural produce and crops. Carbohydrates (sucrose, starch, cellulose, etc.) undergo microbial fermentation to give bioethanol, and this technology is quite ancient. Nowadays bioethanol is a very popular biofuel worldwide especially in Brazil and the USA where its blending of up to 25 % in gasoline is prevalent. Bioethanol production and consumption statistics in terms of cost, feedstocks, plant operational variables, and manufacturing processes has been reviewed (Demirbas 2011). Ethanol can be used in gasoline-driven engines in the form of blended fuel in any proportion. Although ethanol has lower calorific value than hydrocarbons, its high octane rating is used to increase the octane rating of gasoline which is an important parameter for petrol-powered engines. The limitation associated with ethanol use as fuel or a replacement of gasoline is its production from food grade crops and high cost in current scenario where gasoline is available. In addition, its low energy content requires larger fuel tanks as compared to conventional fuels, and water solubility leads to corrosion of engine parts. Butanol has promising potential for being utilized in fuel applications due to higher energy density and lesser water content. Biosynthesis of butanol was carried out through ABE

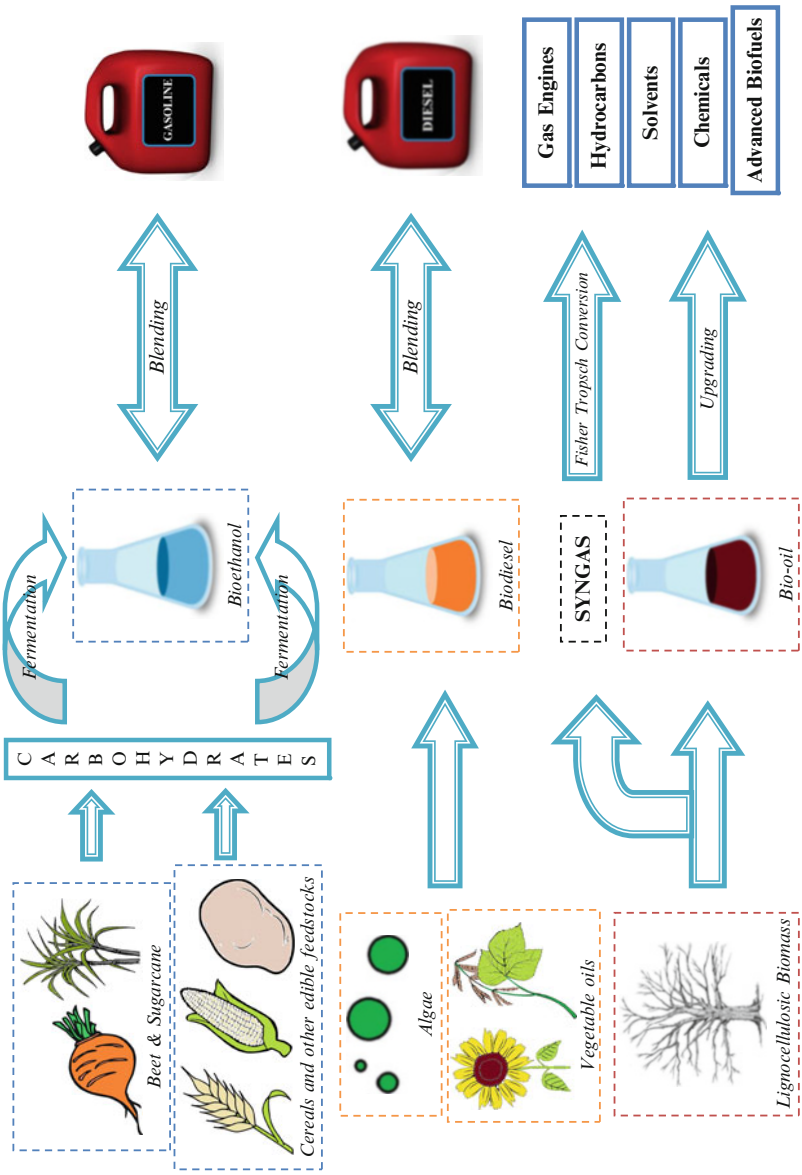

Fig. 1 Schematic presentation for the utilization of biomass to biofuels and other value-added products through pyrolysis

(acetone–butanol–ethanol) with the help of bacterium from *Clostridium* species; however, the process was abandoned commercially after the 1960s. Recently, microbial fermentation of substrates of sustainable origin to produce higher alcohols, short-chain alkanes, free fatty acids, fatty esters, and fatty alcohols as gasoline substitutes has been explored by several scientists (Atsumi et al. 2008; Choi and Lee 2013). First-generation biofuels also comprise biodiesels and green diesels which have gained popularity due to their close similarity with conventional diesel. Biodiesel is readily obtained from oils or fats of plant or animal origin through transesterification. It is primarily composed of methyl esters (or some ethyl), fatty acids (if derived from vegetable oils), and triglycerides of fatty acids (if derived from animal fats such as tallow or lard). Biodiesel is nontoxic, biodegradable, and used as diesel additive to reduce levels of particulates and other toxic emissions (Ma and Hanna 1999). Essential feedstocks for biodiesel production are animal fats, soybean, sunflower, rapeseed, corn, cottonseed, palm, *Jatropha*, *Pongamia*, algae, and many more.

In contrast, second-generation biofuels or advanced biofuels are derived from abundant, renewable, and nonedible biomass comprising wood and agricultural residues broadly called as lignocellulosic biomass. Throughout the human evolution, lignocellulosic biomass has been extensively exploited as a primary renewable energy resource. By the end of the eighteenth century, fossilized energy sources started replacing biomass utilization as a result of increased urbanization, industrialization, and improved lifestyles. Mitigation of environmental pollution, global warming, and other limitations associated with fossil fuel use requires biomass-based economical energy. At the beginning of the twentieth century, a large number of industrial materials such as solvents, dyes, fibers, etc. were obtained from wood biomass/trees and agricultural crops. By around the 1960s and after it, such products were readily obtained from petroleum. Biomass gasification is popular and pretty prevalent as it leads to the formation of *syngas* (also termed *producer gas*) in controlled conditions. It is an important development since the evolved gases may be utilized in several ways such as in gas engines to produce energy and in producing methanol or converted into synthetic fuel via Fischer–Tropsch process. Biomass gasification, however, is associated with the production of tar in variable quantities irrespective of the gasifiers utilized. Pervasive research in this area has improved our understanding and know-how related to chemical and physical properties of biomass and derived products. Engineering solutions were provided from time to time on the basis of outcomes from longitudinal observations which are considered necessary part of process development. Tars have either been recycled or disposed through fractionation or chemical and biological transformations. A simple illustration of the utilization of agricultural and plant biomass for biofuel production and other applications is presented in Fig. 1.

2 Pyrolysis and Lignocellulosic Biomass

Pyrolysis of wood biomass was known to ancient societies where tars and condensates were used for various applications. Ancient Egypt people used pyroligneous acid (wood vinegar) from hardwood as embalming fluids (Austin 1984). In the

olden times, pyroligneous acid was known for its strong sterilizing properties in addition to its widespread use as deodorizer, preservative, and flavoring substance. Since the early 1990s, it has also been used in agriculture as a fertilizer and growth-promoting agent. Wood pyrolysis liquids have always been considered as a wealthy source of essential oils and biopesticides. Detailed chemical characterization of products obtained after thermal decomposition of wood biomass remained unknown for quite some time. Later on fractionation and identification of liquid pyrolysis condensates aimed at developing refining methods were started with the use of available tools of analytical chemistry (Soltes and Elder 1981).

Lignocellulosic biomass is a promising renewable resource for biofuels and numerous value-added chemicals. Pyrolysis is a widely adopted process for the thermal breakdown of biomass feedstocks in the absence of oxygen which leads to the fragmentation of macromolecules and rearrangement of smaller reactive chemicals into stable molecules. The pyrolysis of biomass is either fast or slow and produces gaseous, liquid, and solid products (Goyal et al. 2008). The distribution of formed chemical species among the obtained products usually depends upon utilized feedstocks and pyrolysis parameters such as reactor, residence time, and employed temperatures. Before the middle of twentieth century, the liquid obtained as a result of thermal decomposition (i.e., partial oxidation, steam reforming, or pyrolysis) which is popularly termed *pyrolysis oils* or "*bio-oils*" was considered as an offshoot of these processes, and its uses were restricted to larger extent due to unavailability of efficient procedures of handling and utilization. On the contrary, biomass gasification had larger end use earlier due to the complex chemical composition of bio-oils. Despite several advantages and popularity, biomass gasification is accompanied with tar formation, loss of C–C bond, and eventually energy wastage (Bridgwater 2003). However, as the research progressed in this area, tremendous prospect of bio-oil utilization and upgrading into fuel and chemical production were realized. Major limitations associated with bio-oil utilization, handling, storage, transportation, and refining have been significantly surmounted.

Pyrolysis is a process of thermochemical decomposition of organic matter at high temperatures in the absence of oxygen that leads to changes in chemical and physical properties of the pyrolyzed substance. Pyrolysis of biomass yields bio-oil for which considerable efforts are being continuously put in to develop and improve the upgrading strategies to transform it into prospective engine fuel applications within an integrated bio-oil refinery. Energy content of lignocellulosic biomass feedstocks can be effectively and economically utilized in stationary energy applications such as boilers (or steam generators) in power plants and other utility sectors like commercial or domestic heating purposes. For all other energy requirements, especially fuel related, gaseous as well as liquid pyrolyzed products from lignocellulosic biomass have been explored and put forward. Pyrolysis also produces fuel gases and leads to four- to sevenfold concentration in energy density in bio-oils as compared to raw biomass. On the basis of employed temperatures, heating rates, and residence time, pyrolysis is broadly classified into three types, viz., slow, fast, and flash. The adopted procedure of pyrolysis and employed parameters extensively govern the quality and amount of formed bio-oil.

The demarcation between slow and fast pyrolysis is solely observed on the basis of employed heating rates and vapor residence times. In slow pyrolysis, vapor residence times vary from few minutes to half an hour, whereas in fast pyrolysis, extremely less time (<2 s) is employed. The heating rate in slow pyrolysis usually ranges between 0.1 and 1 °C/min^{-1}, and the pyrolysis temperature varies around 300–700 °C. In contrast, fast pyrolysis typically involves heating rates of 1,000 °C/s or more and pyrolysis temperature up to 1,000 °C. In slow pyrolysis, long vapor residence times inside the reactor provides opportunity to the gaseous products to react, rearrange, and stabilize, thereby producing considerable amounts of char.

During fast pyrolysis, fast heating rates facilitate the formation of small reactive chemical from thermolabile biomass constituents. The formed chemical species instantly condense as a consequence of short vapor residence time which maximizes yields of liquid. In a typical fast pyrolysis process, nearly 60–75 wt% of bio-oil is formed along with solid char and noncondensable gases (in almost equal proportions). The quantity of bio-oil, char, and gases is largely governed by the nature of biomass feedstock. Since the pyrolysis process takes place at fast pace, the reaction kinetics, high heat transfer, thermal stability, and phase separation of formed chemicals determine the overall product distributions. Among several pyrolysis processes, fluidized bed reactors and circulating fluidized bed reactors are efficient and easy to operate. Flash pyrolysis is an improved format of fast pyrolysis which utilizes very high heating rates, generally more than 1,000 °C s^{-1}, and lesser reaction times. In addition to the reactors used in fast pyrolysis, others such as vacuum pyrolysis, rotating cone, entrained flow, ablative, vortex, and twin screw reactors are widely used. However, entrained flow (fluidized bed) reactors are quite widespread.

Pyrolysis leads to significant thermochemical changes within biomass that always depends on how fast and efficiently thermodynamic equilibrium is achieved within a very small time scale. It also governs the chemical reaction kinetics during thermochemical transformations. During pyrolysis, heat is transferred to biomass which leads to the commencement of thermal breakdown of biomass constituents to release volatiles which move rapidly to the cooler region of the reactor and collide with unpyrolyzed biomass and may condense over it to form char. Shorter residence times also allow volatiles to move out and condense. The pyrolysis then proceeds autocatalytically to form other chemicals and intermediates simultaneously along with the formation of volatiles and gases. During the progress of pyrolysis, thermal degradation, rearrangement, dehydration, and so many other chemical processes take place. The biomass pyrolysis process is highly endothermic due to which several ways of transferring heat to biomass are in practice. Combustion of biomass is a process with poor energy generation leading to char formation. Prominent reactor designs which have been employed for biomass fast pyrolysis are based on fixed beds, entrained flow, augers, ablative, rotating cone, fluidized beds, and circulating and bubbling fluidized bed reactors. The pyrolysis of lignocellulosic biomass has been reviewed by several eminent researchers in this area (Bridgwater 1999, 2003, 2004; Bridgwater et al. 1999, 2001; Bridgwater and Peacocke 2000; Peacocke and Bridgwater 1996; Meier and Faix 1999; Vamvuka 2011; Mohan et al. 2006).

These reviews are very much informative and explore fundamental aspects of pyrolysis process, reactor designs, process scale-up, and commercialization. Wide-ranging biomass comprising agricultural and forest scrap have been extensively used for pyrolysis. These include barley straw and hulls, rice husk, wheat straw, corn plant (stalks, leaves, and husks), coconut shells, peanut shells, fruit pulps, woods (waste furniture, eucalyptus, pine, etc.), forage crops, tea waste, bagasse, and many more. In addition to the lignocellulosic feedstocks, sewage sludge, swine manure, and more interestingly waste plastic and printed circuit boards from used computers have also been utilized to produce pyrolysis oil. Algae being a rich source of energy owing to its higher photosynthetic efficiency than trees are a promising feedstock for bio-oils and valuable chemical (Amin 2009).

The lignocellulosic biomass is primarily composed of three major components: cellulose, hemicellulose, and lignin (Fig. 2). Other minor constituents of wood are fats, mucilage, wax, alkaloids, terpenoids, etc. The presence of substantial amount of oxygen in lignocellulosic biomass differentiates it from fossil fuels and significantly lowers its heating value. The proportion of the three major constituents differs from one species of wood to the other. The usual distribution of cellulose,

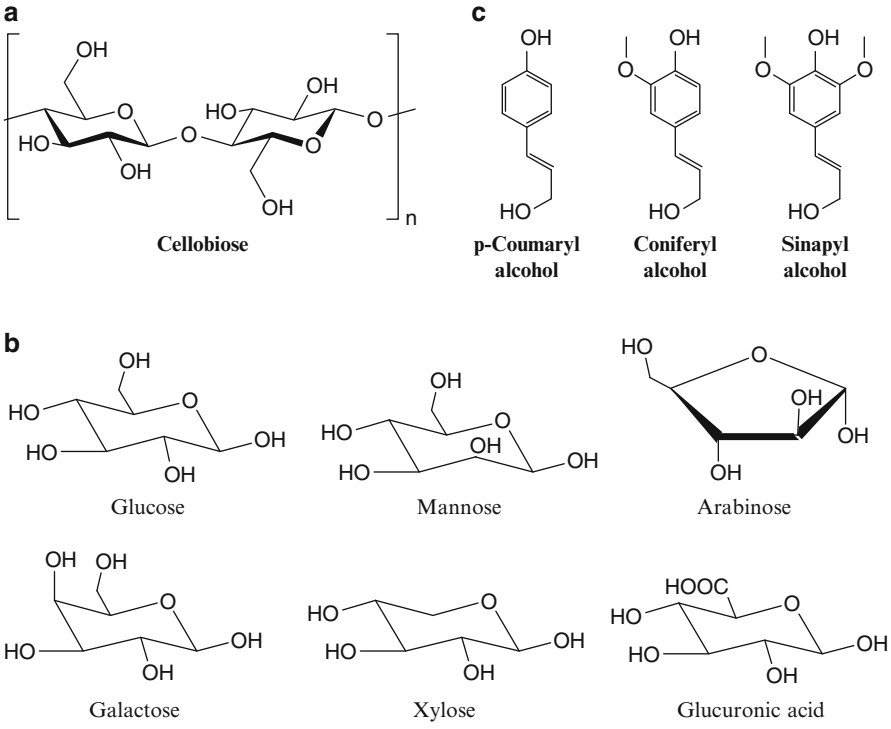

Fig. 2 Chemical structures of (**a**) cellulose, (**b**) hemicellulose monomers, and (**c**) lignin precursors

hemicellulose, and lignin across biomass samples (e.g., softwood, hardwood, straw, husk, stalk, leaves, etc.) roughly ranges between 24–51 %, 16–39 %, and 12–53 %, respectively (Demirbas 1997). Cellulose ($C_6H_{10}O_5$) is a polysaccharide and a prominent constituent of lignocellulosic biomass which is comprised of linear chains of β(1 → 4)-linked D-glucose units as shown in Fig. 2a. Cellulose is the most abundant organic polymer which is biosynthesized naturally and is typically present in cell wall of plants and many other organisms (e.g., algae). In angiosperms, cellulose is the chief component of reaction wood (tension wood). It has several important commercial applications like paper, textile fibers (cotton), filtration and chromatography (thin layer chromatography), conversion to biofuels, etc. Cellulose fibers provide mechanical strength to the wood and comprise considerable dry weight of wood. The fibrous nature of cellulose is due to long chains of glucose units bonded to each other by a long network of hydrogen bonds which exists within the same chain and also across several chains. Cellulose thermal degradation begins at around 240 °C and produces levoglucosan, levoglucosenone, furfural, substituted furans, and several other small molecules comprising aldehydes and ketones.

Hemicellulose is the second major constituent of lignocellulosic biomass and is composed through the combination of several monosaccharides as glucose, mannose, galactose, xylose, arabinose, 4-*O*-methyl glucuronic acid, and galacturonic acid residues. The chemical structures of important hemicellulose constituents (monomers) are shown in Fig. 2b. Unlike cellulose it has a random (branched), amorphous structure with little strength and consists of shorter chains (500–3,000 monosaccharide units) as compared to cellulose (with 7,000–15,000 glucose units). Hemicellulose pyrolysis occurs at temperatures ranging between 200 °C and 260 °C, leading to the formation of volatile organic compounds and smaller amounts of levoglucosan. Deacetylation of hemicellulose leads to the formation of acetic acid which is responsible for acidic nature of derived bio-oils. Lignin on the other hand is the third most abundant constituent of lignocellulosic biomass, and it is mainly concentrated in the compression wood in conifers. It acts as binder for cellulosic components and also provides protection against microbial damage. It is covalently linked to hemicellulose and acts as a strong cross-linker between plant polysaccharides, thereby imparting mechanical strength to the plant. Lignin has a very complex and highly branched chemical structure which contains phenolic, hydroxy-, and methoxy-substituted phenylpropane units. It plays a crucial role in the transportation of water across the plant through stems. During lignin biosynthesis, three common alcohols, viz., paracoumaryl, coniferyl, and sinapyl alcohols (Fig. 2c), undergo oligomerization and polymerization to give lignin whose structure varies depending upon the wood (hardwood and softwood). Lignin decomposes when heated above 280 °C, and its pyrolysis produces phenols and methoxyphenols of which monolignols (guaiacol and syringol) are prominent. Lignin is one of the major sources of phenol-containing species in bio-oils. The pyrolytic products originating from cellulose, hemicellulose, and lignins are schematically shown in Figs. 3 and 4.

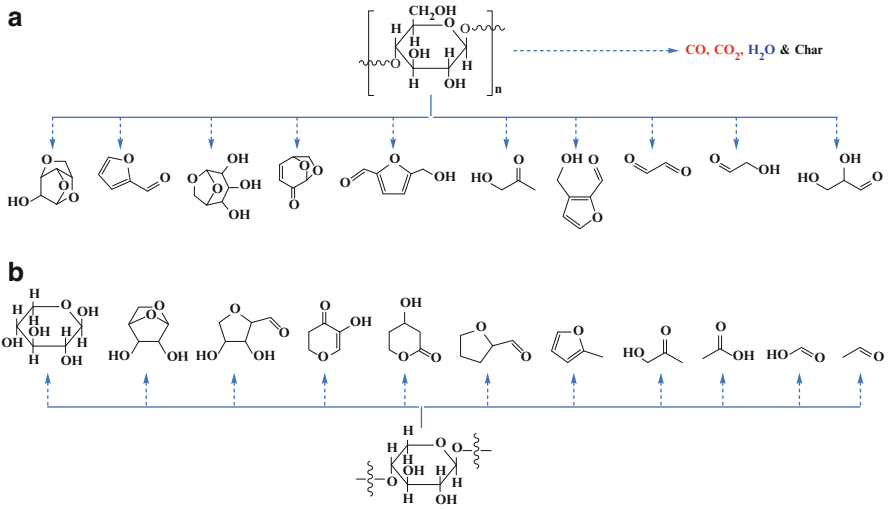

Fig. 3 Prominent pyrolytic products from (**a**) cellulose and (**b**) hemicellulose

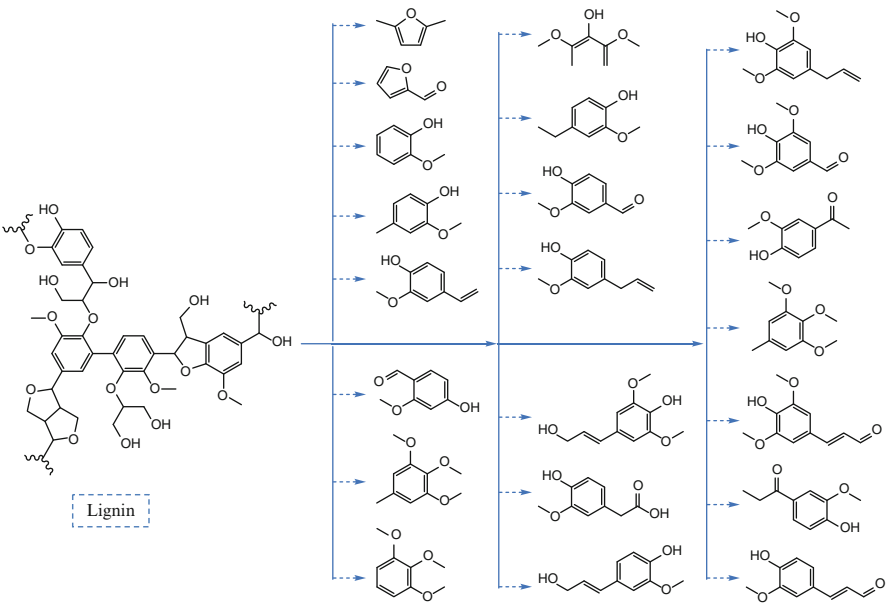

Fig. 4 Pyrolytic degradation of lignin (only important chemicals which typically form during lignin pyrolysis are shown above and do not cover the complete list)

3 Bio-oil

Bio-oil is a dark-colored liquid comprising mixture of organic compounds that serves as mother liquor to a large number of valuable chemicals. It is a complex assemblage of chemicals arising from the pyrolysis involving depolymerization, fragmentation, and rearrangement of cellulose, hemicellulose, and lignin with increase in temperature. Shorter residence times of the formed organic species inside the pyrolysis reactors lead to their condensation to give bio-oil. However, the presence of many highly reactive species results in unusual change in the stability and properties of bio-oils. Chemically, bio-oil comprises water, guaiacols, catechols, syringols, vanillins, substituted furans, furancarboxaldehydes, isoeugenol, pyrones, organic acids, hydroxyaldehydes, hydroxyketones, sugars, and phenolics. Bio-oil physically behaves like microemulsions in which the continuous phase is an aqueous solution solubilizing highly polar components of all origin and dispersed phase that is largely composed of pyrolytic lignin macromolecules and compounds derived from it. Stabilization of the continuous and discontinuous phases is mainly achieved through hydrogen bonding and micelle formation. The complex composition of bio-oils has been explained as multiphase due to the presence of char particles, waxy materials, aqueous droplets, droplets of different nature, and micelles formed of heavy compounds in a matrix of holocellulose-derived compounds and water (Garcia-Perez et al. 2006). The presence of reactive chemicals as acids, aldehydes, ketones, intermediates, etc. causes instability of bio-oil which causes gradual change in the phases due to several organic reactions (oxidation, hydrolysis, substitution, elimination, and rearrangements). Upon prolonged storage, bio-oil undergoes series of chemical reactions triggered by organic acids and intermediates in addition to the re-polymerization of reactive olefinic compounds. Aging reduces bio-oil quality and increases its viscosity which ultimately leads to the separation of continuous and dispersed phases. The presence of substantial amounts of organic acids is the sole cause of bio-oil acidity whose pH ranges between 2 and 4. As a consequence, storage, handling, and transportation of bio-oils become very difficult and challenging. In view of several challenges associated with bio-oil use involving upgrading and value addition, large amount of research has been witnessed in this area. Thorough analytical investigations are essential in every aspect related to bio-oil upgrading to prospective refining applications to yield fuels.

4 Bio-oil Upgrading

Bio-oil upgrading is essential for its value addition and involves series of chemical and engineering processes to overcome drawbacks associated with its use. Bio-oils are useless as fuels except for direct boiler and related applications, e.g., for some turbine or large diesel applications after necessary modifications. Since bio-oil is a source of valuable chemicals and advanced biofuels, its reliable scale-up and refining promise its economic viability and usage at par with conventional fossil fuels. In this direction, upgrading implicitly involves removal of bio-oil acidity

caused by free organic acids (formic, acetic, propionic acid, etc.). Additionally, upgrading is needed to increase thermal stability and reduce volatility and viscosity through oxygen removal and molecular weight reduction. Adequate upgrading of bio-oil is also essential in order to blend it with petroleum fuels. Catalytic cracking, decarboxylation, decarbonylation, hydrocracking, hydrodeoxygenation, and hydrogenation are some important approaches of upgrading biomass pyrolysis oils (Jacobson et al. 2013). Apart from these widespread approaches, organic solvents addition is also utilized for phase separation to improve the bio-oil quality. The role of additives in lowering the viscosity of bio-oils has been investigated to see its effect in imparting storage stability. For this purpose, ethyl acetate, methyl isobutyl ketone, methanol, and several others were screened. Additionally, accelerated aging tests were also conducted at 90 °C to measure the effect of these additives on the studies' parameters (Diebold and Czernik 1997).

Catalytic cracking is a prevalent upgrading process for bio-oils which requires an acidic and hydrophobic catalyst, typically ZSM-5 to produce H_2O, CO_2, or CO under atmospheric pressure. The high temperature employed in this process facilitates cleavage of bonds in large molecules and decarbonylate/decarboxylate the compounds present in biomass pyrolysis oils to form hydrocarbons. The mechanism is similar to reforming of heavily oxygenated bio-oils. One of the key approaches of upgrading also involves the removal of free organic acids (formic, acetic, propionic acid, etc.) through esterification. It is achieved through various catalysts such as solid catalysts, acidic ionic liquid catalysts, acidic ion-exchange-based zeolites (HZSM-5), H-mordenite, H-Y, silicalite, aluminum silicate catalysts, propyl-sulfonated mesoporous silica, etc. Unlike the hydroprocessing processes used for petroleum fuels (hydrotreating, hydrodesulfurization, hydrodenitrification, etc.), hydrodeoxygenation is widely applicable for bio-oils since it lacks nitrogen or sulfur but abundant in oxygen. The catalysts efficiency (in catalytic cracking or hydrodeoxygenation) is adversely affected by the deposition of oligomeric species (derived largely from lignins) present in bio-oil. It has been overcome by co-feeding hydrogen donors such as methanol, tetralin, and decalin. These co-feeds promote cracking as well as hydrogenation reactions and simultaneously dilute the lignins, thereby decreasing polymerization at high temperatures (Gayubo et al. 2009). Various aspects of hydrodeoxygenation and zeolite cracking of bio-oils to engine fuels have been reviewed by researchers and focused upon updates on catalyst development, their performance evaluation, and optimization of efficiencies. Other aspects such as reaction mechanisms, kinetics of crucial reaction parameters, and prospects of process commercialization have also been explored in detail (Mortensen et al. 2011).

5 Bio-oil: Physical Properties

As we discuss about the prospective application of lignocellulosic biomass-derived pyrolysis oil for engine fuel applications, a comparison of bio-oils and petroleum-based fuels becomes obvious. Differences in physical properties of both the liquids depend upon their chemical compositions. Bio-oils derived from all kinds of biomass

(hardwood, softwood, straw, agricultural residues, etc.) are distinctively high in water content, pyrolytic lignins, organic acids, and high oxygen content as a result of which possess very low heating values. Fuel oil qualities and chemical composition are important parameters for fuel engine applications or combustion and rely on some crucial physical properties as stability, homogeneity, heating value, viscosity, liquid density, solid content, and many more. Chemical composition is the fundamental consideration which largely governs all the listed physical properties and ultimately a measure of suitability of the liquid for being used as either boiler/turbine or engine applications. ASTM provides certain guidelines and specifications of pyrolysis liquid produced from biomass. It also specifies about the estimation of a number of physicochemical properties for such liquids for which normative references of the standards developed for characterization of petroleum and lubricants have been given. VTT, Finland, has been carrying out systematic research in this area for more than the past two decades and presented detailed physical properties of pyrolysis oils and methods to determine them (Oasmaa et al. 2005). The detailed guidelines provided have been rigorously formulated after huge analytical work and several round robin exercises in bio-oil analysis. Physical properties of biomass pyrolysis oils are extensively studied and very well documented in the scientific literature.

The lower heating value of bio-oils (18 MJ/kg) remains around 60 % of that of hydrocarbon fuels due to the presence of 50 wt% oxygen (approximately 40 wt% of dry matter) as revealed through proximate analysis of the former. Bio-oils are readily miscible with polar organic solvents (lower alcohols) that demonstrate the presence of high amounts of oxygenated species in it. The presence of more than 300 oxygen-containing chemicals has been reported in bio-oils of which hydroxyaldehydes, hydroxyketones, sugars, carboxylates, and phenolates are prominent (Soltes and Elder 1981). The measurement of physicochemical properties is an important analytical activity for biomass pyrolysis liquids and aimed at ascertaining its usefulness as fuels. The International Energy Agency and European Union jointly conducted several round robin tests for comparing the accuracy of chemical and physical characterization methods for fast pyrolysis liquids (Oasmaa and Meier 2005). During these tests, the measurement of critical properties as water content, viscosity, solid content, pH, stability, elemental analysis, and pyrolytic lignins was carried out. It was, however, realized after the tests that liquid sample handling and accurate estimation of water and density were precise whereas oxygen determination by difference remained variable even as its direct determination was poor. In addition, high variations were obtained for nitrogen, viscosity, pH, and solid measurements. Generally, large variability in the determination of such properties always exists due to complex scenarios. A list of applicable ASTM methods along with the brief detail is given in Table 1.

6 Sample Preparation

Sample preparation plays a crucial role in chemical analysis through instrumental techniques and implies how samples are treated prior to analysis. It is applicable to all the disciplines of chemical and biochemical investigations (Chen et al. 2008).

Table 1 Overview of ASTM standard methods of physicochemical and chemical analysis as applicable for the estimation of bio-oil properties

S. no.	Estimation parameter	Standard method (ASTM)	Basis of determination
1.	Ash content	D482	Thermogravimetry
2.	Boiling range fractionation	D2887	Boiling range distribution by gas chromatography
3.	Carbon residue	D189	Destructive distillation method of carbon residue determination
		D4530	Micromethod (gravimetric analysis at 500 °C under inert nitrogen atmosphere)
4.	Density	D1298	Hydrometer method
		D4052	Digital density meter
		D369 (withdrawn)	Specific gravity by pycnometer method
5.	Elemental analysis	D5373	Estimation of CO_2, H_2O, and NO_x (oxidization of sample containing C, H, and N)
		D5291	Determination of gases obtained after conversion from their respective elements
6.	Fixed carbon/ash content of biochar	D3174	Gravimetric analysis under controlled environment
7.	Flash point	D93	Flash point by specified rate of heating
		D3828	Small-scale cup test in which sample is placed and ignition spark is created
8.	Heating value	D3286 (withdrawn)	Gross calorific value of coal and coke by isoperibol bomb calorimeter
		D240	Heat of combustion by bomb calorimeter method
		D4809	By bomb calorimeter method
9.	Lubricity	D2783	Load under one ball is measured against three fixed balls covered by sample
10.	Pour point	D97	Specified cooling of a heated sample with monitoring of sample movement
11.	Sulfur analysis	D4239	Infrared determination of SO_x after combustion of sample
		D4294	Energy dispersive X-ray fluorescence spectrometric method
		D2622	Wavelength dispersive X-ray fluorescence spectrometric method
12.	Surface tension	D971	Measurement of interfacial tension by the movement of a platinum ring from the surface of fluid with higher surface tension
13.	Solid content	D2276	Particulate contaminant by line sampling (filtration through membrane)
14.	Total acid value	D974	Color-indicator titration
		D664	Potentiometric titration method
		D3339	Semimicro color indicator titration

(*continued*)

Table 1 (continued)

S. no.	Estimation parameter	Standard method (ASTM)	Basis of determination
15.	Viscosity	D88	Saybolt viscosity by viscometer method
		D445	Capillary method through viscometer
		D2170	Flow of liquid through calibrated glass capillary method
16.	Volatiles in biochar	D3175	Weight loss through gravimetric analysis under controlled conditions
17.	Water content	D1744 (withdrawn)	Karl Fischer method–volumetric method
		D95	Distillation method
		E203	Karl Fischer reagent through volumetric method

Mainstream analytical techniques require adequate pretreatment procedures to obtain a suitable sample form which can be analyzed. Sample preparation is often performed to achieve selectivity and/or specificity in analyte identification. It tends to eliminate matrix interferences which may suppress signals from the analyte of interest and removes artifacts encountered in analysis. The selection of a sample preparation method is dependent on the analytical technique being employed for analysis, nature and background of matrix (sample), chemical nature of target analyte, and background material present in the sample. It may sometimes become extremely labor intensive and time consuming yet unavoidable. For bio-oils, sample preparation is indispensable due to its chemical complexity; hence enormous literature reports are available for adopting an appropriate procedure which comprises filtration, centrifugation, phase separation, LLE, SPE, and several others. An overview of available sample preparation methods for chemical analysis of bio-oils is given in Table 2. Every class of naturally occurring biomolecules present in biomass degrades pyrolytically to give simpler organic molecules containing varied functional groups. This complex mixture of extremely polar to nonpolar chemicals presents a tough analytical scenario which can be handled by adopting an adequate cleanup strategy and analytical technique. Important approaches of sample preparation before contemporary chemical analysis may be summarized as follows:

6.1 Chromatography

Adsorption chromatography is widespread among all other chromatographic separations. It is used to obtain various fractions of bio-oil containing chemicals on the basis of polarity. Column chromatography is a prominent method in which the mixture of chemical species is separated over stationary phase held in a tubular column. In bio-oils, column chromatography plays significant role since large number of chemicals (>400) may be present in such complex matrices.

Table 2 Overview of sample preparation methods and their application to bio-oil analysis

S. no.	Sample preparation	Mechanism
1.	***Mechanical phase separation***	
A.	Filtration	Size of particulate matter; difference in solubility
B.	Centrifugation	Separation of solids from continuous phase
C.	Phase separation through water addition	Separation of phases due to difference in polarity
2.	***Solvent extraction***	
A.	Liquid–liquid extraction (LLE)	Difference in solubility between two immiscible liquids
B.	Single-drop microextraction (SDME)	Same as above but with high enrichment factors
3.	***Chromatography***	
A.	Column (normal and reversed phase)	Difference in rate of movement of solutes through stationary phase in column
B.	Solid-phase extraction (SPE)	Adsorption of solute over adsorbent through normal or reversed phase approach
C.	Ion exchange	Interaction of solute with ion-exchange (anion or cation) resins
D.	Size exclusion	Difference in molecular size
E.	High performance thin layer chromatography (HPTLC)	Difference in the rate of movement of solutes through stationary phase on planar support
F.	Solid-phase microextraction (SPME)	Adsorption of analyte over adsorbent fiber followed by thermal desorption
4.	***Electrophoresis***	
A.	Capillary electrophoresis (CE)	Difference in migration of charged species in electric field

The adsorption chromatography can be normal or reversed phase and more applicable to the organic fraction of bio-oils. Close to ASTM D2549 test method for high-boiling petroleum distillates, pentane-soluble fraction of bio-oils can be easily separated over normal phase adsorbents. In the same way, moderate to heavy polar bio-oil organic or aqueous fractions can be segregated on reversed phases (Venter et al. 2006). The miniaturized mode like SPME and HS-SPME are equally popular (Pan and Pawliszyn 1997). In a research work, volatiles from eucalyptus bio-oil and its vacuum-distilled residual product were extracted through HS-SPME (Araujo et al. 2010).

SPE, due to its versatility, efficiency, and simplicity, is an extensively used sample pretreatment tool in almost all the areas of basic and applied science. It is a usual and highly convenient method of choice for sample preparation of complex matrices prior to analysis with sensitive spectroscopic techniques. In the literature, enormous details related to bio-oil pretreatment through SPE involving normal phase, reversed phase, and ion exchange are available. Reversed phase (C_{18}) SPE has been reported for extraction of oxygenates, whereas normal phase was used for polycyclic aromatic hydrocarbons. Other equally important modes of chromatography include ion-exchange and size-exclusion chromatography. Anion exchange and cation exchange have their applications in trapping anions (carboxylates, phosphates,

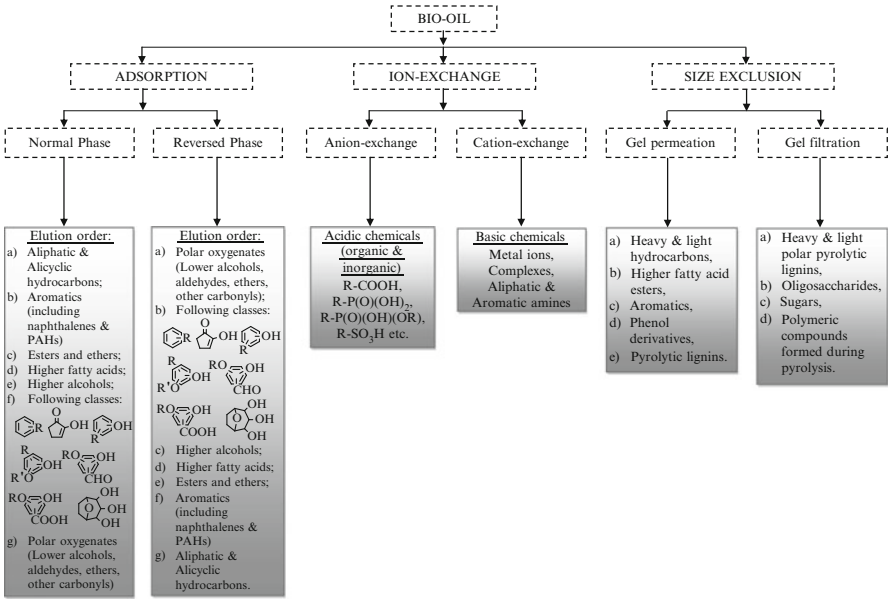

Fig. 5 Schematic presentation of the chromatographic methods of bio-oil fractionation

phosphonates, sulfonates, etc.) and cations (metal ions, cationic complexes, amines, etc.) present in bio-oils. Bio-oil fractionation through ion-exchange and size-exclusion chromatography to segregate components on the basis of their polarity and pK_a is reported. GPC is a popular size-exclusion approach and has been used in the removal of pyrolytic lignins in bio-oil samples (Andersson et al. 2000; Mullen and Boateng 2011). The chromatographic approach of bio-oil fractionation is represented in Fig. 5.

6.2 Liquid–Liquid Extraction

LLE is an efficient method of bio-oil fractionation and allows ionization suppression through pH manipulation and Soxhlet extraction. Optimization of extracting solvent is essential in maximizing the enrichment of target compounds in LLE extracts. Broadly bio-oils need to be separated between two phases, aqueous and organic, by addition of water if necessary. The aqueous phase presumably contains most of the small polar organic chemicals which may be extracted with water immiscible solvents in order of their polarities. Hexane, dichloromethane, chloroform, ethyl acetate, diethyl ether, etc. are some solvents which fractionate the entire bio-oil aqueous portion. Partitioning of analytes is generally governed by factors such as solvent polarity, nature of analytes, functional groups within the compounds, pK_a, and types of physical and chemical interactions (Garcia-Perez et al. 2007). Pyrolysis oils have been fractionated into distinct fractions, i.e., *n*-pentane soluble and insoluble which were further separated chromatographically

over activated silica gel column and eluted successively with pentane, toluene, and methanol to produce aliphatic, aromatic, and polar fractions, respectively (Ozbay et al. 2006).

SFE with CO_2 is also used for thermally labile compounds and serves the same purpose. SDME, SLE, and extraction mediated by advanced material-based adsorbents (carbon nanotubes, graphenes, nanomaterials, molecularly imprinted polymers) appear attractive in the modern research. These methods are considered green due to the miniaturized formats and associated with high enrichment factors. They also offer efficiency with the requirement of very little sample size. However, limited or very scarce information is available on such approaches due to the complexity/nonuniformity of sample matrix and restricted applications at higher scale. HPTLC has also been shown to separate anhydrosugars (levoglucosan, cellobiosan, glucose, arabinose, xylose, cellobiose, etc.) (Tessini et al. 2011). Ion-exchange methods are widely used for the removal of bio-oil acidity which involves elimination of organic acids (acetic and formic acids) formed during the thermal breakdown of lignocellulose. Anion-exchange resins (e.g., Dowex-22) are convenient options in addition to the ionization suppression caused by pH change. Neutralization of such acids with calcium oxide followed by filtration of the precipitate has also been used. Inorganic contents of bio-oil comprise of metals, their oxides, and complexes with several ligands and ions. Solvent-based extraction method for bio-oil is shown in Fig. 6. AAS and AES through ICP are highly

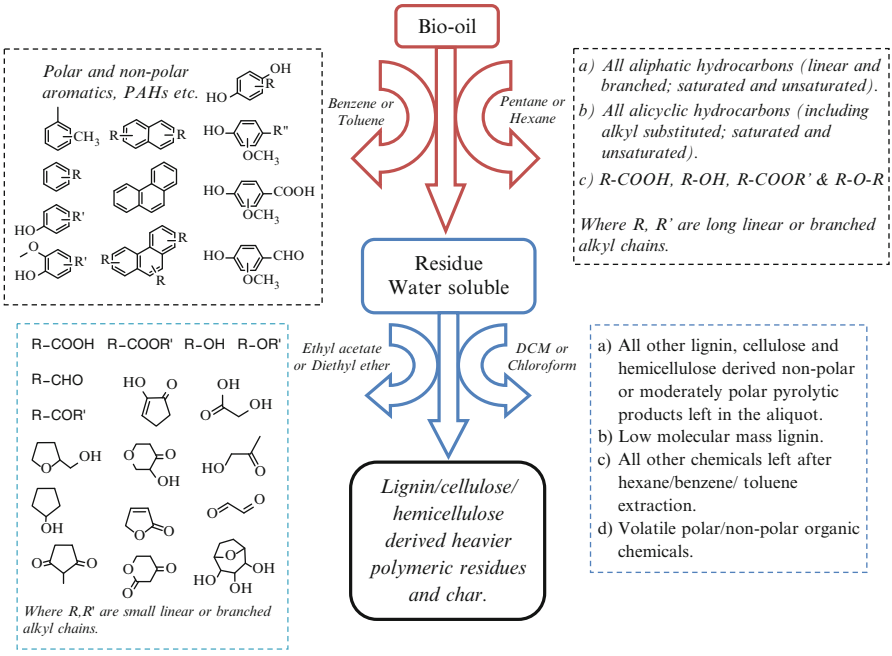

Fig. 6 Schematic presentation of liquid–liquid extraction plan for fractionation of bio-oil chemical components

appropriate instrumental procedures of analysis. Its sample preparation usually involves acid digestion and alkali fusion followed by dissolution.

Chemical derivatization is an essential aspect of sample preparation and highly crucial especially for chromatographic methods. The goal of derivatization is to primarily impart thermal stability to the labile (and polar) compounds which thereby enhances their chromatographic properties. Besides chromatographic applications, other purpose of derivatization is to improve the sensitivity of certain detection techniques. These techniques include mass spectrometry (especially soft ionization based) and NMR analysis with certain nuclei. Derivatization may sometimes become tedious and time consuming and often introduces error in the quantitative estimations. It is a potential source of unwanted artifacts in certain analytical methods. In bio-oil analysis, all the conventional and nonconventional methods of sample preparation are widely applicable due to the availability of varied chemical species into it. However, literature still lacks rigorous sample preparation for the characterization of complex matrices such as bio-oils unless extensive upgrading research is involved.

7 Chemical Characterization of Bio-oils

Chemical characterization of bio-oils remained a challenge before the early 1990s, due to the complex nature of matrix. Highly viscous nature due to macromolecules produced from lignins and sugars and highly polar chemicals caused difficulty in the complete analysis. Drastic variations in the chemical composition of biomass feedstocks are imperative in understanding chemical nature of the formed pyrolysis liquid. Adequate sample pretreatment is absolutely essential in view of the presence of a large number of chemicals arising due to pyrolysis. Furthermore, monitoring of upgrading approaches implies understanding of chemical processes and reactions at molecular levels. Due to these reasons, the need for complete chemical characterization of bio-oils is crucial in understanding the involved processes at molecular levels. Tools of analytical chemistry provide comprehensive solutions in this field and engage all the aspects like thermogravimetry, electroanalytical methods, volumetry, and spectroscopy. Separation processes act as backbone for the entire chemical analysis and encompass most of the activity covered in sample preparation. Typically, analytical techniques which have been widely employed for bio-oil analysis are GC, GPC, GC $\times$ GC, HPLC, GC–MS, LC–MS, FTIR, NMR, and TGA. Bio-oil analysis unequivocally involves synchronization in results obtained from several analytical techniques and methods. In other words, analytical data should complement each other, leading to unambiguous identification and quantification of any chemical component. GC-based detection inevitably requires volatility of analytes so that gases, non-polars, and chemical derivatives of polar organic compounds present in bio-oils are largely amenable to this technique. To deal with moderately polar to highly polar thermolabile compounds, HPLC-based detection is generally more common. Molecular weight distribution of compounds within a sample is reliably estimated with GPC. Mass spectrometer

being a very powerful detector to the chromatographic systems is ideal for identification, quantification, behavior studies of molecules, and advanced gaseous phase reactions.

Hyphenated analytical techniques as GC–MS, LC–MS, and GC-FTIR immensely contribute in the area of bio-oil analysis as witnessed through the huge amount of literature reports which started appearing since the early 1990s. FTIR reveals the presence of functional groups in the sample which helps in narrowing down on present chemical compounds and establishing molecular structure. NMR essentially determines the nature and types of covalently bonded nuclei (hydrogen or carbon) within a molecule. It provides an estimated number of such nuclei in a sample under observation through peak area integrations. Thus, spectroscopic analysis offers valuable structural insights of such complex matrix. In contrast, physicochemical characterization of bio-oil largely relies on bulk property measurements and is expressed as functions of respective chemical properties. Bio-oil production and upgrading research require monitoring of the progress and fate of chemical reactions that is very useful in developing molecular-level understanding of bio-oils and upgraded products.

7.1 Thermal Analysis

Biomass thermal profiling is highly crucial in ascertaining the quality of bio-oil for which thermoanalytical techniques represented by TGA and DTG are commonly used. TGA helps in determining plausible thermal behavior of the biomass feedstocks and products derived from it. It involves change of sample mass against time or temperature which is monitored at a specified rate of heating. Valid thermodynamic values can be concluded by the application of Arrhenius equation corresponding to separate slopes of constant loss of mass for a sample. The abundance of particular lignocellulosic components in the biomass samples governs bio-oil properties which can be reliably predicted by analyzing TGA profiles of employed biomass feedstock. It has been observed generally that the lower the parent biomass lignin content, the better is the quality of obtained bio-oil. Bio-oil and its organic fractions have some volatility, and its vapors comprise collection of true vapors and minute droplets (aerosols) along with a large number of nonvolatile compounds formed during or along with vapor condensation in an irreversible manner. Through TGA, estimation of properties such as evaporation, thermal decomposition, and combustion becomes easy. It is known that volatile and lighter product is considered better for combustion; hence TGA data of biomass and bio-oil is useful in optimizing pyrolysis conditions, improving bio-oil quality and energy content, since the measured mass losses are related to the volatility and molar masses of fractions under investigation. TGA/DTG has been extensively used in comparing evaporation and cracking behavior of bio-oils derived from different feedstocks (Vitolo and Ghetti 1994).

Thermal analysis of various biomass samples and bio-oils derived from them typically shows three major steps of weight losses:

(i) Loss of water at around 100 °C
(ii) Loss of volatiles and smaller organic compounds (from lignin pyrolysis) at 250–350 °C
(iii) Complete combustion of all kinds of organic matter at 350–500 °C

These losses provide valuable information about sample reactivity which shows that wood with lower lignin showed higher reactivity. They also suggest amounts of tar which one should expect from pyrolysis of dissimilar biomass samples. TGA coupled to FTIR is an essential technique in determining the composition of biomass sample in terms of the presence of functional groups and change in composition during or after pyrolysis. Roughly, 65–70 % (w/w) volatiles, 27–29 % (w/w) fixed carbon, and around 3 % (w/w) ash content have been estimated in biomass samples which is subject to moderate variations depending upon the nature of plant biomass (Kanaujia et al. 2013, 2014). The yield of pyrolysis liquids, sometimes, has been reported to be influenced by ash content in the feedstocks, and it has been reported that the higher the ash concentration, the lower is the yield of bio-oil. Apart from feedstock analysis, thermal stability of bio-oil has been monitored systematically by Adjaye et al. in 1992 where fresh bio-oil sample was distilled at different temperatures under vacuum and its physicochemical properties (density, viscosity, distillation characteristics, composition, etc.) were measured. The important observations of this study were related to yields of oxygenated compounds, such as acids, ethers, alcohols, and volatile aliphatic hydrocarbons with moderate thermal stability, which decreased with increase in temperatures above 200 °C. In contrast, concentration of aromatic hydrocarbons, naphthenes, aldehydes, and ketones remained at maximum at around 175 °C (Adjaye et al. 1992). Qiang et al. compared TGA and DTG curves for bio-oil analyzed in nitrogen and air and compared its thermal behavior. The similarities in combustion profiles were observed in nitrogen as well as air at temperature less than 400 °C, while burning of char residues occurred in air above 400 °C. DTG curves of bio-oil heated in nitrogen and air showed two and three distinct peaks, respectively, which indicated that first two peaks corresponded to the loss of volatiles in both the atmospheres whereas third peak in the latter was due to burning of char residues (Lu et al. 2008). It is largely observed that DTG curves serve as good indicators of boiling point distribution and overall composition of bio-oils. In addition, a very interesting application of TGA exists for catalyst characterization in which it determined the amounts of deposits over bio-oil upgrading catalysts. These catalysts, as discussed previously, have been employed to upgrade and obtain a variety of value-added end products from bio-oil by various processing methodologies.

7.2 Fourier Transform Infrared Spectroscopy

Infrared spectroscopy is one of the most conclusive and simple methods of structure elucidation which is extensively utilized to reveal the presence of various functional groups in molecules. It has significance in light of the examination of the

Table 3 Typical FTIR peak assignments for biomass pyrolysis oils and its fractions

S. no.	Wave number (cm^{-1})	Vibrations	Functional group assignment
1.	1,100–1,000	C–O stretch	Carboxylic acid, ester, ethers, and alcohols
2.	1,200–900	C–O and C–O–C stretch	Polysaccharides (pyrolytic cellulose and hemicelluloses)
3.	3,100–2,850	C–H stretch	Aliphatic and olefinic hydrocarbons
4.	3,300–3,400	O–H stretch	Bonded or nonbonded hydroxyl groups and water
5.	1,600–1,800	C=O stretch	Carboxylic acids, esters, aldehydes, and ketone groups
6.	1,600–1,450	C–C stretch	Aliphatic and aromatic species
7.	1,640–1,600	C=C stretch	Olefinic and aromatic species
8.	700–850	C–H bend out of plane	Aromatic ring structure
9.	2,350–2,000	C≡C stretch	Alkynes

fundamental vibrations and associated rotational–vibrational structure within a molecule. FTIR is highly useful in the qualitative and quantitative component analysis, thermal deterioration, aging, and QA/QC studies in almost all areas of contemporary chemical and biological research. A huge number of literature information indicates enormous applications of FTIR in every aspect of bio-oil research. The interpretation of IR spectra is straightforward and generally shows peaks above 3,050 cm^{-1} corresponding to C–H stretching vibration for the presence of aliphatic hydrocarbons. Peak above 3,100 cm^{-1} indicates C–H stretch for olefinic compounds, between 3,300 and 3,400 cm^{-1} corresponding to O–H stretching vibrations caused by carboxylic acids and/or alcohols. Peaks between 1,450 and 1,600 cm^{-1} indicate a C–C stretching vibration caused by aliphatic or aromatic structure. The C=O peak is seen between 1,600 and 1,800 cm^{-1}, indicating either carboxylic acid/ester or aldehydes/ketone groups. C–O stretching peak (in acids, esters, ethers, and alcohols) is seen between 1,000 and 1,100 cm^{-1}. Aromatic rings are also determined by the presence of C–H "out of plane" bands in the range of 700–850 cm^{-1}. The details of important FTIR vibrations and their functional group assignments are shown in Table 3.

TG-FTIR, as discussed in the previous section, is a useful hyphenated tool for determining online changes in the chemical composition within a bio-oil during its formation or upgrading. It has been used widely to predict and optimize factors which influence pyrolysis (Kanaujia et al. 2014). Pyrolysis of wheat straw and other agricultural residues including coconut shell, sugarcane bagasse, corn stalks, peanut shell, etc. has been explored with TG-FTIR (Souza et al. 2009). Bio-oils from other feedstocks such as rapeseed cake, apricot, peach pulp, pine wood, linseed, etc. have been analyzed to divulge usual functional groups. Characterization of bio-oil fractions with organic solvents shows usual functional group distribution and suggests oxygen content and their distribution. In one of the studies, bio-oils obtained from subcritical and supercritical water liquefaction of wood biomass

largely contained heavy oils among other compounds such as hydrocarbons, aldehydes, ketones, hydroxybenzenes, and esters (Qian et al. 2007). In another well planned pyrolysis and upgrading study, bio-oil was produced from soybean stalk (and deoxy-liquefied in a closed reactor) was shown to possess low oxygen content, high H/C ratio, and high heating value. In this study, dissociation of O–H and recombination of C–H bonds into hydrocarbons were proposed which was accompanied by the release of CO and CO_2. This bio-oil was analyzed with FTIR and typical oxygenates were observed. Upgrading of this bio-oil yielded final product which was distilled and decolorized over activated clay to obtain high-quality biofuel (Li et al. 2008). The existence of -NO_2 and N–H functional groups with signals between 1,550 and 1,490 cm^{-1} has been identified in bio-oil obtained from rice husk (Lu et al. 2008). Analysis of bio-oil from lignin pyrolysis in attenuated total reflectance (ATR) mode revealed the predominance of hydrocarbon structures in addition to small amounts of phenols and carboxyl groups. Fiber optic-based near IR system working on reflection–absorption phenomenon has been used for predicting water content in bio-oils by applying multivariate analysis to the spectral information containing different water content.

The reactivity of various carbonyl functionalities in bio-oils has been predicted on the basis of FTIR analysis and considered important for upgrading process. Carbonyl distribution in bio-oil is a function of composition of feedstock (hemicellulose, cellulose, and lignin) and an indicator of aging. FTIR is important in ascertaining the aging of bio-oils since reactive organic species that form during pyrolysis have a strong tendency to repolymerize, rearrange, or fragment to form stable products. It leads to deterioration of quality which creates difficulty in bio-oil storage, handling, transportation, and upgrading. FTIR is employed to monitor this transformation effectively, and several studies have been carried out in this direction. In one such experiment, bio-oil quality during thermal treatments or long-term storage at ambient temperatures was evaluated which showed the presence of C–H out of plane deformation vibration of terminal olefins (signal at 880 cm^{-1}) indicating commencement of thermal deterioration process (Xu et al. 2011). Increase in C–O stretch (phenols, carboxylic acids, esters, and ethers) and C=O (carbonyl) functional groups suggests aging for which accelerated aging studies have been carried out to establish the stability of bio-oils. Upgraded bio-oils have also been emulsified with biodiesel and monitored for stability through FTIR. Biochars obtained after pyrolysis can be routinely analyzed with FTIR to determine its nature which usually comprises N–H, C–H, C–C, and C–O–C bonds. In this way, FTIR finds immense application in all aspects of bio-oil research due to its simple, straightforward, and conclusive nature that is useful in process scale-up.

7.3 Gas Chromatography

GC is a highly versatile separation technique extensively employed for volatile and thermally stable organic compounds. Widespread utilization of a variety of available detectors has exponentially expanded the dimensions of GC, mass selective

detection being one of the most popular. Regular modifications to this technique have led to the evolution of other efficient formats such as GC × GC, static headspace extraction-GC, and fast GC. In itself, GC is a vast area of contemporary research including petroleomics. Since bio-oils contain a large number of chemicals with varying volatility and polarity, GC employs several variables that cater requirements for their analysis. Polar organic compounds often require chemical derivatization prior to GC-based analysis, and bio-oils contain numerous such chemicals which originate from thermal degradation of lignins, hemicelluloses, and celluloses. GC-FID is generally used for estimating biomass pyrolysis/gasification condensates as well as other formed organic components. In an interlaboratory proficiency test conducted by the International Energy Agency and European Union, GC analysis was included as a primary technique for bio-oil characterization. In this round robin activity, bonded cross-linked mid-polar capillary column containing 14 % cyanopropyl-phenyl was the recommended stationary phase. In the same test, participating laboratories reported GC-FID analysis of organic acids, aldehydes, ketones, alcohols, sugars, phenols, and PAHs (Oasmaa and Meier 2005). Analyses of acetic acid, aldehydes, sewage sludge pyrolysis liquid, bio-oil fractions, etc. have been carried out with GC-FID. Bio-oil extracts obtained with LLE, SPE, and column chromatography have also been analyzed showing that GC-FID is a method of choice for bio-oil characterization. Similarly, gases evolved during pyrolysis are easily identified and quantified with GC-TCD. These uncondensed gases comprise CO, CO_2, CH_4, H_2, C_2H_4, C_2H_6, C_3H_6, C_3H_8, C_4H_8, and C_4H_{10}, and their analysis is largely preferred by GC-TCD or GC-FID.

Group-type analysis of oxygenated organic compounds in bio-oils is conveniently accomplished with GC × GC with usual detectors. Recently, GC × GC has progressed quickly and emerged as a powerful analytical tool for analysis of organic compounds. It offers discrete separations of the sample components which are separated firstly in one dimension (column) and then subsequently separated in second dimension (column) with the help of modulators. In other words, the separation occurs on a long nonpolar column where the components are separated on the basis of their boiling points. The separation on this column is assisted by another column through a modulator which collects the effluents in a defined elution range and then inject this fraction over another short (polar or intermediate polar) column which helps separate the components on the basis of their polarities under isothermal conditions. Excellent results are obtained if the two or more separation mechanisms are independent of each other. It is for this reason the technique is termed multidimensional GC or GC × GC. This technique is valuable in the separation of large number of compounds with very close boiling points such as in bio-oils. Pyrolytic products from lignocellulosic biomass along with the upgraded products have been analyzed with GC × GC-FID and GC × GC-HRMS. Time of flight mass spectrometer (TOFMS) is the detector of choice for GC × GC which is helpful in identifying components having close molecular weights. Pyrolytic products obtained from biomass have been analyzed with GC × GC-TOFMS to reveal more than 100 (or 300) chemicals which vary from one feedstock to other (Sfetsas et al. 2011).

7.4 Nuclear Magnetic Resonance

The NMR is essentially employed in monitoring experiments on solid, liquid, and gaseous samples, over a wide temperature span. NMR plays a remarkable role in the structural characterization of petroleum fractions and a crucial tool for carrying out group-type analysis which directly measures aromatic and aliphatic carbon (^{13}C) and hydrogen distributions (^{1}H). It provides vital information on the structural layout of carbon and hydrogen groups within a molecule with standard methods of ^{13}C NMR studies on coal and asphaltenes being its important applications. In the area of bio-oil research, the advantage of using NMR is that whole bio-oil can be dissolved and analyzed in a suitable solvent, and qualitative assessment of the oxygen-containing functionalities can be determined from the integration of appropriate regions of the ^{13}C and ^{1}H spectra.

Bio-oils are largely studied with ^{13}C NMR particularly due to large chemical shift regions. ^{13}C and ^{1}H NMR offer reasonable trade-off between functional group identification and analytical measurement effort. Accuracy, however, depends upon the selection of chemical shift regions, baseline compensation, and correction for incomplete longitudinal relaxation effects. NMR had been used in the past to provide confirmation of chemical functional groups in bio-oil. In the 1980s, the concern was the solubility of components in deuterated acetone or chloroform along with the poor quality of ^{13}C spectra owing to low signal-to-noise ratio and imperfect baseline attenuation. The presence of high molecular weight compounds also remained a challenge in NMR analysis. Several studies were carried out in the past which provided a lot of information on pyrolytic lignins and bio-oils. Evaluation of pyrolysis conditions and changes in product formation profile has been performed through ^{1}H NMR which helped in optimizing pyrolysis parameters and manipulating the yields of components such as aromatics, paraffins, and olefins. Chemical species present in bio-oils derived from lignin pyrolysis have been identified through NMR with ^{1}H, ^{13}C, and ^{31}P to determine the extent of lignin degradation. Polar functionalities present in bio-oils are comprised mainly of hydroxyl and carboxyl groups that can be quantified using ^{31}P NMR after derivatizing them with phosphorus-containing reagent such as 2-chlorophosphalone or 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. In an investigation, ^{31}P was used for determining three principal forms of phosphitylated phenolic hydroxyls present in lignins (*p*-hydroxyphenyl, guaiacyl, and syringyl structures) along with other groups such as hydroxyls, carboxylic acids, and two diastereomeric forms of arylglycerol-beta-aryl ether units. ^{31}P NMR has advantage over other nuclei due to the absence of the interference of peaks from other nuclei and can be studied in a wide chemical shift scale. DEPT NMR is useful in the quantitative classification of carbon and hydrogen. DEPT quantifies protons and carbon atoms in off-resonance-decoupled ^{13}C spectra to extract information on the types of molecules that are found in the matrix. In a study, biomass fast pyrolysis oils were characterized with ^{13}C and DEPT NMR to determine functional group compositions related to their energy content. The spectra were compared with gasoline and diesel and divided into distinct regions

according to the chemical shift ranges appropriate for various functional groups. The spectral intensities of each region were quantified, and carbon–proton substitution numbers were determined. Additionally, chemometrics was used to extract more useful information from spectra (Strahan et al. 2011).

Several NMR studies related to bio-oil analysis have been carried out with ^{13}C NMR which suffers from low sensitivity due to its low natural abundance and long relaxation times. Few important observations have thrown light on the process part of bio-oil production on the basis of ^{13}C NMR experiments. It has been proposed on the basis of ^{13}C NMR analysis that carbohydrate fraction decreases and aromatic fraction increases as the pyrolysis temperature is increased in a fluidized bed reactor. It has also been observed that pyrolysis oil from kraft lignin showed removal of aliphatic hydroxyl, carboxyl, and methoxyl groups during pyrolysis. On the other hand, ^{1}H NMR is sensitive and essential for determining hydrogen distributions in crude bio-oil obtained from various biomass feedstocks. Thermal stability of whole bio-oil, its ether-soluble fraction, and biodiesel blends are reported in addition to monitoring of structural changes in the blends measured with ^{1}H and ^{13}C NMR experiments, respectively (Jiang et al. 2011). Interestingly, it has been proposed that the presence of very broad peak in the spectra results due to the presence of polymeric material with slow molecular motions (Mullen and Boateng 2011). Proton NMR was also used for water estimation in bio-oil at par with conventional methods like Karl Fischer titration and azeotropic distillation. Heteronuclear single quantum correlation NMR (HSQC-NMR) has been shown to provide chemical shift assignment for 27 different types of C–H bonds present in bio-oils and indicated the presence of two different types of methoxyl groups in samples. Levoglucosan, furfurals, and phenols were the major components in all samples; however, most aliphatic and aromatic C–H bonds were produced from lignin component (Ben and Ragauskas 2011).

7.5 Mass Spectrometry

Mass spectrometry (MS) provides unequivocal elemental and structural composition of chemical species. Quadrupole mass analyzers are affordable, rugged, and commonly available and, hence, are used extensively in the unambiguous analysis of liquid pyrolysis products. A wide range of ionization modes and analyzers have expanded the scope of mass spectrometry, which is applicable to all the areas of chemical and biological research. Briefly, among the available ionization techniques, EI in positive mode and at 70 eV is largely used for characterization purposes due to the existence of huge mass spectral database. Soft ionizations such as CI, ESI, APCI, FI, FAB, and MALDI are used for thermolabile molecules. Other less common one is DESI which is manifested by DART-based mass spectrometers. Tremendous possibilities exist in the area of chemical and biochemical analysis with the available analyzers and ionization methods in mass spectrometry. A wide range of analyzers such as magnetic sector, quadrupole, time of flight, ion traps, orbitrap, and ICR are being used currently.

Py–GC–MS is highly useful in understanding the process of biomass pyrolysis and obtained products. It comprises thermal decomposition of biomass feedstocks under defined conditions followed by GC–MS analysis. There are two formats of Py–GC–MS, namely, online and offline methods. In online pyrolysis, the pyrolytic products directly enter into the GC inlet where they are analyzed after being separated. On the contrary, offline pyrolysis involves trapping of the formed pyrolytic products on adsorbents, after which it is eluted with solvents before introducing into the GC columns. The online method is good and provides valuable information on the formed pyrolytic products, but it is associated with challenges such as deterioration of liners and columns due to high molecular weight polar products. It often leads to artifacts and imprecise/inaccurate analytical data. Offline method on the other hand is advantageous because it enables analyst to adopt suitable column and sample pretreatment steps (including derivatization) and leads to accurate results. A large amount of work has been carried out with Py–GC–MS in which upgrading of bio-oils and determinations of bio-oil compositions of different pyrolytic lignins obtained from different feedstocks are involved (Johnson et al. 2009).

Low-resolution mass spectrometry exhibited by quadrupole-based instruments has been widely used for chemical identification for bio-oil analysis and upgrading. It is highly suitable for the characterization of small molecular weight organic compounds from aliquots obtained after sample preparation. GC is used for sample introduction and separation followed by data acquisition in EI mode. The prerequisite for GC–MS analysis is the extraction of the liquid condensate with suitable organic solvents (LLE) or SPE. Bio-oil characterization and stability analysis have identified more than 100 individual compounds including oxygenates and hydrocarbons. Oxygenated compounds mostly included acids, cyclic alcohols, aliphatic alcohols, aldehydes, cyclic ketones, substituted furans, ethers, and alkyl-substituted methoxy phenols. Hydrocarbons consisted mainly of long-chain paraffins, olefins, aromatics, cycloparaffins (non-condensed and condensed), and polycyclic aromatic hydrocarbons (Adjaye et al. 1992). Carbon range distributions have also been estimated in pentane soluble bio-oil fraction which showed the presence of *n*-alkanes, *n*-alkenes, and branched hydrocarbons with the majority of linear hydrocarbons being distributed in the range of C_{11}–C_{29}. Semiquantitative estimation of hydrocarbon distribution showed roughly 72.87 % *n*-alkanes, 16.44 % *n*-alkenes, and 1.84 % branched hydrocarbons. Bio-oil from cyclone reactor has been analyzed with GC–MS and found to contain: (i) holocellulose-derived compounds, viz., propanol, acetic acid, propanone, and furfural; (ii) lignin-derived compounds, viz., phenol, cresol, guaiacol, xylenol, methyl guaiacol, ethyl guaiacol, syringol, vanillin, and eugenol; and (iii) PAHs such as pyrene, fluorene, naphthalene, phenanthrene, biphenyl, and anthracene (Lede et al. 2007). Other PAHs which have been identified by several other researchers include alkylated benzofurans, indenes, and naphthalene-dominated PAHs. Around 63 PAHs have been confirmed to be found in bio-oils among which some of them were also quantified. Bio-oil derived from algal biomass has also been characterized with GC–MS (Zhou et al. 2010).

ESI-based mass spectrometers are typically used as LC–MS in order to obtain molecular weight information of polar and thermolabile molecules. Molecular mass distribution and all polar chemicals present in bio-oil obtained from lignocellulosic biomass have been reported with LC–MS. Positive and negative ESI-MS have revealed around 320 peaks in a bio-oil sample with mass distribution ranging between 100 and 1,100 mass units complemented through size-exclusion chromatography (Harris et al. 2011). Chemometrics along with the LC–MS is generally preferred for fingerprinting of bio-oil components and petroleum products. MALDI-TOFMS, LDI-TOFMS, and temperature-resolved analytical pyrolysis field ionization mass spectrometry Py-FI-MS are also used for this purpose.

HRMS is a valuable analytical approach of mass spectrometry which is expensive and more sophisticated and, hence, is not very common. HRMS plays a significant role in accurate mass measurement which deconvolutes molecular information from complex matrix like petroleum where as much as 250 chemicals have been known to have same nominal masses. It is the mass analyzer which is responsible for resolving the power of the mass spectrometer which refers to the ability to separate two mass spectral peaks differing by accurate masses. Analyzers which help in achieving high resolving power are double-focusing magnetic sector, TOF, and FT-ICR. Excellent reviews have been written in this area dealing with the fundamentals and basic into advanced applications of HRMS in various R&D sectors (Burlingame et al. 1996). Bio-oils have high degree of compositional complexity; hence the resolving power of MS to extract accurate molecular-level information is useful. HRMS has been employed to evaluate bio-oil composition using LDI and linear ion trap–orbitrap analyzer. Comparison of high-resolution data obtained from orbitrap and FT-ICR-MS has been performed to establish the minimum resolution required for unambiguous detection of analytes. Furthermore, the orbitrap analyzer was reported to provide sufficient resolution which could determine chemical compositions for over 100 compounds (Smith and Lee 2010). Similarly, high-resolution orbitrap, quadrupole TOF (QTOF), and FT-ICR-MS in negative ESI modes were also compared. Heteroatom class distribution and double bond equivalence have also been performed with FT-ICR-MS to obtain clearly distinguished phenols derived from lignin. Analysis of bio-oil at 8,000 resolution with TOF at different electron beam energies provided molecular weight distribution. Inorganic mass spectrometry for the determination of common elements in the biomass ash is preferred with ICP-based technique such as AES, AAS, and MS. ICP-MS is preferred over others and is used for a variety of applications in bio-oil characterization and upgrading including algal biomass-derived liquids (Jena and Das 2011).

7.6 Other Analytical Techniques

Owing to the diversity and complexity of lignocellulosic biomass pyrolysis oils, there are very limited literature applications of other instrumental analytical techniques besides those discussed above. Only few studies have been undertaken for

bio-oil characterization with such techniques which have proven capabilities in other areas of modern research. Micellar electrokinetic chromatography is similar to capillary electrophoresis and has been used for the analysis of phenolic fraction of bio-oil. In capillary electrophoresis, components of a sample are separated by differential partitioning between micelles (pseudo-stationary phase) and a surrounding aqueous buffer solution (mobile phase). HPLC analysis with retention index and ultraviolet detection is reported, for polar bio-oil components such as aldehydes after derivatization with suitable reagents, in addition to multidimensional LC which is optimized for estimation of phenols (Andersson et al. 2000). Size-exclusion chromatography with ultraviolet and retention index detector has been used to obtain average molecular weight, dispersity, and molar mass at maximum peak height. Laser-induced fluorescence has been used to simulate and monitor the effect of ultraviolet exposure on the chemical stability of bio-oil produced from pine lumber, pine bark, oak bark, and oakwood. It was proposed that phenols present in the bio-oil showed chemical instability by giving time-dependent fluorescence intensities (Tripathi et al. 2010). Other less used analytical techniques include XRD and SEM which have been used for biochar characterization.

8 Conclusion

The presence of a very large number of compounds with varied functional groups in bio-oils has necessitated the development of universal extraction and analytical protocols. Widespread utilization of advanced analytical methods has enhanced our understanding about the composition and correlation of several variables associated with quality, upgrading, and stability. Feedstock, pyrolysis process, reactor design, pressure and temperature conditions, etc. are responsible for overall quality and stability of the bio-oil. The research in this direction is academic in nature; however, huge investment across the globe has promising prospects. The European Committee for Standardization is developing quality standards for pyrolysis products for the development of necessary test methods to determine relevant properties. Finland along with other countries has already invested in developing an integrated pyrolysis and upgrading refinery which would take shape in the coming years. Analytical insights have simplified overall bio-oil understanding that ranges from compositional revelations to modeling and chemometric approaches in the process optimizations. Chromatography and spectroscopy offer various approaches of instrumental analysis of which sample preparation is extremely crucial. Nanomaterials exhibit extraordinary physical, chemical, mechanical, and electrical properties and therefore have ample scope in sample pretreatment and catalyst formulations. In sample preparation, their enormous adsorption potential can be utilized as such or with covalent/non-covalent modification of surfaces. Magnetic nanoparticles with or without functionalization are useful in selective extractions and other desired sample pretreatments. Principles of immunoaffinity chromatography in the form of molecularly imprinted polymers may be explored for specific refining applications.

A concerted effort involving funding bodies, industry, and academy is absolutely essential for bio-oil value addition and to circumvent the increasing burden on limited petroleum reserves. Tools of analytical chemistry offer unequivocal identification and quantitation of target analytes and the matrix as well. Spectroscopic analysis provides vital structural insights and dominates over others, whereas determination of physicochemical properties is based on the classical methods of analysis. Discussion on all the aspects of bio-oil characterization is presented to emphasize and enumerate important findings plus the importance of correlation between feedstock nature and bio-oil quality. Unstable chemical species in bio-oils are revealed by TGA points toward the development of efficient extraction protocols which must be adopted for further production, processing, and storage of bio-oil. Ongoing use of mass spectrometry for bio-oil analysis is apparent by the presence of enormous literature and has necessitated the need for interlaboratory proficiency testing (round robin) so that uniform analytical guidelines and results evaluation criteria evolve in due course of time. Since mass spectrometric results provide conclusive evidence of compounds in fresh and aged bio-oil samples, therefore storage and stability issues associated with it must be appropriately addressed. Other techniques like NMR, FTIR, and chromatography have already simplified the analysis not only in this area but modern research as a whole.

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HPLC Method for Microanalysis and Pharmacokinetics of Marine Sulfated Polysaccharides, Propylene Glycol Alginate Sodium Sulfate

42

Yi-Ting Xue, Chun-Xia Li, Xia Zhao, and Hua-Shi Guan

Contents

Abstract

With the development of glycobiology and glycochemistry, bioactive polysaccharides are more and more widely used in the clinical area due to their anticoagulation, antitumor, immune regulation, antivirus, and other activities. As a marine sulfated polysaccharide, propylene glycol alginate sodium sulfate (PSS) has been commonly used as a heparinoid drug for the treatment of ischemic cerebrovascular and hyperlipidemia diseases in China for nearly 30 years. The reliable high-performance liquid chromatography (HPLC) with pre-column derivatization and post-column fluorescence derivatization methods is developed for the determination of the β-D-mannuronic acid (M)/α-L-guluronic acid (G) ratio of PSS and microanalysis of PSS in rat plasma, respectively. These methods provide the basis of the quality control, qualitative, and quantitative analysis. Especially, these methods can be used for microanalysis of other polysaccharide drugs in vivo and pharmacokinetic study.

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Polysaccharide • Propylene glycol alginate sodium sulfate • Pharmacokinetics • Microanalysis • Pre-column derivatization • Post-column derivatization

Abbreviations

ADME	Absorption distribution, metabolism, and excretion
$AUC_{0\text{-}t}$	Area under the curve from zero to t
CL	Plasma clearance
C_{max}	Maximum concentration
Emission	λem
Excitation	λex
F	Absolute bioavailability
FD	Fluorophotomeric detector
FITC	Fluorescein isothiocyanate
G	α-L-Guluronic acid
GC	Gas chromatography
^{3}H	Tritium
HPLC	High-performance liquid chromatography
IS	Internal standard
M	β-D-Mannuronic acid
MRT	Mean residence time
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
PABA	*p*-Aminobenzoic acid
PGS	Polyguluronate sulfate sodium
PMP	1-Phenyl-3-methyl-5-pyrazolone
PMS	Polymannuronate sulfate sodium
PSS	Propylene glycol alginate sodium sulfate
PTFE	Polytetrafluoroethylene
SD	Standard deviation
$T_{1/2}$	Half-life
TFA	Trifluoroacetic acid
T_{max}	Time point of maximum concentration
UVD	Ultraviolet detector
Vd	Volume of distribution

1 Introduction

PSS is a marine sulfated polysaccharide composed of (1→4) linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) (Fig. 1), which is prepared by hydrolysis, esterification and sulfation of sodium alginate from brown seaweeds (Guan 1999; Rahelivao et al. 2013). PSS is a heparinoid-active drug and has good anticoagulant, hypolipidemic, hypoglycemic activities, and other

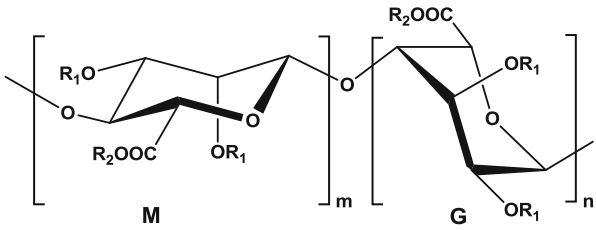

Fig. 1 Chemical structure of PSS, $R_1 = H$, SO_3Na, $R_2 = Na$, or $CH_2CH(OH)CH_3$

functions (Li et al. 2011, 2014). In the 1980s, it was first authorized for clinical application in China, and it is mainly used for the prophylaxis and treatment of ischemic cardiovascular and cerebrovascular diseases.

The previous work has shown that the bioactivities of PSS are mainly related with uronic acid composition (M/G ratio), degree and pattern of sulfation, and weight-average molecular weight (Lin et al. 2007; Fan et al. 2011). Among them, M/G ratio of PSS is one of the most important structural factors (Wu et al. 2014) for its activities and identification. There is an urgent need to develop a reliable and sensitive method for determination of the M/G ratio of PSS for quality control and clinical purposes.

Pharmacokinetics is usually carried out by studying the absorption, distribution, metabolism, and excretion (ADME) of drugs in vivo and in vitro. It is essential to guide the clinical administration including dose regulation, regimens, plasma concentration monitoring, and evaluation of the interactions among medicines (Zeng et al. 2013). Pharmacokinetic study plays an important role in making objective evaluation of existed drugs, designing new drug delivery systems, optimizing dosing regimen, and directing clinical medication (Liu and Sun 1999; Zhong 2000).

With the development of polysaccharide drugs, it is a great challenge for the analysis of polysaccharide drugs in vivo due to their structural complexity and heterogeneity (Wang 2007; Lv 2008). The pharmacokinetic analysis and microanalysis of polysaccharide drugs in biological samples of polysaccharide drugs have become huge obstacle in the pharmaceutical analysis field. The reasons are mainly elaborated in the following aspects. Firstly, the chemical structure of polysaccharide is complex. The lack of chromophoric and fluorophoric groups reduces specific ultraviolet absorption; therefore, it is difficult to use conventional sensitive detectors to analyze. Secondly, polysaccharide has large molecular weight, and it is often composed of multiple components. Hence it is difficult to use specific standardization methods to make quantitative detection. In addition, there are many endogenous polysaccharides in the organism to interfere with analysis of polysaccharide drugs. These offer great obstacle in pharmacokinetic study of polysaccharide drugs. In this chapter, a reliable HPLC with pre-column derivatization and post-column fluorescence derivatization methods was developed for the determination of the M/G ratio of PSS and microanalysis of PSS in rat plasma, respectively. These methods provide the basis of the quality control, qualitative, and quantitative analysis. Especially, the HPLC methods can be used for microanalysis of polysaccharide drugs in vivo and pharmacokinetic study.

2 The Pre-Column Derivatization Method for Analysis of M/G Ratio of PSS

Nuclear magnetic resonance (NMR) has been widely used in determining the M/G ratio of alginate (Heyraud et al. 1996; Buranaosot et al. 2009; Aida et al. 2010). However, it is not suitable for the analysis of sulfated alginate derivatives because the presence of sulfate groups affects the resolution of anomeric proton signals. The method for M/G ratio determination of alginate by anion exchange liquid chromatography (Gacesa et al. 1983) is also not suitable for the microanalysis of PSS, alginate sulfated derivatives, because the lactone forms of uronic acids interfere with the quantification of M and G.

A reversed-phase HPLC method using 1-phenyl-3-methyl-5-pyrazolone (PMP) as a pre-column derivatization reagent for the separation and analysis of M and G was developed by our group. This method is rapid, accurate, and sensitive for determination of the M/G ratio in PSS, and it is suitable for quality control and microanalysis of PSS.

The pre-column derivatization method refers to samples reacting with derivatization reagents into derivatives with chromophoric and fluorophoric groups in advance, following chromatographic separation and detection. This method mainly applies the sensitive ultraviolet detector (UVD) and the fluorophotomeric detector (FD).

2.1 Degradation of Samples

Polysaccharide degradation includes chemical degradation (such as $NaNO_2$ degradation, acid degradation, and oxidative degradation), physical degradation (ultrasonic method and radiative method), and biodegradation (specific enzyme hydrolysis and nonspecific enzyme hydrolysis) (Chen and Wu 2008).

For the determination of M/G ratio, PSS should be hydrolyzed into monosaccharides primarily. The average molecular weight of PSS is about 10 KDa, and it is usually hydrolyzed by chemical degradation methods. Wu et al. (2014) studied four methods of hydrolysis of PSS, including dilute sulfuric acid (0.1 mol L^{-1}) degradation (at 120 °C for 4 h), trifluoroacetic acid (TFA, 2 mol L^{-1}) degradation (at 110 °C for 6 h) (Wang et al. 2009), microwave degradation (1,600 W at 130 °C for 15 min) (Hu et al. 2013), and conventional acid hydrolysis method (with 80 % H_2SO_4 at 20 °C for 18 h and then 2 mol L^{-1} H_2SO_4 at 100 °C for 6 h) (Haug and Larsen 1962). Comparing with the results of ^{1}H-NMR analysis, the M/G ratios of PSS degraded by the microwave method or dilute sulfuric acid method were approximate with a low loss of uronic acid. But the microwave method showed the poorest repeatability. Dilute sulfuric acid hydrolysis was selected as the best method for the hydrolysis of PSS, with low uronic acid loss and good repeatability.

The dilute sulfuric acid hydrolysis method is carried out as follows: 600 μL PSS solution (8 mg mL^{-1}) is added in a sealed glass ampoule, adding 200 μL water and

200 μL dilute sulfuric acid (0.5 mol L^{-1}), hydrolyzed in 120 °C for 4 h, and then neutralized with 500 μL NaOH (0.4 mol L^{-1}) after it is cooled to room temperature. In the last, PSS hydrolysates are kept at −50 °C until used for derivatization and analysis.

2.2 Derivatization of Samples

Due to the strong polar, similar structure, and lack of optical activity in polysaccharide, gas chromatography (GC) was applied to determine the composition of polysaccharide previously. But more and more researchers focus on the HPLC method for the qualitative and quantitative analysis. Usually monosaccharide derivatization with ultraviolet or fluorophotomeric groups is required for separation and detection by HPLC (Bao et al. 2002; Xing et al. 2011). Currently, monosaccharide derivatization reagents include PMP (Honda et al. 1989), *p*-aminobenzoic acid (Hao et al. 2007), *p*-anisidine (Ma et al. 2012), 2-aminopyridine (Tomiya et al. 1987), and so on.

The majority of derivatization is based on the reductive amination reaction. In this process, acid was added to catalyze the reaction for a long time which prompted the growing unstable products and influenced the next analysis (Suzuki et al. 2001). PMP can react with reducing sugars in mild conditions without the addition of acid, and the derivative products have strong absorption in 245 nm. Therefore, PMP has been widely used in the analysis of polysaccharide composition.

Wang et al. (2009) optimized the derivatization conditions for PSS, PMS, and PGS, three marine drugs with different uronic acid components. With the development of analytical techniques, the derivatization method of PSS was further improved (Wu et al. 2014).

The PMP derivatization method is carried out as follows: 100 μL of 0.3 mol L^{-1} NaOH is added in 100 μL of PSS hydrolysate and the mixture is blended. One hundred twenty microliter methanolic solution of PMP (0.5 mol L^{-1}) is added and blended and then the final mixture is heated to 70 °C and incubated for 1 h. After the reaction mixture is cooled to room temperature, it is neutralized with 100 μL of 0.3 mol L^{-1} HCl and then extracted with chloroform (500 μL each) for four times. The aqueous layer is filtered through a 0.22 μm micron membrane filter before HPLC analysis.

2.3 HPLC Analysis of Samples

After PSS was hydrolyzed and derivatizated with PMP, it was separated at the optimal chromatographic conditions by HPLC to determine the M/G ratio. The M/G ratio of PSS was calculated by comparing the peak area values of M or G to each calibrated standard curve. Calibration curves of M and G were constructed by plotting the peak areas against the concentrations of their monosaccharide standards.

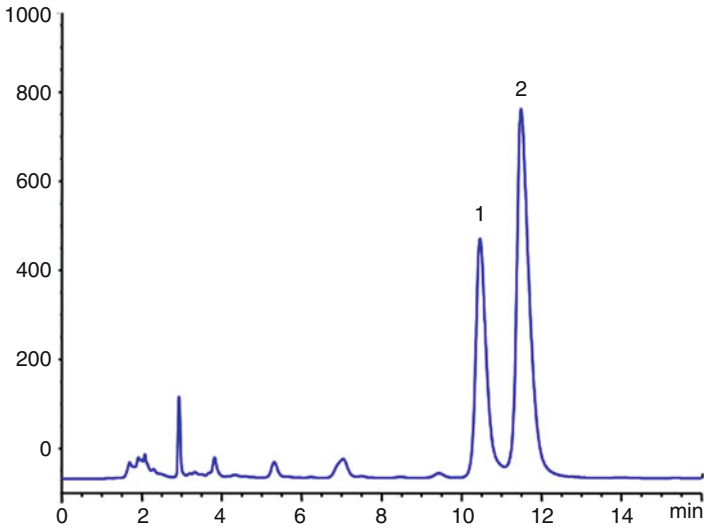

Fig. 2 The chromatogram of M/G ratio of PSS, 1-guluronic acid (*G*), and 2-mannuronic acid (*M*)

The separation conditions of M and G derivatives were investigated by changing the pH value of the phosphate buffer, the proportion of acetonitrile, the flow rate, and loading quantity of the sample (Wu et al. 2014). The chromatogram was shown in Fig. 2.

The optimal chromatographic separation of derivatives is carried out as follows: A HPLC system with UVD and a C18 column (150 × 4.6 mm, 5 μm) is used for HPLC analysis. Chromatographic separation of PMP derivatives is carried out using 0.1 mol L^{-1} phosphate buffer (pH = 7.0) and acetonitrile at a ratio of 83:17 (v/v, %) as a mobile phase at a flow rate of 0.8 mL min^{-1}. The temperature of the column is maintained at 30 °C and detected by UVD at 245 nm.

To demonstrate that the proposed HPLC method is optimal, we employed a desulfurization strategy (Falshaw and Furneaux 1998) to overcome the influence of the sulfate group on the resolution of anomeric proton signals. We determined the M/G ratio of PSS by the NMR technique using its intermediate (low-molecular-weight alginate) and desulfurization products. Results showed that the M/G ratios of PSS determined by the HPLC method were in good accordance with those obtained by the traditional nondestructive ^{1}H-NMR method using desulfurization products (Wu et al. 2014).

This showed that the HPLC method is accurate in determining the M/G ratio of PSS. The M/G ratio results for PSS samples that were collected from different manufacturers in China were shown in Table 1 (Wu et al. 2014). There were obvious differences in the M/G ratios for the ten PSS samples, which likely resulted from the differences in raw material alginates. This means it is necessary to strengthen the quality control of PSS.

Table 1 M/G ratios of PSS samples that were collected from different manufacturers in China

Sample	Peak area (average, mAu*min)		Peak area ratio	M/G ratio	RSD(%) ($n = 3$)
	G	M			
1	76.49	181.91	2.38	1.26	1.62
2	68.90	164.98	2.39	1.26	0.83
3	72.71	165.44	2.28	1.21	1.60
4	128.18	224.39	1.75	0.93	1.81
5	127.01	240.08	1.89	1.00	1.34
6	132.81	238.29	1.79	0.95	1.10
7	112.84	233.91	2.07	1.10	0.74
8	124.35	204.55	1.65	0.87	1.54
9	106.58	227.59	2.14	1.13	0.63
10	89.94	174.61	1.94	1.03	0.53

3 The Post-Column Derivatization Method for Pharmacokinetic Analysis of PSS

For pharmacokinetics of polysaccharide, the analytical methods include isotope labeling method (Laforest et al. 1991), immunoassay (Wang et al. 2011), bioassay (Mischke et al. 2012), chromatography (Lin et al. 2010), and mass spectroscopy (MS) (Pan et al. 2009). PSS possesses few chromophoric or fluorophoric groups. The sensitivity of analysis of PSS in biological samples by measuring the refractive, scattering indexes or using colorimetric assays is relatively poor. Due to the limitations of traditional analysis techniques and methods, there are not many suitable methods to study the pharmacokinetic characteristics of PSS. The labeling method of tritium (^{3}H) was firstly applied to early pharmacokinetic study of PSS (Liu et al. 1990). However, taking into account the radioactivity of ^{3}H, it was not applied to pharmacokinetic study in the human body. We also developed a fluorescent labeling method to microanalyze PSS and study its pharmacokinetic parameters with fluorescein isothiocyanate (FITC) (Li et al. 2014). The fluorescent labeling method was of high sensitivity and safety with relatively low cost. However, this method was complicated and time-consuming and also could not be applied to pharmacokinetic studies in human body.

The post-column derivatization method refers to add reagents into the liquid chromatographic effluent to increase detection sensitivity of sample bands or to enhance sensitivity with respect to interfering bands which overlap sample bands of interest, using one or more hollow fibers immersed within mobile reagent which is permeated through the walls of the fibers and, thus, ultimately diffused into the column effluent (Davis 1985). The post-column HPLC system was showed in Fig. 3. This method has advantages of convenience, good reproducibility, continuous reaction, and is easy to realize automatic analysis (Yang 2008).

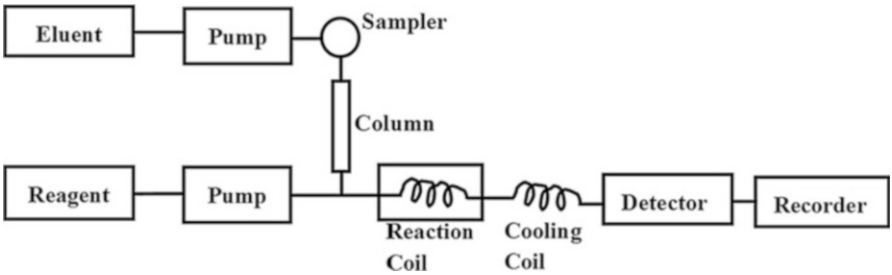

Fig. 3 The flow diagram of the post-column HPLC system

The post-column fluorescence derivatization reagents mainly include aliphatic amines, guanidines, amidines, and so on (Sun 2008). Nozal et al. employed post-column derivatization of carbohydrates with ethanolamine-boric acid prior to their detection by HPLC (Nozal et al. 1992). Coquet et al. realized trace level detection of reducing saccharides using HPLC by selective post-column fluorogenic reaction with benzamidine (Coquet et al. 1991). Sun et al. (2012) selected guanidine as a fluorometric reagent for the determination of oligosaccharide HS203 in beagle dog blood plasma by HPLC post-column fluorescence derivatization method. In 2013, Li et al. (2013) also chose guanidine hydrochloride as a post-column derivatizing reagent to detect PSS in rat plasma by HPLC method.

Based on the above research work, a highly sensitive and effective HPLC method with post-column fluorescence detection was developed to determine PSS in rat plasma. This method, using guanidine hydrochloride as the fluorescence derivatization reagent, was validated to have high accuracy, precision, and reproducibility. Plasma samples were prepared by a simple and fast ultrafiltration method. Isocratic chromatographic separation was performed on a TSKgel G2500PWxL column with the mobile phase of 0.1 M sodium sulfate. Analyte detection was achieved by FD at 250 nm (excitation, λex) and 435 nm (emission, λem).

3.1 Animal Administration and Sampling

Healthy Wistar rats (male and female, 1:1), 200–220 g in body weight, were used for the study of pharmacokinetics of PSS.

Rats were randomly divided into two groups, the oral administration group of PSS and the intravenous injection group of PSS. All the rats were fasted overnight prior to dosing. PSS was dissolved in sterile saline and administered to the rats by a single oral gavage (50 mg kg^{-1}) and a single injection via the tail vein (25 mg kg^{-1}).

The blood samples (about 0.3 mL) collected from the tip of the tail at predetermined time intervals up to 48 h after oral administration and 24 h after intravenous injection were placed into sodium citrate microcentrifuge tubes. The blood samples were centrifuged at 3,000 rpm for 15 min and the separated plasma (100 μL) was stored at −50 °C until analysis.

3.2 Pretreatment of Blood Sample

Except for the drugs, biological samples contain many endogenous substances, including protein, polypeptide, fatty acids, pigments, and lipids. These interfering substances with larger molecular weight cannot only plug the column (Masuda et al. 1996) but also influence the determination of drugs if the separation of drugs with these macromolecular substances is not good. Accordingly, pretreatment of biological samples (serum, tissue, urine, etc.) is deemed the foundation of pharmacokinetic study. Pretreatment of biological samples should fully consider the physicochemical properties, the chemical composition, the protein binding rate, the type of matrix interference, the stability, the pollution of pretreatment process, the suitable solvents of chromatographic system for the tested samples, and so on (Masuda et al. 1996; Danielson et al. 1999).

Pretreatment of biological samples is mainly to remove protein. In order to quantitatively analyze drugs in biological samples, the combination between protein and drugs must be destroyed and drugs can be collected as much as possible (Nojiri et al. 2000). Sun et al. (2012) carried out the ultrafiltration method to remove protein of oligosaccharide HS203 blood samples, established a simple and convenient method of oligosaccharide pharmacokinetics.

In 2013, Li et al. (2013) carried out the ultrafiltration method to remove protein of PSS blood samples, laying the foundation of polysaccharide pharmacokinetics. In the pretreatment procedure, PSS was released completely from the blood plasma and almost all plasma proteins were retained in Ultra-0.5 centrifugal filter (30 kDa, MWCO). The method involving centrifugal ultrafiltration was convenient, reproducible, and high recoverable, and the endogenous substances in the plasma did not interfere the detection of PSS in HPLC analysis. Since PSS is a kind of uronic acid derivative, D-glucuronic acid was selected as IS due to its high recovery, suitable retention time, and similar structure to the analyte.

This pretreatment of centrifugal ultrafiltration method is carried out as follows: To a 100 μL portion of plasma sample, 50 μL of water and 10 μL D-glucuronic acid (IS, 3.0 mg mL^{-1}) solution are added. Then the mixtures are vortexed for 3 min and transferred into a 30 KD Ultra-0.5 centrifugal filter device. After centrifugation at 12,000 rpm for 20 min, the aliquot (20 μL) of the supernatant is submitted to the HPLC system.

3.3 HPLC Analysis of Samples

To explore high-sensitivity detection method for PSS, the derivatization conditions were optimized, including the selections of columns and the fluorescence derivatization reagents, and the IS was carried out to determine the analytes in the plasma (Li et al. 2013).

The column selection was based on the properties of PSS and relevant literature of similar polysaccharide drugs. Based on size exclusion and distribution, TSKgel G3000PWxL and TSKgel G2500PWxL columns were tried, which are different in

the exclusion limits of molecular weights. The glucose in the plasma was separated excellently from PSS and IS by the TSKgel G2500PWxL column, so it was chosen as the analytical column. Sodium sulfate (0.1 M) was chosen as the appropriate mobile phase after testing several mobile phases, and the appropriate flow rate was established at 0.5 mL·min^{-1}.

Then benzamidine and guanidine hydrochloride were selected as fluorescence derivatization reagents as a check. The experiments showed that guanidine hydrochloride had high sensitivity compared with benzamidine. Therefore guanidine hydrochloride was used as the fluorescence derivatization reagent.

In order to improve the sensitivity of the method, three derivatization reaction factors were tested: the concentration of guanidine hydrochloride, the reaction temperature, and the concentration of sodium hydroxide. As a result, 0.1 M guanidine hydrochloride, 120 °C, and 0.5 M sodium hydroxide were chosen as the optimal reaction conditions.

The post-column HPLC analysis was performed on a Dionex UltiMate™ 3000 HPLC system, which was equipped with a pump (DGP-3600SD), an autosampler (WPS-3000SL), a column compartment (TCC-300RS), and a fluorescence detector (FD-3100). The chromatogram of blood sample was shown in Fig. 4.

The optimal chromatographic separation is carried out as follows: A HPLC system with FD and TSKgel G2500PWxL column is used for HPLC analysis. The chromatographic separation is performed on a TSKgel G2500PWxL column (300 × 7.8 mm, 7 μm) at 30 °C. The mobile phase is 0.1 mol·L^{-1} Na_2SO_4 at a flow rate of 0.5 mL min^{-1}. In the post-column procedure, a 0.5 mol L^{-1} NaOH solution containing 0.1 mol L^{-1} guanidine hydrochloride is passed through polytetrafluoroethylene (PTFE) reaction coil (0.5 mm i.d. × 10 m) thermostated at 120 °C and then the mixture is added to the column effluent at a flow rate of 0.3 mL min^{-1}. Subsequently, a 0.5 mol L^{-1} NaOH solution at a flow rate of 0.3 mL min^{-1} is added

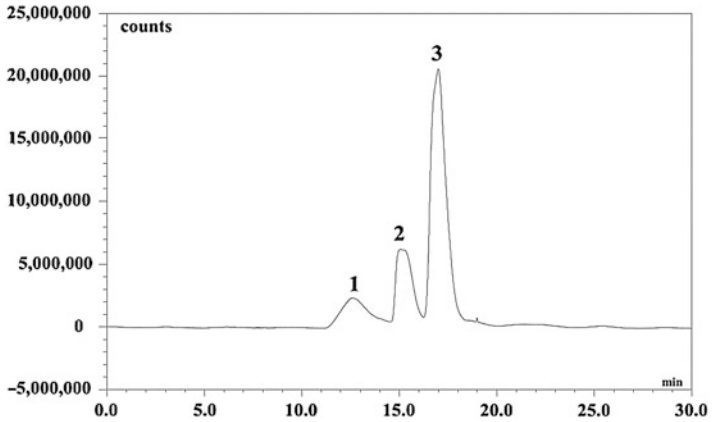

Fig. 4 The chromatogram of blood sample, 1-PSS, 2-D-glucuronic acid, and 3-glucose in the plasma

Table 2 Pharmacokinetic parameters ($n = 7$) of PSS after a single oral gavage (Ig) and a single intravenous injection (i.v.) of PSS, respectively

	PSS	
Pharmacokinetic parameters	i.v.	Ig
T_{max} (h)	–	0.50 ± 0.00
C_{max} (μg·mL^{-1})	1217.21 ± 361.74	22.91 ± 4.67
$T_{1/2}$ (h)	3.11 ± 0.62	35.96 ± 16.65
$AUC_{0\text{-}t}$ (h*μg·mL^{-1})	1315.52 ± 314.33	251.22 ± 30.25
Vd (mL·kg^{-1})	0.087 ± 0.021	5.59 ± 1.96
CL (mL*kg·h^{-1})	0.020 ± 0.0039	0.12 ± 0.032
MRT (h)	2.59 ± 0.71	55.96 ± 23.30
F (%)	9.55	

to cool down the mixture in another PTFE coil (0.25 mm i.d. × 3 m) with an ice bath. Fluorescence detection is carried out at λex of 250 nm and λem of 435 nm.

The validated method described above was successfully applied to the determination of PSS in rat plasma. Calculations were performed with the pharmacokinetic program PKSolver. The pharmacokinetic parameters of PSS were characterized and the limit of lowest detection (LLOD) was 250 ng mL^{-1} which indicated that this method was of high sensitivity. The pharmacokinetic parameters and concentration-time curve were shown in Table 2 and Fig. 5.

All the pharmacokinetic parameters in this study were similar to those obtained previously by the fluorescent labeling method (Li et al. 2014). Taking into account the simplicity of the method and the suitability for human pharmacokinetic study, the HPLC method with post-column fluorescence detection was shown to be the optimal method to determine PSS and to study its pharmacokinetic characteristics in biological samples.

4 Conclusion

With the deep development of chemical glycobiology, the research and development on carbohydrate-based drugs attract more and more attention, especially the bioactive polysaccharides. Because of the structure complexity and interference of endogenous glycan, the microanalysis of polysaccharide drugs in vivo and in vitro is a big challenge. Although the development of modern analytical techniques offers favorable support for pharmacokinetic study of polysaccharide, the current pharmacokinetics of polysaccharide is still lacking of comprehensive, systematic, and in-depth study. The reported study related with pharmacokinetics of polysaccharide is relatively few. The isotope labeling method is once most widely used, and biological method is also explored. Currently chromatography has been becoming more and more important in pharmacokinetics study with its sensitivity, simplicity, non-toxicity, and other characteristics. However, new suitable HPLC method is demanded for the microanalysis of polysaccharide drugs. In this chapter, HPLC with pre-column derivatization and post-column fluorescence derivatization

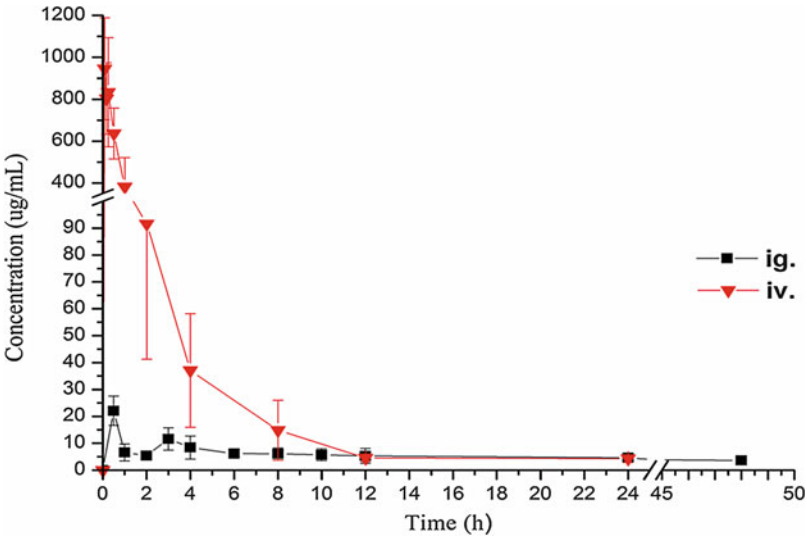

Fig. 5 The concentration-time curves of PSS in rats after a single oral gavage (ig) and a single intravenous injection (i.v.) of PSS, respectively. Each data point represents the mean ± sd ($n = 7$)

methods was developed for the determination of the composition of PSS and microanalysis of PSS in rat plasma. These methods provide the basis of the quality control, qualitative, and quantitative analysis for polysaccharide drugs.

But these methods cannot answer all the problems of microanalysis of polysaccharide drugs, especially the pharmacokinetics. Taking into account the accuracy sensitivity, simplicity of the methods, and the suitability for human pharmacokinetic study, innovative methods are still demanded for the detection and quantification of polysaccharide drugs.

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Determination of Must and Wine Polysaccharides by Gas Chromatography-Mass Spectrometry (GC-MS) and Size-Exclusion Chromatography (SEC)

43

Zenaida Guadalupe, Belén Ayestarán, Pascale Williams, and Thierry Doco

Contents

Abstract

Polysaccharides are one of the major classes of macromolecules found in wines. They play a critical role in stabilizing other molecules in solution and thus are able to modify both the wine processing and organoleptic properties. Detailed analyses of these polysaccharides are essential to know their physicochemical properties and biological functions.

We present analytical techniques not only to define the fine chemical structures of individual wine polysaccharides but also to estimate the overall polysaccharide composition of must and wines. The procedure covers the preparation of the sample, together with gas chromatography-mass spectrometry-based methods, for both the analysis of monosaccharides as their volatile trimethylsilyl methyl glycoside derivatives and methylation analysis to determine linkage positions between monosaccharide residues as their volatile partially methylated alditol acetate derivatives. We also provide a protocol for estimating the global

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content of wine polysaccharides by using size-exclusion chromatography with refractive index detector (SEC-RID). This is a rapid and simple method that can be used routinely in a reasonably equipped laboratory. Analysis complexity and time will vary depending on the method used, and the duration ranges from 2 days for a global polysaccharide estimation to 2 weeks for a carboxyl reduction/methylation linkage analysis.

Keywords
Wine • Polysaccharides • PRAG • Arabinogalactans • RG-II • Glucans • Mannoproteins • Methylation • GC-MS • SEC

1 Introduction

Polysaccharides are one of the main groups of macromolecules in wines. They can be present in concentrations ranging from 200 to 1,500 mg L^{-1} depending on winemaking stage, winemaking practices, vintage, variety, and *terroir*.

Wine polysaccharides originate both from grapes and microorganisms acting during the winemaking. Major wine polysaccharides include (i) polysaccharides rich in arabinose and galactose (PRAG), which comprise arabinans, arabinogalactans, and arabinogalactan proteins (AGP) and originate from grape berry cell walls; (ii) rhamnogalacturonans types I and II (RG-I and RG-II), which also arise from grape berry cell walls; and (iii) mannoproteins (MP) and glucans (GL) which are released by yeast during fermentation and during the aging of wines on lees. Therefore, Vidal et al. (2003) found that the polysaccharides from a Carignan Noir wine were composed of 42 % AGP, 35 % MP, 19 % RG-II, and 4 % RG-I, and Guadalupe and Ayestarán (2007) found values of 50 % AGP, 30 % MP, and 15 % RG-II in young red Tempranillo wines and 37 % AGP, 45 % MP, and 15 % RG-II in Tempranillo wines aged in oak. In a recent study, values of 35 % PRAG, 35 % MP, and 25 % GL were found for white and rosé sparkling wines made from different grape varieties (Martínez-Lapuente et al. 2013).

Wine polysaccharides play a critical role in stabilizing other molecules in solution, preventing or limiting aggregation and flocculation and thereby haze formation (Waters et al. 1994; Moine-Ledoux and Dubourdieu 1999; Dupin et al. 2000; Lomolino and Curioni 2007; Schmidt et al. 2009) and tartrate salts crystallization (Gerbaud et al. 1997; Moine-Ledoux and Dubourdieu 2002). Wine polysaccharides have also been described for their detrimental role in filterability (Belleville et al. 1991, 1992; Vernhet et al. 1999), their influence on the fermentation flora (Guilloux-Benatier et al. 1995; Guilloux-Benatier and Chassagne 2003), and their interaction with aromatic compounds (Dufour and Bayonove 1999; Chalier et al. 2007) and other molecules responsible for wine flavor, color, and foam (Riou et al. 2002; Vidal et al. 2004; Poncet-Legrand et al. 2007; Martínez-Lapuente et al. 2013). However, it has been shown that not all polysaccharides have the same behavior with respect to wines. Their influence on wine processing and sensory properties will depend not only on their quantity but also on the type of

polysaccharide. It has been shown that AGP have greater influence on the filtration procedures than MP (Ribéreau-Gayon et al. 2006) which are more efficient at reducing protein haze in white wines (Waters et al. 1994; Moine-Ledoux and Dubourdieu 1999; Dupin et al. 2000). RG-II is a stronger accelerator of hydrogen tartrate crystallization than RG-I, accelerating crystallization at low concentrations and inhibiting it at high concentrations (Gerbaud et al. 1996). AGP, on the other hand, have no effect on this phenomenon (Ribéreau-Gayon et al. 2006). Among the MP classes present in wine, some have been found to act as protective factors with regard to tartrate salts precipitation (Gerbaud et al. 1997; Moine-Ledoux and Dubourdieu 1999), and it has also been described that MP can adsorb toxic ochratoxin A from wines (Bejaoui et al. 2004; Baptista et al. 2004; Ringot et al. 2005; Caridi et al. 2006) and that the dimer RG-II /boron can form complexes with di- and trivalent cations which could reduce the level of toxic cations in wines (Pérez et al. 2003). There are also important differences with regard to the effect of wine polysaccharides on the organoleptic properties of red wines. It has been shown that RG-II, MP, and AGP have different influences on the aggregation of proanthocyanidins (Riou et al. 2002; Mateus et al. 2004; Carvalho et al. 2006; Poncet-Legrand et al. 2007) and, therefore, have varied influences on the wine gustatory structure, fullness, and body (Vidal et al. 2004; Quijada-Morín et al. 2014). Interactions between aroma compounds and MP have also been described (Dufour and Bayonove 1999; Chalier et al. 2007). In the case of sparkling wines, some authors have correlated the foam properties of grape juices, base wines, and sparkling wines with the polysaccharide content (Andrés-Lacueva et al. 1996, 1997; López-Barajas et al. 1998a; Girbau-Sola et al. 2002a, b). A connection between the molecular weight and composition of polysaccharides and foaming characteristics has also been shown (Moreno-Arribas et al. 2000; López-Barajas et al. 2001), and some authors have identified the yeast MP released during autolysis as the molecules responsible for the improvement of foaming properties (Nuñez et al. 2005, 2006; Abdallah et al. 2010; Coelho et al. 2011). However, it has been shown that not all mannoproteins have the same behavior (Nuñez et al. 2006; Coelho et al. 2011). Moreover, MP plays other roles in sparkling wines since they contribute to the flocculation of yeast strains (Caridi 2006) and improve their elimination from the bottle during disgorging.

Considering all the potential effects of wine polysaccharides in both wine processing and quality, several attempts have been done during the last years in order to increase their content into the wines. The amount of polysaccharides released in red wines depends on the winemaking process, and low temperature techniques during the winemaking (cold prefermentative maceration, dry ice addition, grape skin freezing) have been applied in order to degrade the cell walls and achieve greater extraction of polysaccharides (Apolinar-Valiente et al. 2013, 2014). The efficiency of using commercial pectinases to increase the extraction of wine polysaccharides and oligosaccharides has also been studied (Ayestarán et al. 2004; Guadalupe et al. 2007; Doco et al. 2007; Ducasse et al. 2010a, 2011; Apolinar-Valiente et al. 2013). The effect of adding commercial mannoproteins or other yeast extracts and autolysates in the winemaking has also been studied in literature

(Comuzzo et al. 2006; Guadalupe and Ayestarán 2008; Pozo-Bayón et al. 2009; Guadalupe et al. 2010; Del Barrio-Galán et al. 2011). Moreover, many types of these products are currently used as technological adjuvants in the enological industry in order to increase the content of mannoproteins in the wines and thus improve their overall stability, modify the mouth-fell properties and color of red wines, and improve the aromatic intensity of white wines or the foam properties of sparkling wines. Taking into account the growing tendency in the use of these commercial products, different studies have focused on the evaluation of methodologies to increase the content of mannoproteins into the wines. The effect of the addition of mannoprotein overexpressing yeast strains during the alcoholic fermentation of still wines was analyzed by Guadalupe et al. (2007, 2010); thermosensitive autolytic mutants of *S. cerevisiae* made to increase the release of polysaccharides have been employed during the alcoholic fermentation (Giovani and Rosi 2007) and the secondary fermentation of sparkling wine production (González et al. 2003; Nuñez et al. 2005); and different technological approaches have been applied to accelerate the autolytic process during wine aging on lees (Doco et al. 2003a; Nuñez et al. 2005; Pati et al. 2010; Fernández et al. 2011).

In conclusion, many groups have focused their research on wine polysaccharides during the last decade, trying to isolate and characterize them; studying their evolution from grape to wine and during the winemaking and aging; analyzing their functions, mechanisms of action, and factors affecting their content; or trying to modulate their content into the wines. However, many of these researches have shown contradictory results, and there are still many aspects regarding polysaccharide structural characterization, functions, and mechanisms of action that still remain unknown. The difficulty in separating and purifying all of these compounds has resulted in them being less studied than other wine compounds such as polyphenols, polypeptides, or proteins. Therefore, the simple study of wine polysaccharides is difficult and time-consuming, and an adequate determination of the different polysaccharide families requires analytical methodologies which involve complex multistep procedures.

To determine the content of grape, must, or wine polysaccharides, all the methods proposed begin with an extraction step by direct precipitation with either ethanol acid, concentration-precipitation, dialysis, or ultrafiltration. After the extraction step, two alternatives can be chosen to analyze the polysaccharides in the extract: (i) using rapid and simple methods to estimate the global content of polysaccharides and (ii) using more complex and time-consuming methods to quantify specific families of polysaccharides. Direct quantification of wine polysaccharides is usually based on the precipitation of total wine colloids, followed by traditional colorimetric assays (Segarra et al. 1995) or by the determination of peak areas by size-exclusion chromatography (Dubourdieu et al. 1986; López-Barajas et al. 1998b; Guadalupe et al. 2012). However, these global methods do not allow the quantification of the different families of polysaccharides present in wines, which can only be estimated by assessing its monosaccharide profile. Several methods have been proposed for the identification and quantification of grape and wine monosaccharides: high-performance anion-exchange chromatography with

pulsed amperometric detection (HPAEC-PAD) (Arnous and Meyer 2009), Fourier transform infrared spectroscopy (FTIR) (Coimbra et al. 2005; Boulet et al. 2007), and gas chromatography (GC). When GC is used, two different detectors have been used: flame ionization detector (FID) and mass spectrometry detector (MS). Undoubtedly, gas chromatography coupled with mass spectrometry (GC-MS) after hydrolysis and monosaccharide silylation is of general acceptance due to its high sensitivity combined with its ability to achieve efficient separation of complex mixtures and structural characterization.

We present in this chapter analytical techniques to quantify must and wine polysaccharides. We provide a simple and rapid protocol for estimating the global content of wine polysaccharides by using of size-exclusion chromatography with refractive index detector (SEC-RID). We also present more complex techniques not only to define the fine chemical structures of individual wine polysaccharides but also to estimate the overall polysaccharide composition of must and wines. The procedure covers the preparation of the sample, together with gas chromatography-mass spectrometry-based methods, for both the analysis of monosaccharides as their volatile trimethylsilyl methyl glycoside derivatives and methylation analysis to determine linkage positions between monosaccharide residues as their volatile partially methylated alditol acetate derivatives. Firstly, an understanding of the structure of the different polysaccharide families is essential.

2 Wine Polysaccharides: Origin and Structure

Wine polysaccharides are grouped in two families according to their origin: those originating from grape berry cell walls and those released by microorganism, which include yeast, bacteria, and fungal grape contamination such as *Botrytis cinerea*. Exogenous polysaccharides such as arabic gum and carboxymethyl cellulose could also be present in several commercial wines as they are authorized as additives. This origin diversity leads to polysaccharide families that are different in composition and structure, and a deep knowledge of their composition is essential for their quantification. Hence, during the past decade, many works have focused on the isolation and structural characterization of polysaccharides from different must and wine matrices.

Grape polysaccharides include polysaccharides rich in arabinose and galactose (PRAG), which comprise arabinans, type I arabinogalactans and type II arabinogalactan proteins (AGP), rhamnogalacturonans type I (RG-I) and type II (RG-II), and homogalacturonans (HL). These polysaccharides are released from the pectic network of berry cell walls under the action of several endogenous and exogenous enzymes during the earlier stages of winemaking (Guadalupe and Ayestarán 2007). The cell wall of grape skins is built of three general layers: (i) the middle lamella, which binds the cell together and is mainly composed of pectin, (ii) the primary cell wall, and (iii) the secondary cell wall. Figure 1 shows a model of a primary plant cell wall, which consists of several interconnected matrices composed of structural proteins and polysaccharides, which make up about 90 % of the dry weight of primary cell walls. Such matrices include a

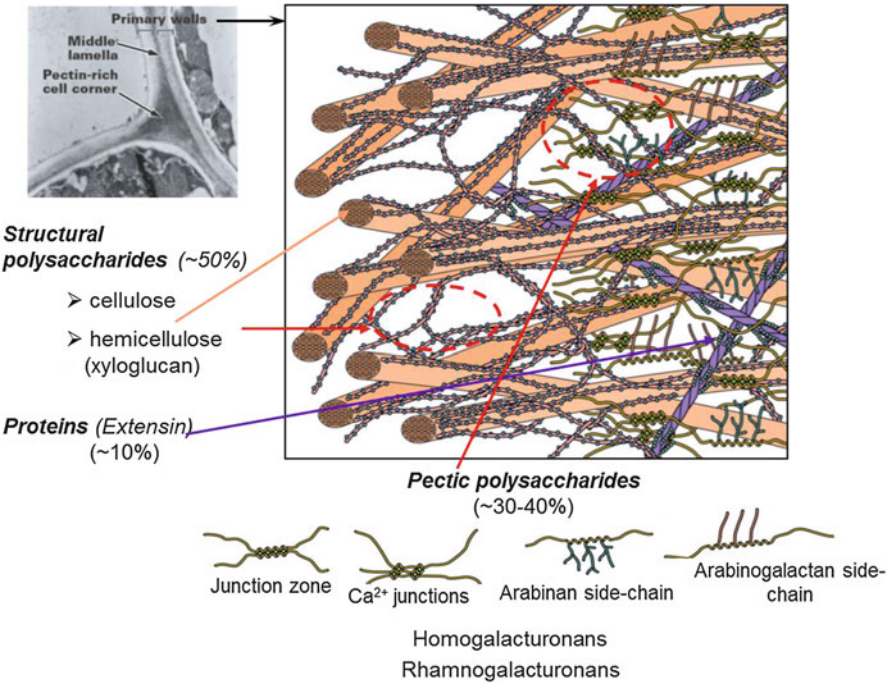

Fig. 1 Model of a primary plant cell wall. The percentage of the dry weight of each compound is shown

cellulose and associated hemicelluloses framework that is embedded in a matrix of the pectin polysaccharides composed of homogalacturonan, rhamnogalacturonan II, and rhamnogalacturonan I, with a diverse range of arabinogalactan and arabinans side chains (Vidal et al. 2003). The secondary cell wall is largely made of cellulose microfibrils (40–80 %) and also contains hemicelluloses and some lignin (Pinelo et al. 2006).

Cellulose is composed of α-(1–4)-linked D-glucose. In grape cell walls, hemicellulosic polysaccharides consist mainly of xyloglucans whose structures are based on a β-(1–4) D-glucan backbone in which about 75 % of the glucose residues carry β-D-Galp-(1 → 2)-α-D-Xylp(1 → 6) or α-L-Fucp(1 → 2)- β-D-Galp-(1 → 2)-α-D-Xylp(1 → 6) side chains (Doco et al. 2003b; Pinelo et al. 2006). Although these structural glucosyl polysaccharides are major in both the skin and pulp cell wall of grape berries (Vidal et al. 2001), and they are found in important amounts in must samples (Guadalupe and Ayestarán 2007), they are detected in very low quantities in wine samples as they precipitate during the winemaking (Guadalupe and Ayestarán 2007). Homogalacturonans, which are linear chains of α-(1–4)-linked D-galacturonic acid (Fig. 2), can make up to 80 % of the pectic polysaccharides in grapes, but they are also present in wines in very low amounts (Guadalupe and Ayestarán 2007) as they are probably fragmented by polygalacturonases during winemaking (Vidal et al. 2001). RG-I molecules are characterized

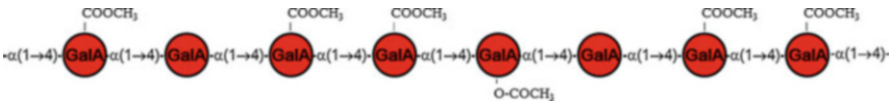

Fig. 2 Chemical structure of the homogalacturonan (HL). *GalA* galacturonic acid

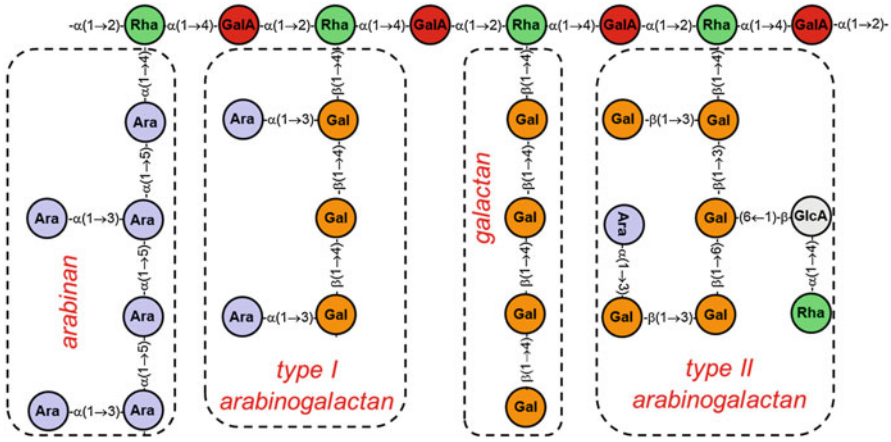

Fig. 3 Chemical structure of the type I rhamnogalacturonan (*RG-I*). *Ara* arabinose, *Gal* galactose, *GalA* galacturonic acid, *GLcA* glucuronic acid, *Rha* rhamnose

by a backbone of alternating galacturonic acid and rhamnose residues and side branches that are arabinans, galactans, and arabinogalactans (Fig. 3). These polysaccharides are also abundant in grape berries, but they are also insignificant in wines due to their poor solubility (Vidal et al. 2001). RG-I isolated from grapes show mainly arabinans and type II arabinogalactan side chains (Vidal et al. 2001). However, RG-I isolated from wines, with molecular weights ranging from 45 to 50 kDa, contain side chains of type I and II arabinogalactans and only a small proportions of arabinans (Vidal et al. 2003). RG-II structure is largely conserved along plant species and consists of a short (1 → 4)-α-D-galacturonan backbone branched with four different side chains containing primarily rhamnose, arabinose, and galactose and also different rare sugars such as aceric acid or 3-*C*-carboxy-5-deoxy-L-xylose, apiose, Dha or 3-deoxy-D-lyxo-2-heptulosaric acid, Kdo or 3-deoxy-D-manno-octulosonic acid, and 2-*O*-methyl-fucose and 2-*O*-methyl-xylose (Fig. 4), which allow for its identification and quantification. RG-II, which accounts for less than 3 % of the berry cell wall (Vidal et al. 2001), is one of the major polysaccharides in musts and wines (Vidal et al. 2003; Guadalupe and Ayestarán 2007), where it is mainly found in the form of dimmers cross-linked by borate-diol esters, with an average molecular weight of around 10 kDa. As previously described, arabinans and arabinogalactans are side chains of the RG-I backbone arising from their degradation by pectinases. Type I arabinogalactans have a backbone of (1 → 4)-β-D-galactan and are not present in wine polysaccharides

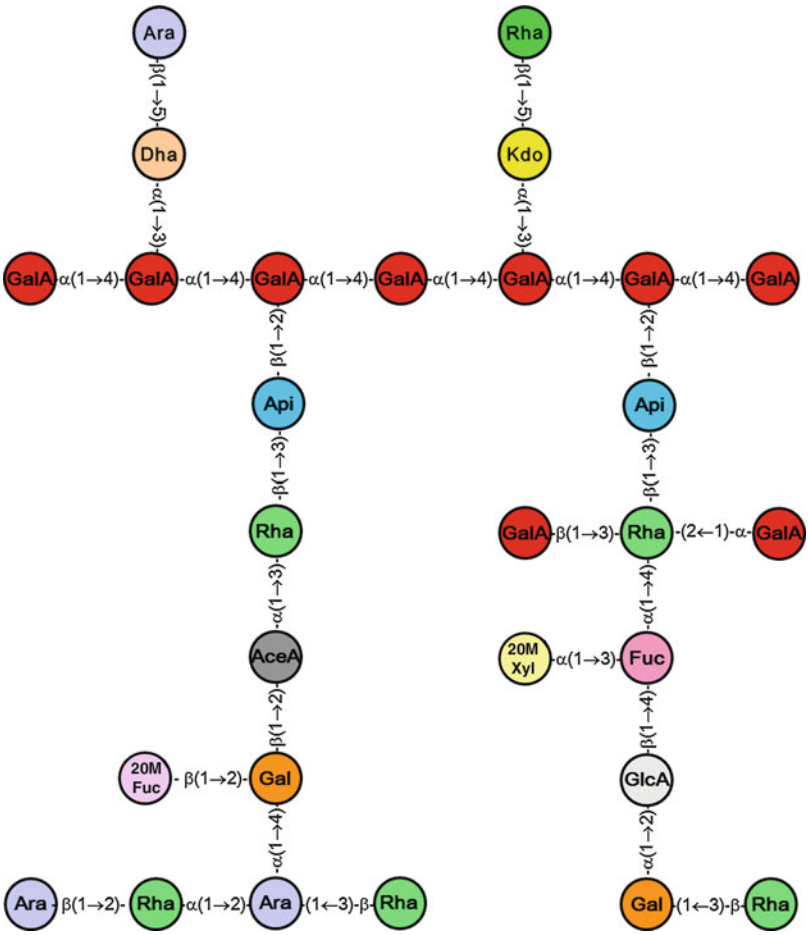

Fig. 4 Chemical structure of the type II rhamnogalacturonan (*RG-II*). *AceA* aceric acid (3-C-carboxy-5-deoxy-L-xylose), *Ara* arabinose, *Api* apiose, *Dha* 3-deoxy-D-*lyxo*-2-heptulosaric acid, *Fuc* fucose, *Gal* galactose, *GalA* galacturonic acid, *GlcA* glucuronic acid, *Kdo* 3-deoxy-D-manno-octulosonic acid, *Rha* rhamnose, *2OMFuc* 2-*O*-methyl-fucose, *2OMXyl* 2-*O*-methyl-xylose

(Doco and Williams 2013). Type II arabinogalactans are in fact arabinogalactan proteins (AGP) and represent more than 35 % of total red wine polysaccharides (Pellerin et al. 1995; Vidal et al. 2003; Guadalupe and Ayestarán 2007). Their common structural feature is a (1 → 3)-β-D-galactan backbone with (1 → 6) linked β-D-galactan side chains highly substituted by arabinofuranosyl residues (Fig. 5). Typical AGP isolated from wines commonly contain less than 5 % protein and show a molar mass from 50 to 260 kDa (Pellerin et al. 1995; Vidal et al. 2003; Doco and Williams 2013). AGP are localized in soluble forms within the cell walls and can be membrane anchored although this has not been demonstrated in the cell wall of grape berries (Doco and Williams 2013).

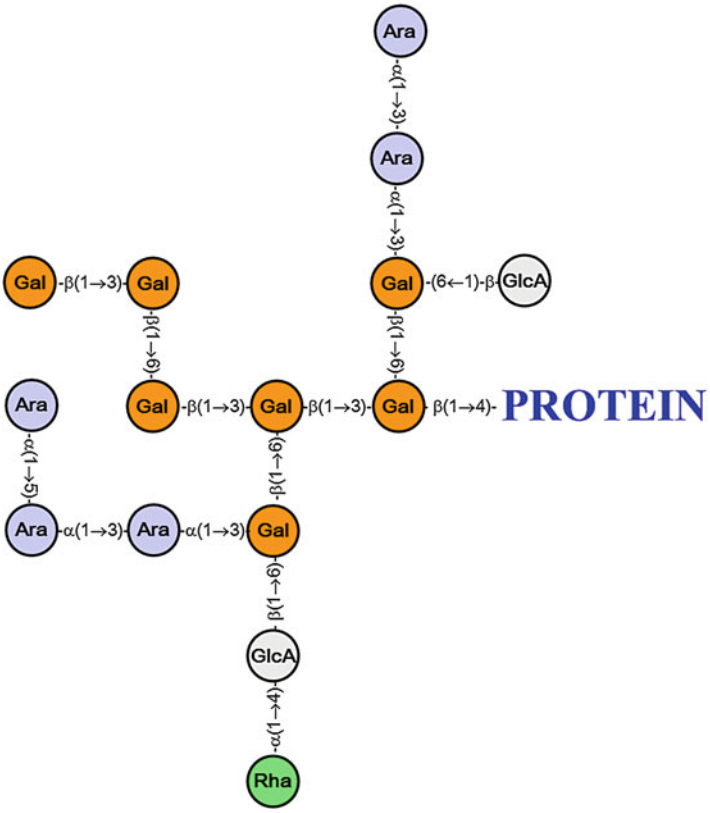

Fig. 5 Chemical structure of the type II arabinogalactan or arabinogalactan protein (*AGP*). *Ara* arabinose, *Gal* galactose, *GalA* galacturonic acid, *GLcA* glucuronic acid, *Rha* rhamnose

Polysaccharides from yeasts include mannans and mannoproteins (MP) and glucans (GL). They arise from the cell wall of *Saccharomyces cerevisiae*, and they can account for up to 90 % of the dry weight of their cell wall (Fig. 6). Mannoproteins produced by yeasts are the second most abundant family of polysaccharides in wine (Vidal et al. 2003; Guadalupe and Ayestarán 2007). These polysaccharides, which can account for up to 50 % of the cell wall dry mass of *S. cerevisiae*, are located in the outermost layer of the cell wall, where they are linked by β-1,3 glucan chain to the innermost, fibrous layer formed of β-1,3 glucan chains and chitin (Klis et al. 2006). These polymers, with highly variable sizes, are glycoproteins, often highly glycosylated, with carbohydrate fractions consisting mainly of mannose (>90 %) and glucose (Fig. 7). Two classes of mannoproteins have been described in wines. The first group is released by the action of a β-1,3 glucanase upon the wall during aging of wine on lees in the presence of nonmultiplying cells (autolysis); the second group is released by yeast when actively growing during alcoholic fermentation of grape must. These last compounds, with highly variable sizes from 5 to more than 800 kDa (Doco et al. 2003a),

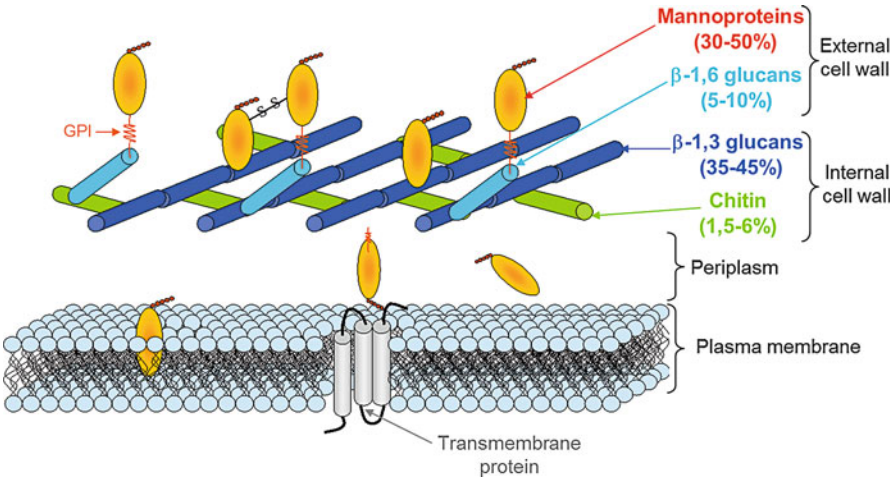

Fig. 6 Model of a yeast cell wall. The percentage of the dry weight of each compound is shown

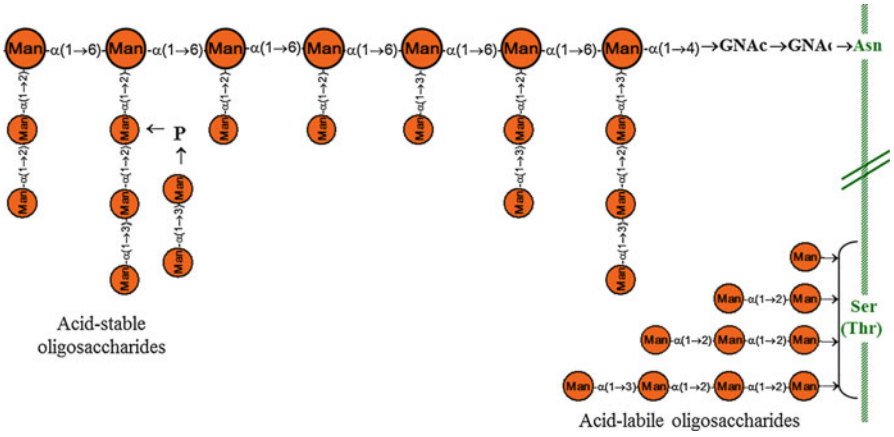

Fig. 7 Chemical structure of yeast exocellular mannoproteins. *Asn* asparagine, *GNAc N*-acetyl-glucosamine, *Man* mannose, *P* phosphate, *Ser* serine, *Thr* threonine

are similar to those from yeast cell walls, except for a lower protein content (Saulnier et al. 1991; Rosi et al. 2000). Glucan molecules are polysaccharides of D-glucose monomers linked by β-(1 → 3) and β-(1 → 6) glycosidic bonds, the β-1,3 glucans being the most abundant. These compounds, with a molecular weight ranging from 25 to 270 kDa (Klis et al. 2006), are only major polysaccharides in sparkling wines (Martínez-Lapuente et al. 2013) and wines aged on lees (Fernández et al. 2011).

3 Methods of Sample Preparation

In this section, we will only describe the methodologies for obtaining polysaccharides from musts, juices, or wines. We will not present the protocols carried out to obtain the polysaccharides from the cell walls of grape berries, which have already been described recently by other authors (Apolinar-Valiente et al. 2010).

Many studies have dealt with soluble polysaccharides present in must, grape juices, or wines, but they differ in the procedures used for the preparation of the samples, polysaccharide recovery, and the analytical methods for the quantification of polysaccharides (Table 1). According to the most often reported procedures, musts are usually obtained at the laboratory scale by grinding berries in a Waring commercial blender or from industrial processes in wineries where free-run juices are obtained by crushing or pressing grapes. Wines are usually obtained from wineries or experimental wine cellars and sometimes from microvinifications carried out in research laboratories. Moreover, polysaccharides have been isolated either from turbid and clarified musts, even during fermentation, or from wines at all stages of the winemaking process: during alcoholic fermentation, post-maceration, malolactic fermentation, wine aging in oak, wine aging in bottles, wine aging on lees, or second fermentations in sparkling wines.

3.1 Isolation of Polysaccharides from Musts and Wines

To determine the content of must or wine polysaccharides, all the methods begin with an extraction step by direct precipitation with ethanol acid, concentration-precipitation, dialysis, or ultrafiltration.

The ultrafiltration membranes covering a wide range of molecular weight cutoffs (MWCO) that overlap the molecular weight distribution of wine polysaccharides make this operation particularly appropriate to separate the wine polysaccharides. Applications of ultrafiltration membranes in enology have been described in the review of El Rayess et al. (2011). However, dialysis is a method of choice in comparison to the ultrafiltration methods because there is no problem of membrane fouling which affects the operating costs and limits the application of the filtration membranes. Whether for dialysis or ultrafiltration membranes, the most important parameter is the pore size of the membrane, whose MWCO should be at around 10 kDa to retain the carbohydrate macromolecules and remove small sugars, salts, monomeric polyphenols, etc.

The most common method used to extract polysaccharides in must and wine samples are the alcohol precipitation in an acidic medium (Segarra et al. 1995; López-Barajas et al. 1998b; Vidal et al. 2003; Ayestarán et al. 2004; Doco et al. 2007; Guadalupe et al. 2007, 2012; Apolinar-Valiente et al. 2014). Factors affecting the yield of polysaccharide precipitation have been studied in detail by Ayestarán et al. (2004) and Guadalupe et al. (2012), who optimized the conditions regarding the temperature and time of precipitation. Moreover, the concentration of

Table 1 Principal references concerning the extraction of polysaccharides from musts and wines and their quantitative methods of analysis

Origin of samples	Polysaccharide extraction	Quantitative method	References
Winery	Ethanol precipitation	Gravimetry	Usseglio-Tomasset (1976)
Waring blendor	Chemical extraction	*m*HDP and carbazole	Robertson (1979)
Heat treatment			
Winery	Concentration and dialysis	GC analysis of aldonitrile acetate and carbazole	Villetaz et al. (1982)
Must in anaerobic conditions	Ethanol precipitation and chemical extraction	GC analysis of the alditol acetates and *m*HDP	Mourgues et al. (1984)
Winery	None	HPSEC	Dubourdieu et al. (1986)
Waring blendor	Discoloration on CC6 column	Phenol, GC of alditol acetates and *m*HDP	Brillouet (1987)
	Dialysis		
Winery	Ethanol precipitation	Phenol and *o*HDP	Segarra et al. (1995)
Winery and during fermentation	Discoloration on CC6 column	GC of alditol acetates and *m*HDP	Doco et al. (1996)
	Dialysis	HPSEC	
Winery	Ethanol precipitation	Phenol and *o*HDP	López-Barajas et al. (1998b)
		HPSEC	
INRA experimental station	Ethanol precipitation	HPSEC	Vidal et al. (2003)
		GC-MS of TMS	
Winery	Ethanol precipitation	HPSEC	Guadalupe and Ayestarán (2007)
		GC-MS of TMS	
INRA experimental station	Ethanol precipitation	HPSEC	Doco et al. (2007)
		GC of alditol acetates	
CIVC (Epernay)	Successive ultrafiltrations	GC of alditol acetates	Abdallah et al. (2010)
		NMR spectroscopy	
INRA experimental station	Discoloration on CC6 column	HPSEC	Ducasse et al. (2010a)
	SEC column	GC of alditol acetates and GC-MS of TMS	
Winery	Ethanol precipitation	HPSEC	Guadalupe et al. (2012)
		GC-MS of TMS	
Dois portos experimental station	Successive ultrafiltrations	Phenol	Resende et al. (2013)
	Ethanol precipitation		

the sample prior the precipitation with ethanol acid was found to be critical to ensure the quantitative precipitation of all soluble polysaccharides since some polysaccharide families such as RG molecules or HL precipitated only partially in non-concentrated samples. They concluded that it was necessary to concentrate must samples three times and wine samples five times to ensure the adequate extraction of all polysaccharide families. Therefore, wine samples are firstly concentrated five times under reduced pressure or using centrifugal evaporators. Thereafter, precipitation of polysaccharides is carried out by adding four volumes of cold 96 % ethanol containing HCl (0.3 M), and samples are kept for 18 h at 4 °C. Samples are centrifuged, the supernatants discarded, and the pellets obtained washed by absolute ethanol several times to remove interfering materials. The precipitates are finally dissolved in ultrapure water and freeze-dried. These precipitates contain the total polysaccharide extract.

Although the methodology described above constitutes a simple and rapid procedure to obtain polysaccharides and it has shown very good values of repeatability in both wine and must samples, i.e., 3.02 % and 2.89 %, respectively (Ayestarán et al. 2004), other chemical compounds present in the samples can complicate the further analysis by GC-MS. In general, the complexity of the method used to obtain the polysaccharide extract will depend on the type of starting material and on the purpose of the analysis. For musts or grape juices, a preliminary step is usually carried out to remove the great excess of sucrose present, and total colloids are firstly recovered from musts or juices by different extraction procedures such as dialysis or ultrafiltration membranes (Vidal et al. 2000). For red wine samples, and depending on the aim of the analysis, the polysaccharide extract (or directly the wine prior the precipitation step) has to be depigmented to remove the interference of anthocyanins. It is important to take into account that the amount of glucose residues present in the extract originates not only from the polysaccharides but also from other molecules such as condensed anthocyanins, which are present in red wines in quite important amounts. Therefore, if we want to use the content of glucose to estimate the content of glucans, a previous depigmentation by decolorization of the sample is needed, which can be achieved by the use of polyamide columns. Finally, our objective could be to achieve a further separation of polysaccharides from oligosaccharides or even a separation of the different polysaccharide families. In this sense, a method has been recently proposed in order to isolate any complex carbohydrate present in musts, juices, or wines. Firstly, the *contaminant molecules* are removed by a separation mechanism mainly based on hydrogen bonds, and then the polysaccharides are fractionated according to their molecular sizes (Ducasse et al. 2010a). Wines are first partially depigmented onto a column of NN polyamide SC6 previously equilibrated with NaCl 1 M. Neutral and acidic polysaccharides and oligosaccharides are eluted with 2 bed volumes of 1 M NaCl, while polyphenolic materials are retained in the column. The eluted fraction is then concentrated under vacuum. Polysaccharides can be then separated in different fractions according to their molecular weight by high-resolution size-exclusion chromatography on a Superdex 75-HR column (1.3 × 30 cm) or a Superdex-30 HR column (1.6 × 60 cm) equilibrated at 1 mL min^{-1} with 30 mM

ammonium formiate pH 5.6. The elution of polysaccharides is followed by refractive index detector, and the different polysaccharide fractions (Ayestarán et al. 2004; Guadalupe and Ayestarán 2007) or oligosaccharide fractions (Ducasse et al. 2010a) are collected according to their elution times (Fig. 8). The isolated fractions are freeze-dried, redissolved in water, and freeze-dried again for three times to remove the ammonium salt.

3.2 Purification of the Different Families of Wine Polysaccharides

The isolation and purification of the different families of wine polysaccharides (PRAG, RG-I, RG-II, MP, and GL) is essential to know their chemical structures and to determine their physicochemical properties. However, these studies are limited due to the diversity of wine polysaccharides, their relatively low concentration, the recovery of sufficient amounts, and the time-consuming methods needed to obtain purified and well-defined polysaccharides.

Most methodologies to obtain purified wine polysaccharides have been based on gel permeation chromatography, ion-exchange chromatography, or affinity-exchange chromatography techniques alone or in combination. Few studies describe the fractionation and purification of *pure* polysaccharides (Villetaz et al. 1981; Llaubères et al. 1987; Brillouet et al. 1990; Doco and Brillouet. 1993; Pellerin et al. 1993, 1995, 1996; Waters et al. 1994; Ciezack et al. 2010; Vidal et al. 2003; DOCO and Williams 2013. Vidal et al. (2003) summarize the techniques of fractionation and purification to obtain a collection of wine polysaccharides in sufficient amounts to allow the determination of their structural characterization and intrinsic properties (Fig. 9).

The most common method used to purify polysaccharides is size-exclusion chromatography (SEC), in which macromolecules in solution are separated by their size and, in some cases, their molecular weight. Sephacryl-S400 HR or Sephacryl-S200 HR (cross-linked copolymer of allyl dextran and *N*,*N*′-methylene bisacrylamide) gel permeation columns equilibrated in 50 mM sodium acetate buffer pH 5 are used to obtain different fractions of wine polysaccharides. However, in most cases, a first step of anion-exchange chromatography is performed before SEC in order to separate charged and non-charged wine polysaccharides. Briefly, in cation-exchange chromatography, positively charged molecules are attracted to a negatively charged solid support, while in anion-exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. To separate polysaccharides of musts, juices, and wines, the anion-exchange chromatography techniques are used with different medium gels. In Vidal et al. (2003), the support was a Fractogel EMD DEAE 650 (M) equilibrated with 50 mM sodium citrate buffer pH 4.6. An unbound fraction was recovered, and the bound polysaccharides were eluted by stepwise gradient of NaCl (10, 50, 150, and 250 mM in the starting buffer). For some

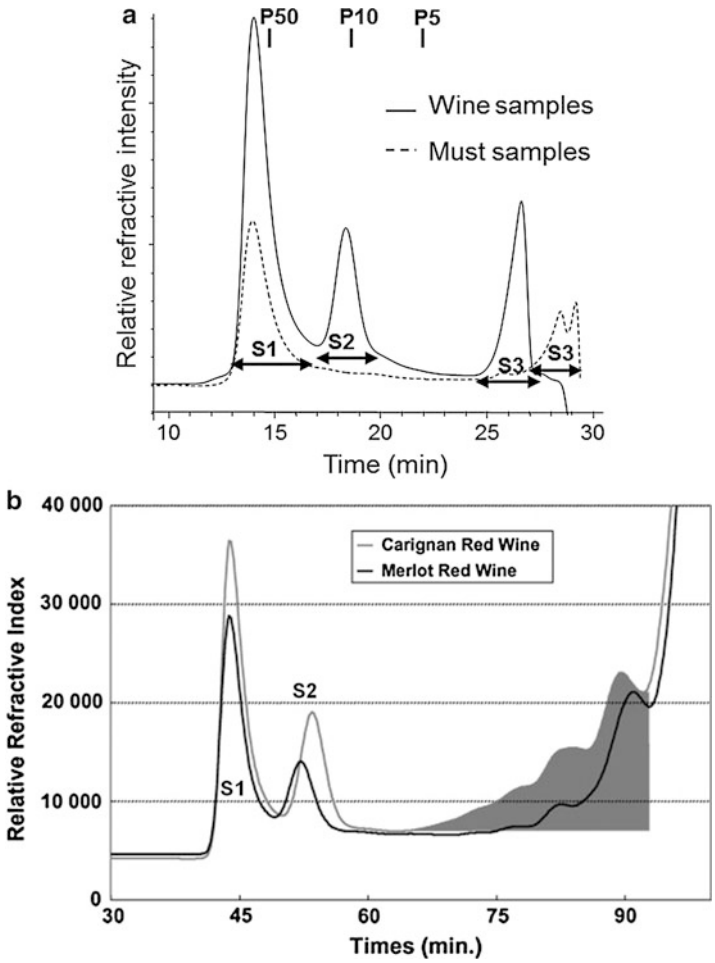

Fig. 8 Fractionation by high-resolution size-exclusion chromatography of polysaccharide and oligosaccharide fractions on (**a**) Superdex 75-HR columns and (**b**) Superdex-30 HR columns. (**a**) Molecular weight distributions of fractions S1, S2, and S3 isolated from Tempranillo wines and musts by SEC-RID on a Superdex 75-HR column (Guadalupe and Ayestarán 2007). Elution times of pullulan standards are also shown (P-5, Mw = 5.9 kDa; P-10, Mw = 11.8 kDa; P-50, Mw = 47.3 kDa). Fraction S1 corresponds to AGP and MP with an average molecular weight of 110 kDa; fraction S2 corresponds mainly to RG-II dimers with an average molecular weight of 10 kDa and smaller AGP and MP; fraction S3 corresponds to HL, monomeric RG-II, and low-molecular-weight fragments of AGP and MP. (**b**) Molecular weight distributions of fractions isolated from Carignan and Merlot red wines by SEC-RID on a Superdex-30 HR column (Ducasse et al. 2010b.). The hatched area corresponds to the oligosaccharide fraction and the non-hatched to the polysaccharide fraction. Fraction S1 corresponds to AGP and MP with an average molecular weight of 110 kDa; fraction S2 corresponds mainly to RG-II dimers with an average molecular weight of 10 kDa and smaller AGP and MP

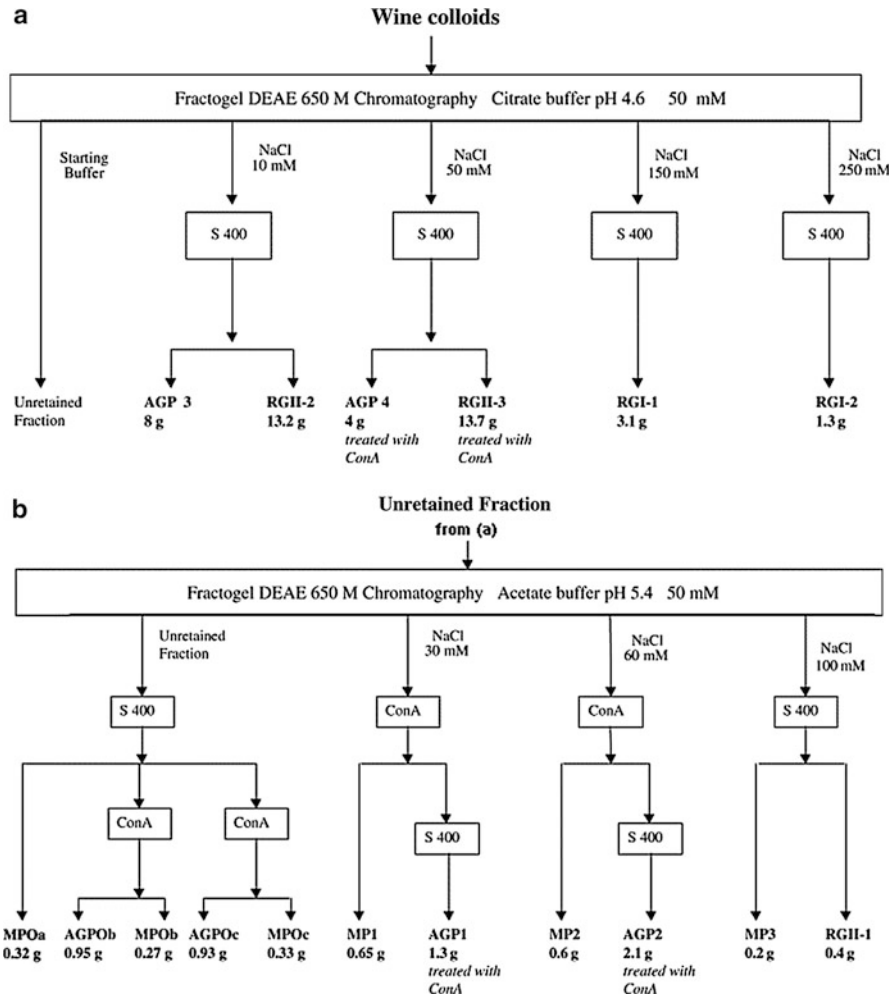

Fig. 9 Scheme of fractionation of the totality of wine polysaccharides by anion-exchange, affinity, and size-exclusion chromatography according to Vidal et al. (2003)

polysaccharides, such as rhamnogalacturonans, the steps of ion-exchange and gel permeation chromatography are sufficient to obtain purified polysaccharides. But for others like PRAG or MP, these two steps are not sufficient, and further separation by affinity-exchange chromatography is needed. Mannoprotein fractions can be obtained using their ability to bind to concanavalin A lectin immobilized on a Sepharose column. Concanavalin A lectin binds molecules that contain α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues with available C-3, C-4, or C-5 hydroxyl groups. Therefore, mannoprotein fractions are loaded on the column equilibrated in 50 mM sodium acetate buffer pH 5.6 containing 150 mM NaCl, 1 mM $NaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The desorption of the wine

mannoproteins is carried out using two bed volumes of the same buffer containing 100 mM of methyl-α-D-mannopyranoside.

The comprehensive fractionation of wine polysaccharides described by Vidal et al. (2003) has allowed obtaining the major classes of polysaccharides present in musts and wines, including xyloglucans, mannoproteins, arabinans and type I and II AGP, and type I and II rhamnogalacturonans. The characterization performed on the purified, isolated polysaccharides in order to know their chemical fine structure by the analysis of the monosaccharide composition and glycosidic linkages by GC-MS is described below.

4 Methods of Analysis

In general, the method used will be dependent on the purpose of the analysis: (i) to know the polysaccharide composition of must or wine samples by quantifying the content of specific polysaccharide families (see Sect. 4.1), (ii) to know the chemical fine structures of concrete polysaccharides isolated from musts or wines (see Sect. 4.2), and (iii) to estimate the global content of polysaccharides in a must or wine sample (see Sect. 4.3).

4.1 Quantification of Polysaccharide Families by GC-MS After Methylation and Derivatization Analysis

4.1.1 Methylation and Derivatization Analysis

The different families of polysaccharides can only be estimated by assessing its monosaccharide profile. The monosaccharide composition of the polysaccharide extracts can be determined both quantitatively and qualitatively by their conversion to their trimethylsilylated methyl glycoside derivatives (TMS) obtained after acidic methanolysis and derivatization.

Methanolysis, which yields methyl glycosides, is a useful technique for determining neutral, acidic, and amino sugars simultaneously. Thereafter, the conversion of methyl glycosides to volatile derivatives can be done by the formation of methyl ethers, acetates, and trimethylsilyl ethers (TMS). TMS derivatization after methanolysis has proven to be simple, quantitative, and suitable for hydrocolloids which have uronic acids. Resolution of multiple peaks of TMS derivatives has been significantly improved by the use of fused-silica capillary columns in the GC system. In the procedure we describe, all the free hydroxyl groups of the polysaccharide are methylated, and, following hydrolysis of the methylated polysaccharide, the partially methylated sugars released are derivatized to convert the methyl glycosides to their TMS derivatives to increase their volatility (Fig. 10). This conversion will result in the formation of several components (derivatives of the α- and β- and pyranose and furanose forms) for each sugar. Finally, TMS can be analyzed by GC with MS detection and the monosaccharides identified by their spectra and retention time relative to the internal standard. We typically use *myo*-inositol as the internal standard as it is not expected to be in the sample.

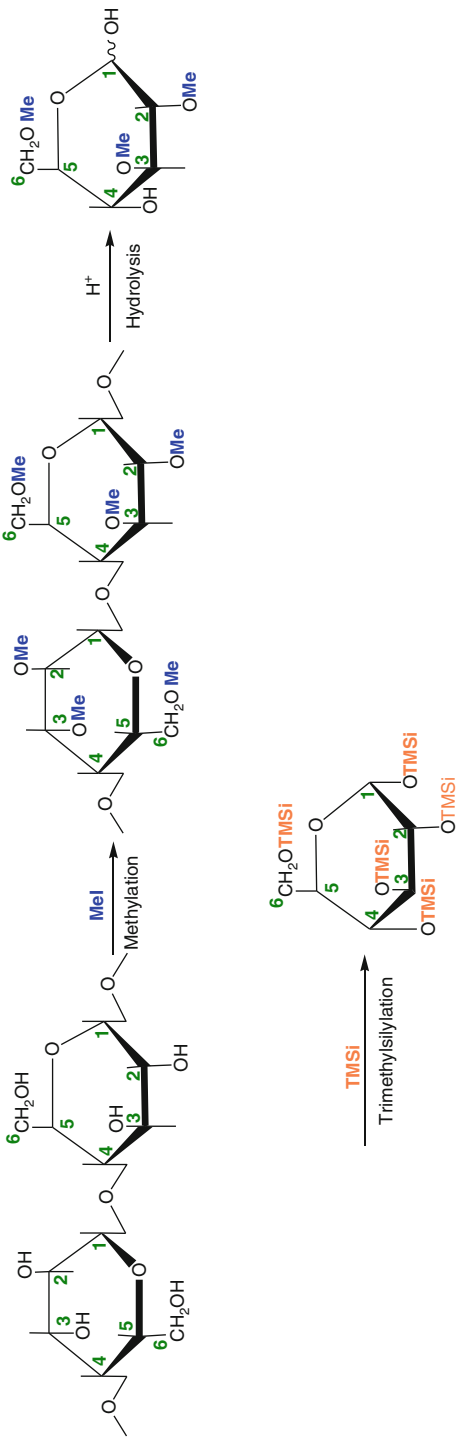

Fig. 10 Methylation and derivatization reactions to obtain trimethylsilylated methyl glycoside derivatives (*TMS*). The methylation positions of the free hydroxyl groups in the polysaccharides are shown (for simplicity, H atoms have been omitted from the top diagram). After acid methanolysis, the methyl glycosides are converted to volatile derivatives by the formation of trimethylsilyl ethers

The hydrolysis and derivatization conditions used are crucial to achieve a complete hydrolysis and TMS conversion. The protocol is described in detail by Guadalupe et al. (2012). Therefore, the polysaccharide fractions are treated with the methanolysis reagent (MeOH containing HCl 0.5 M), and the reaction is conducted in nitrogen atmosphere at 80 °C for 16 h. Thereafter, the excess of reagent is removed using a stream of nitrogen gas. The conversion of the methyl glycosides to their TMS derivatives is performed by adding a mix of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10:2:1 v/v) to the dried material. The reaction is carried out at 80 °C for 30 min and the reagent removed using a stream of nitrogen gas. A solution of derivatized *myo*-inositol is then added as internal standard, and the derivatized residues are extracted with hexane. GC-MS is performed with these solutions, and samples are injected in duplicate. Standard carbohydrates are also converted to their corresponding TMS derivatives and analyzed by GC-MS in order to obtain patterns for identification and the standard calibration curves.

The chromatographic column used is a Teknokroma fused-silica capillary column (30 m × 0.25 mm × 0.25 μm) of phase 5 % phenyl and 95 % methylpolysiloxane. The oven program starts at an initial temperature of 120 °C which was increased at a rate of 1 °C min^{-1} to 145 °C and then to 180 °C at a rate of 0.9 °C min^{-1} and finally to 230 °C at 40 °C min^{-1}. The GC injectors are equipped with a 3.4 mm ID and maintained at 250 °C with a 1:20 split ratio. The carrier gas is helium (99.996 %) at a flow rate of 1 mL min^{-1}. Ionization is performed by electron impact (EI) mode at 70 eV. The temperatures used are 150 °C for the MS Quad, 230 °C for the MS Source, and 250 °C for the transfer line. The identification of the peaks is carried out by comparing retention times and mass spectra with those obtained by injections of pure standards.

Typical GC-MS chromatogram for a wine polysaccharide extract is shown in Fig. 11. MS fragmentation patterns reported in Doco et al. 2001 can be used to identify those monosaccharides for which no commercial standards are available. Calibration curves of L-fucose, L-rhamnose, 2-*O*-methyl D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, Kdo, D-galacturonic acid, and D-glucuronic acid are used for quantification; 2-*O*-methyl fucose, aceric acid, Dha, and apiose can be quantified using the 2-*O*-methyl xylose calibration curve. The different monosaccharides are quantified in selected ion monitoring (SIM) mode, selecting the appropriate number of ions for each compound (*m*/*z*) in one segment from 3 to 65 min. D-galacturonic acid, L-rhamnose, L-fucose, D-galactose, D-glucose, D-mannose, and D-xylose are quantified with 204 ion; D-glucuronic acid, L-arabinose, Kdo, 2-*O*-methyl-L-fucose, Dha, and aceric acid with 217 ion; 2-*O*-methyl D-xylose with 146 ion; apiose with 191 ion; and *myo*-inositol with 305 ion. For all the spectra, these ions show the highest signal/noise ratio and are selected for recording SIM mode chromatograms.

The methodology described above has provided good values of quantification and detection limits and suitable values of repeatability, reproducibility, and overall recoveries. Repeatability and reproducibility values ranged from 1 % to 14 %. Limits of detection were below 1.0 μg for all monosaccharides, and limits of

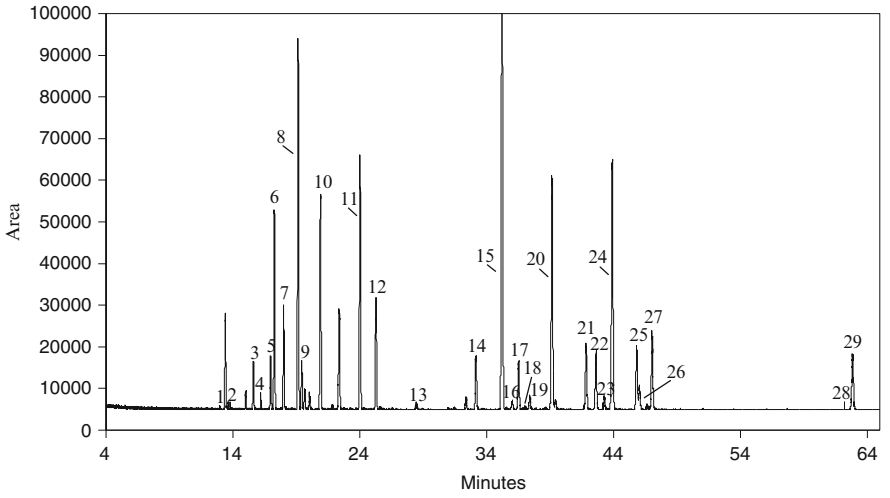

Fig. 11 GC-MS chromatogram obtained for a wine polysaccharide extract obtained after methylation and derivatization analysis. Peak identification: aceric acid (*peak 1*), 2-*O*-Me-fucose (*peak 2*), 2-*O*-Me-xylose (*peak 3*), apiose (*peak 4*), arabinose (*peak 5, 6, 7, 10, 11*), rhamnose (*peak 8*), fucose (*peak 9*), xylose (*peak 12, 13, 14*), mannose (*peak 15, 19*), galacturonic acid (*peak 16, 23*), Dha (*peak 18*), galactose (*peak 20, 21, 22*), glucose (*peak 24, 26*), glucuronic acid (*peak 25, 27*), Kdo (*peak 28*), *myo*-inositol (*peak 29*)

quantification were below 1.8 μg. Moreover, a recovery study of the whole method, including sample preparation, provided absolute recoveries between 81 % and 116 % for different wine samples, very good values taking into account the multistep procedure. The comparison of the results obtained by GC-MS with those obtained by GC-FID (GC coupled to flame ionization detector) revealed that both methods were suitable for determining the content of monosaccharides in wine samples, but MS detector showed to be more sensitive and selective than FID, allowing lower detection limits which may be useful when quantifying rare RG-II monosaccharides which are present in musts and wines in very low concentrations. A further advantage of the MS detection is that it provides unambiguous identification and quantification of the complex mixtures of monosaccharides present in grape-derived beverages.

4.1.2 Polysaccharide Composition Estimation

The content of each polysaccharide family can be estimated from the concentration of their individual glucosyl residues which are characteristic of structurally identified wine polysaccharides. The PRAG, mainly arabinogalactans, arabinogalactan proteins, and arabinans, can be estimated from the sum of galactose and arabinose residues. All of the mannose content can be attributed to yeast mannoproteins, and the content of glucans can be estimated from the content of glucose residues if the anthocyanins have been previously removed in the polysaccharide extract. The RG-II content can be calculated from the sum of its diagnostic monosaccharides, which represent approximately 25 % of the RG-II molecule. Taking into account

the molar ratios of the RG-II described by Pellerin et al. (1996) and Vidal et al. (2003) (1 residue of 2-*O*-methyl fucose, 3.5 rhamnose, 2 arabinose, 2 galactose, 1 glucuronic acid, and 9 galacturonic acid), the remaining part can be attributed to the presence of PRAG in the case of arabinose and galactose, and the remaining galacturonosyl residues can be used to estimate the content of oligomers of homogalacturonans (HG). The content of total polysaccharides can be estimated from the sum of PRAG, MP, GL, RG-II, and HG.

4.2 Structural Characterization of Polysaccharides by the Analysis of Glycosyl-Linkage Composition

Must and wine polysaccharides are known to be heterogeneous with respect to their chemical fine structure and size. Information obtained from isolated and purified polysaccharides enables us to deduce their fundamental structures. We will describe in this subsection analytical techniques to deduce the chemical fine structure of concrete polysaccharides isolated from musts and wines.

The analytical procedure to elucidate the chemical structures of the polysaccharides begins with methylation. Methylation analysis provides information about the linkage position on the sugar sequence. In this procedure, all the free hydroxyl groups of the polysaccharide are methylated, and, following hydrolysis of the methylated polysaccharide, the partially methylated sugars released are reduced and acetylated to yield partially methylated alditol acetates (PMAA). These volatiles can be separated, identified, and quantified by GC-MS. The linkage position (s) of each individual sugar in the parent polysaccharide is deduced by identifying PMAA, but it is not possible to work out the position or anomeric configuration of these residues in the polymer (Pettolino et al. 2012).

Complete methylation results in the methylation of all free hydroxyls not involved in the glycosidic linkage (Fig. 12). After acid hydrolysis, the partially methylated monosaccharides that are released are reduced with $NaBD_4$, which simultaneously opens the sugar ring to form the alditol and tags the anomeric C atom (C1) with a deuterium atom. However, information on the anomeric configuration (α/β) of the glycosyl residue is lost during this reduction step. To increase the volatility of the derivatives for GC separation, the partially methylated alditols are acetylated with acetic anhydride. In this way, C atoms were involved in the glycosidic linkage, and the ring carries acetyl groups (Fig. 12). Finally, the PMAA sugar derivatives are injected directly into a GC-MS system, wherein diagnostic fragmentation is generated by electron impact ionization.

Two of the most common reagents for methylation for monosaccharide linkage are methylsulfinyl carbanion in DMSO (Hakomori 1964) and the solid sodium hydroxide (NaOH) in DMSO method of Ciucanu and Kerek (1984). Both methods involve the preparation of very powerful bases for the ionization of the high-pKa hydroxyl groups of monosaccharide residues. Once ionized, the polysaccharide is treated with CH_3I, which results in the methylation of the hydroxyl groups. Once all free hydroxyl groups in the sugar are protected by persistent blocking with methyl

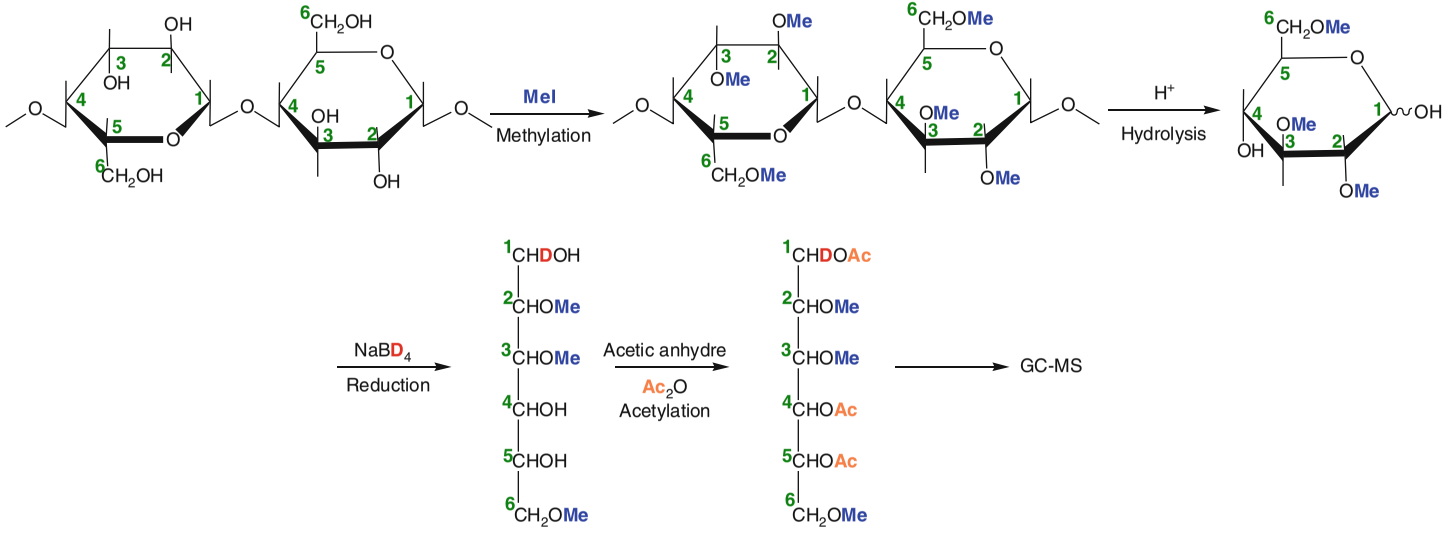

Fig. 12 Methylation reactions to obtain partially methylated alditol acetates (*PMAA*). The methylation positions of the free hydroxyl groups in the polysaccharides are shown (for simplicity, H atoms have been omitted from the top diagram). Thus, hydroxyls not involved in the glycosidic linkage are methylated. After acid hydrolysis, the partially methylated monosaccharides that are released are reduced with $NaBD_4$, which simultaneously opens the sugar ring to form the alditol and tags C atom number 1 with a deuterium atom. To increase the volatility of the derivatives, the partially methylated alditols are acetylated with acetic anhydride and separated by GC

group, the hydrolysis is carried out either with trifluoroacetic acid, which is volatile and therefore easily removed, or sulfuric acid. Hydrolysis conditions are crucial and can vary depending on the nature of the sample and its component sugars.

When polysaccharides contain uronic acids, methylation analysis becomes more difficult. The alkaline conditions used for methylation analysis could cause β-elimination. Glycosidic linkages adjacent to uronic acids are difficult to hydrolyze efficiently/quantitatively, and hence the uronic acids are not detectable as alditol acetates unless they are prereduced to their neutral sugar counterparts. This problem is resolved by a chemical reduction of the carboxyl group by lithium triethylborodeuteride or carbodiimide. Among acetylation methods, the addition of acetic anhydride to form alditol acetates is commonly used.

A method for classical elucidation of the chemical structures of polysaccharides isolated from wines and grapes is summarized below. The polysaccharide fractions are submitted to the reaction of per-*O*-methylated, and the methyl-esterified carboxyl groups are then reduced with lithium triethylborodeuteride prior to hydrolysis, reduction, and acetylation. For the methylation reaction, the fraction of polysaccharide dissolved in DMSO is methylated using methyl sulfinyl carbanion and methyl iodide (Hakomori 1964). Half of the methylated sample is then carboxyl reduced with lithium triethylborodeuteride (Pellerin et al. 1995). Thereafter, both methylated and carboxyl-reduced samples are hydrolyzed with 2 M trifluoroacetic acid (75 min at 120 °C). The released methylated or carboxyl-reduced monosaccharides are converted to their corresponding alditols by treatment with $NaDH_4$ to obtain the PMAA (Harris et al. 1984). PMAA are finally analyzed by GC-MS using a DB-1 capillary column (30 m × 0.32 mm i.d., 0.25 μm film); temperature program starts at 135 °C for 10 min, then 1.2 °C min^{-1} to 180 °C, with H_2 as the carrier gas (Vidal et al. 2001). The identity of each methyl ether is confirmed by electron ionization MS (EI-MS), and their areas are corrected by response factors. Figure 13 shows the fragmentation of PMAA during EI-MS (Pettolino et al. 2012).

It is important to stand out that methylation analysis provides only information about the linkage position on sugar sequence. Complete structures should be built up with additional analytical results obtained with modern techniques such as fast atom bombardment mass spectrometry (FAB-MS), matrix-assistant laser desorption ionization (MALDI-MS), electrospray ionization spectrometry (ESI-MS), and one and two (multi)-dimensional NMR spectroscopy.

4.3 Estimation of the Content of Total Polysaccharides by SEC-RID

Size-exclusion chromatography (SEC) is the technique commonly used to determine the molecular distribution of must and wine polysaccharide extracts according to their molecular mass. On the other hand, some authors have also used this procedure to obtain an estimation of the content of total polysaccharides in must or wine samples (López-Barajas et al. 1998b; Guadalupe et al. 2012).

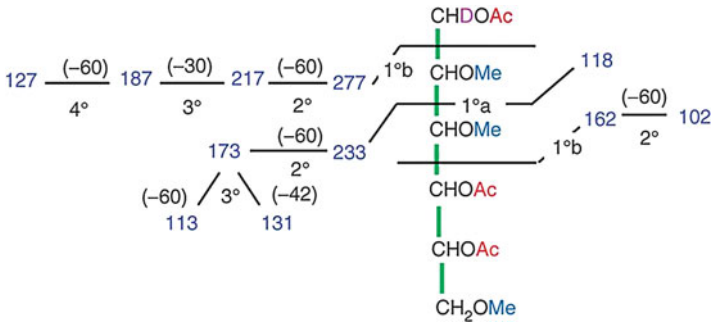

Fig. 13 Fragmentation of *PMAA* during electron ionization MS. Primary fragmentation of *PMAA* occurs in a particular order of preference, namely, (i) between two methoxylated carbons (either fragment carries the charge), (ii) between a methoxylated carbon and an acetoxylated carbon (the methoxy fragment carries the charge), and (iii) between two acetoxylated carbons. Secondary fragmentation results in the following fragments: acetic acid (H_3C-COOH) $m/z = 60$, methanol (CH_3OH) $m/z = 32$, ketene ($CH_2 = C = O$) $m/z = 42$, and formaldehyde (H_2CO) $m/z = 30$ (Pettolino et al. 2012)

The size-exclusion chromatography system is formed by an isocratic pump and two serial exclusion columns connected to a refractive index detector (RID). The size-exclusion columns more frequently used in literature for separating polysaccharide extracts coming from musts and wines are two serial 250 and 500 Ultrahydrogel columns (López-Barajas et al. 1998b, 2001; Palomero et al. 2007, 2009) and two Shodex OHpak SB-803 and SB-805 columns (Doco et al. 1996; Guadalupe and Ayestarán 2007; Guadalupe et al. 2012; Gil et al. 2013), which differ in their exclusion limit. Shodex and Ultrahydrogel columns are different in both their packing material and exclusion limits. Therefore, the Shodex OHpak SB columns are made of polyhydroxymethacrylate gel, while the Ultrahydrogel columns consist of a gel of a cross-linked hydroxylated polymer and contain some residual carboxyl. Both packing materials are suitable for the analysis of aqueous-soluble samples such as oligosaccharides, polysaccharides, and cationic, anionic, and amphoteric polymers and proteins. It is expected that samples or standards are separated in these columns according to their molecular weight. However, López-Barajas et al. (1998b) observed ionic interactions between anionic compounds, such as pectins and polyacrylic acids standards, and the cross-linked hydroxylated polymer of the columns. In order to eliminate these interactions, an eluent with a greater ionic strength must be used, ranging from 0.01 to 0.1 M $NaNO_3$ (Palomero et al. 2007). When the Shodex OHpak KB columns are chosen, the eluents most commonly used are 0.1 M $LiNO_3$ with a constant flow of 1 mL min^{-1} (Guadalupe and Ayestarán 2007; Apolinar-Valiente et al. 2013, 2014) or NH_4HCO_2 (from 30 mM to 50 mM) at 0.6 mL min^{-1} (Gil et al. 2013; González-Royo et al. 2013).

The apparent molecular weights (Mw) of polysaccharide extracts in the SEC chromatograms are compared with those of known pullulan standards of different molecular weights, and the calibration equation is then deduced as log Mw = a − b

Table 2 Retention times and calibration curve equations constructed with dextran (Ds), polyacrylic acids (PACs), and pectins (PECs) pullulan standards using two serial 250 and 500 Ultrahydrogel columns, according to López-Barajas et al. (1998b)

	M_r	T_R mean[a] (min)
Dextrans (Ds) y = 1E + 24x − 11, 78	D-580000	37,08
	D-267000	37,27
	D-150000[b]	39,90
	D-71000	41,36
	D-39000	43,61
	D-11000	49,29
Polyacrylic acids (PACs) and pectins (PECs) y = 5E + 18x − 89, 037	PAC-450000[c]	35,7
	PEC-82000	36,9
	PEC-34000[d]	37,74
	PEC-17000	38,5
	PAC-8000[e]	47,71
	PAC-5100	49,43
	PAC-2000	51,46

x = retention time (min)
y = molecular mass
[a]Mean was obtained with $n = 6$
[b]Reference standard for response factors of dextrans (*Ds*)
[c]This standard was not included in the calibration curve
[d]Reference standard for response factors of pectins (*PECs*)
[e]Reference standard for response factors of polyacrylic acids (*PACs*)

(t_r = column retention time at peak maximum, a = intercept, b = slope). Table 2 shows the calibration curve equations made with dextrans, pectins, and polyacrylic acids (López-Barajas et al. 1998b). It can be observed that the retention times of both pectins and polyacrylic acids standards differ from those expected, which is attributed to the ionic interactions.

Figure 14a shows the SEC-RID chromatograms of the polysaccharides produced in a model medium by autolysis of a non-Saccharomyces wine yeast using two serial Ultrahydrogel 250 and Ultrahydrogel 500 columns. The retention times of pullulan dextran standards of different molecular weight are also shown: P-5, Mw = 5.9 kDa; P-10, Mw = 11.8 kDa; P-20, Mw = kD kDa; P-50, Mw = 47.3 kDa; P-100, Mw = 112 kDa; P-200, Mw = 212 kDa; P-400, Mw = 404 kDa; and P-800, MW = 788 kDa. Figure 14b shows the chromatograms of the polysaccharide extracts from three wine samples using the serial columns OHpak KB-803 and KB-805 (Guadalupe et al. 2012). These authors describe that the first fraction corresponds to molecules with an average molecular weight higher than 47.3 kDa (average of 212, 112, and 50 kDa), attributed to a complex mixture of high-molecular-weight AGP from grape berries and high-molecular-weight MP from yeasts. The polysaccharides with an average molecular weight of 12 kDa (P10), eluting in the second fraction, correspond to grape RG-II dimers and lower-molecular-weight AGP and MP. Signals eluting after P5 correspond to a molecular weight of less than 6 kDa, and they are attributed to oligosaccharides and small

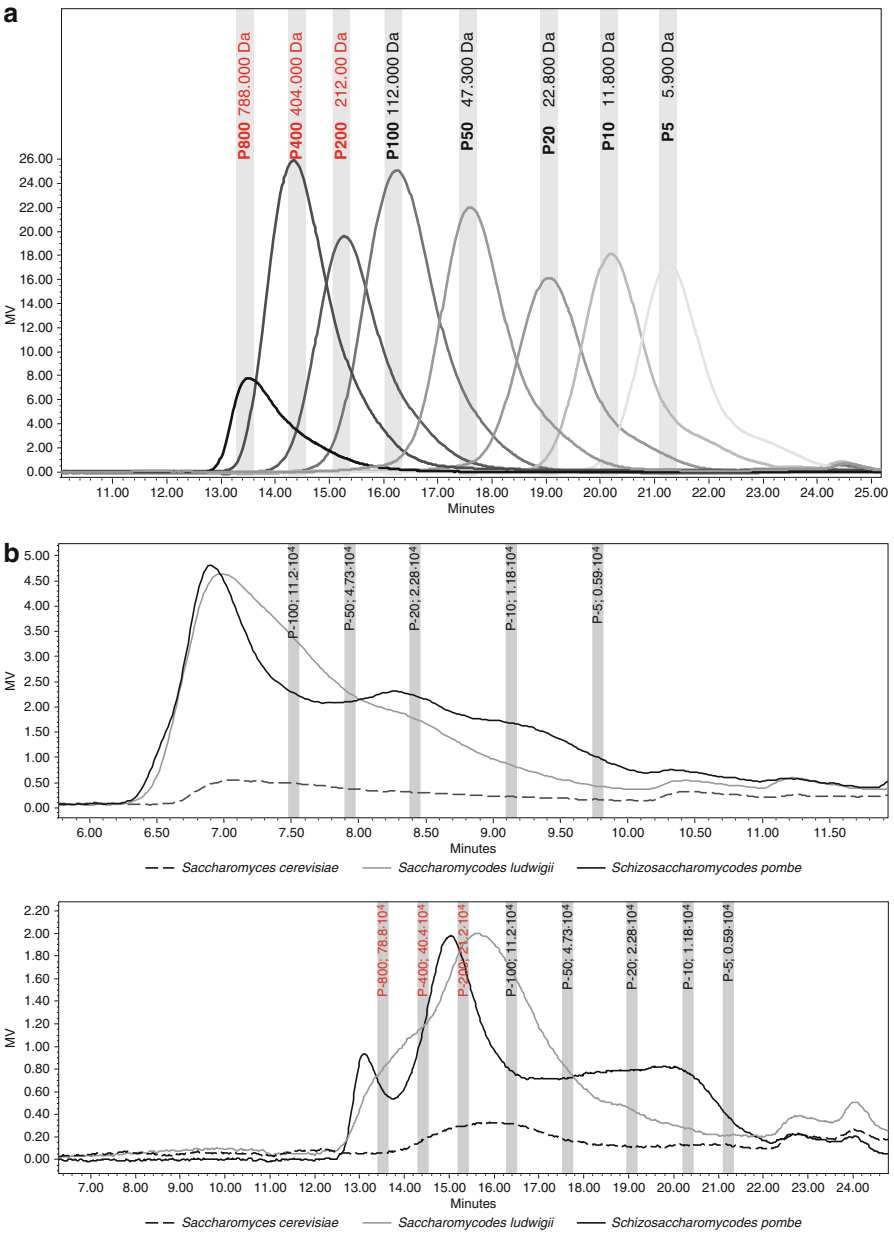

Fig. 14 SEC-RID chromatograms obtained for a wine polysaccharide extract on (**a**) Ultrahydrogel 250 and Ultrahydrogel 500 columns and (**b**) Shodex OHpak KB-803 and KB-805 columns. (**a**) SEC-RID chromatograms of polysaccharides produced by *Saccharomyces cerevisiae* G3(7) (*dashed black line*), *Schizosaccharomyces pombe* (*continuous black line*), and *Saccharomyces ludwigii* (*continuous gray line*) at 58 days of aging over lees. Chromatogram obtained using two serial Ultrahydrogel 250 and Ultrahydrogel 500 columns (Palomero et al. 2009). The retention

fragments of AGP, MP, and RG-II monomers. On the other hand, the chromatogram obtained by the Ultrahydrogel columns shows an early elution peak that corresponds to biopolymers of over 788 kDa (Fig. 14a). It is important to stand out that this chromatogram corresponds to a model media, and these high-molecular-weight signals are not usually obtained in wine samples.

The detailed protocol to quantify the amount of total polysaccharides on the basis of the peak areas from the SEC-RID chromatograms is described in detail by Guadalupe et al. (2012). In this procedure, signals eluting after P5 are not taken into account, and total polysaccharides are estimated from the sum of signals with a higher molecular weight higher than 6 kDa. Polysaccharide contents are estimated using calibration curves constructed with dextran pullulan standards whose peaks best match with those obtained for the samples. It is important to point out that the quantity of polysaccharides obtained by SEC-RID is just an estimation because the polysaccharide extract is not pure and there may be other contaminants such as salts, proteins, or phenolics. These compounds elute together with polysaccharides when these SEC columns are used. Therefore, Guadalupe et al. (2012) observed that the polysaccharide content estimated by SEC was considerably lower than that obtained with GC-MS. However, a good correlation was found between the two methods ($r = 0.746$, $p < 0.05$), indicating that the former could serve as a rapid and simple method for total wine polysaccharide estimation. It is important to notice that SEC-RID provided almost half of the value obtained by the GC-MS method, and thus it led to an underestimation of real wine polysaccharides, although it could be valid for comparative purposes.

5 Conclusions

Polysaccharides play a critical role in both the wine processing and quality, and their influence depends on their structure, composition, and distribution. Detailed analyses of these polysaccharides are essential to know their chemical, physicochemical, and sensory properties. Many researches have focused on wine polysaccharides during the last decade, trying to isolate and characterize them; studying their evolution from grape to wine and during the winemaking and aging; analyzing their functions, mechanisms of action, and factors affecting their content; or trying to modulate their concentrations into the wines. However, these studies are limited due to the diversity of wine polysaccharides, their relative low concentration, the recovery of sufficient amounts, and the time-consuming methods needed for their analysis. The difficulty in separating, purifying, and quantifying all of these

Fig. 14 (continued) times (*bands*) for the pullulan standards are shown. (**b**) SEC-RID chromatograms of total soluble polysaccharides in three wine samples. Chromatogram obtained using two serial Shodex OHpak KB-803 and KB-805 columns (Guadalupe et al. 2012). Elution times for pullulan standards are shown

compounds has resulted in them being less studied than other wine compounds such as polyphenols, polypeptides, or proteins. Therefore, an adequate determination of the different polysaccharide families requires analytical methodologies which involve complex multistep procedures.

We present in this chapter analytical techniques that are useful for the study of must and wine polysaccharides. We describe procedures not only to define the fine chemical structures of individual wine polysaccharides but also to estimate the overall polysaccharide composition of must and wine samples. The procedure covers the preparation of the sample, together with gas chromatography-mass spectrometry-based methods, for both the analysis of monosaccharides as their volatile trimethylsilylated methyl glycoside derivatives and methylation analysis to determine linkage positions between monosaccharide residues as their volatile partially methylated alditol acetate derivatives. We also provide a protocol for estimating the global content of wine polysaccharides by using of size-exclusion chromatography with refractive index detector (SEC-RID). This is a rapid and simple method that can be used routinely in a reasonably equipped laboratory. Analysis complexity and time will vary depending on the method used, and the duration ranges from 2 days for a global polysaccharide estimation to 2 weeks for a carboxyl reduction/methylation linkage analysis. In general, the complexity of the method used will depend on the type of starting material and on the purpose of the analysis: (i) to know the polysaccharide composition of must or wine samples by quantifying the content of specific polysaccharide families, (ii) to know the chemical fine structures of concrete polysaccharides isolated from musts or wines, and (iii) to estimate the global content of polysaccharides in a must or wine sample.

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NMR Spectroscopy for the Determination of Mucoadhesive Properties of Polysaccharides

44

Gloria Uccello-Barretta, Federica Balzano, and Federica Aiello

Contents

Abstract

Applications of nuclear magnetic resonance (NMR) spectroscopy to the structural characterization of polysaccharides with mucoadhesive characteristics are described together with some limited cases of uses in the detection of affinity to mucin. Structural characterization spans from quantification of monomeric units of polysaccharides following degradation procedures to the development of reliable analytical protocols directly on the intact polymeric materials. In these last cases several problems can be solved such as the identification of derivatization sites on monosaccharide units and determination of average length of derivatizing pendants. For non-covalent modifications, the more difficult aspect is the detection of conformational changes. Mucoadhesivity can be determined by exploiting the possibility to detect changes of affinity to mucin of small probe molecules due to the mucin–polysaccharide interaction.

Keywords

NMR • Mucoadhesivity • Polysaccharides • Relaxation parameters • Affinity

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Abbreviations	
AG	Arabinogalactan
BSM	Bovine submaxillary mucin
CD	Cyclodextrin
Ch	Chitosan
Ch–AT	Chitosan–atorvastatin
CMCh	Carboxymethyl chitosan
CMCh-*g*-CM-β-CD	Carboxymethyl chitosan-*g*-carboxymethyl-β-cyclodextrin
CMEH	L-cysteine methyl ester hydrochloride
CM-β-CD	Carboxymethyl-β-cyclodextrin
CS	Chondroitin sulfate
DOSY	Diffusion-ordered spectroscopy
DP	Dexamethasone 21-phosphate
DS	Diclofenac sodium salt
GluGal	Glucose and galactose
HA	Hyaluronic acid
HTCC	*N*-(2-hydroxyl)-propyl-3-trimethylammoniumchitosan chloride
KT	Ketotifen fumarate
NMR	Nuclear magnetic resonance
NP	Nanoparticle
OCM-Ch	6-*O*-carboxymethylchitosan
PEGAc	Polyethylenglycol acrylate
PLLA	Poly(L-lactide)
QACh	Quaternary ammonium chitosan
TMCh	*N*,*N*,*N*-trimethylchitosan
TSP	Tamarind seed polysaccharide
Xyl	2-*O*-galactosylxylose
Xyl_t	Terminal xylose

1 Introduction

The role of NMR spectroscopy in the field of structural, conformational, and stereochemical characterization of high and low molecular weight compounds and their supramolecular aggregates is definitely well recognized, and its potentials can be fully exploited in the field of the characterization of new materials of proved mucoadhesivity. Polysaccharides, in particular, come from natural sources and, therefore, require accurate preliminary characterizations. Furthermore, many of them can be subjected to covalent or non-covalent modifications aimed to improve their mucoadhesivity: in the first case the derivatization degree needs to be defined together with the distribution of derivatizing groups on the different sites of repeating units. Non-covalent modifications constitute a useful alternative approach

to the enhancement of mucoadhesion, which exploits the strong sensitivity of polysaccharide conformation to aggregation processes with other polymeric materials or specific excipients. The use of NMR spectroscopy in all these areas is almost always resolutive and gives quite accurate structural information, which may contribute to the comprehension of the complex phenomenon of mucoadhesion at a molecular level.

The application of spectroscopic techniques for the in vitro evaluation of mucoadhesivity is even more challenging and usually relies on the detection of supramolecular aggregation processes of potentially mucoadhesive materials with mucin.

Based on abovesaid premises, the subject of the present review is not only very complex but also very articulate since it covers applications areas of NMR spectroscopy, which are too wide to be exhaustively reviewed all together. Therefore, although fully aware that in the literature there are many valuable contributions regarding the topics which will be discussed, however we selected just a limited number of examples which are better suited from the illustrative point of view.

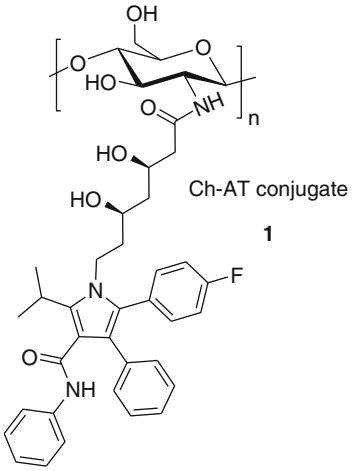
2 Characterization of Polysaccharides and Their Derivatives

A common approach to the accurate quantitative determination of the monomeric composition of polysaccharides is based on chemical degradation processes followed by NMR quantitative analysis. However, the concomitant degradation of monosaccharides could affect reliability of the quantitative analyses. An elegant approach to solving this problem has been recently proposed by de Souza et al. (2013), who described a universally applicable method for the hydrolysis of several kinds of polysaccharides, based on the Saeman hydrolysis, followed by NMR quantification. The hydrolyzed mixture was analyzed by ^{1}H NMR with maleic acid as internal standard for the quantitative analysis, comparing the integrated areas of the maleic acid protons and the anomeric proton resonances. A correction for degradation of monosaccharides was then applied by subjecting a standard mixture represented in the polymer and containing a known amount of glucose, galactose, mannose, glucosamine, xylose, and arabinose to the same conditions. This correction resulted in a very accurate and reproducible method with relative deviations down to 1 %.

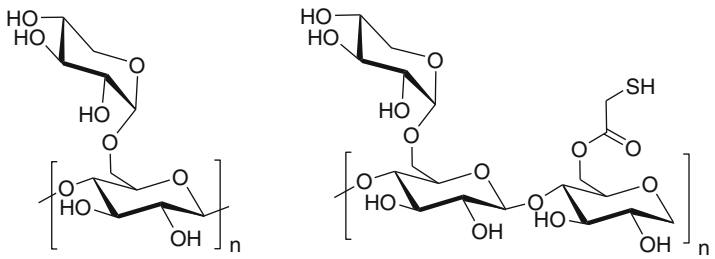
The application of NMR spectroscopy to covalent modifications of polysaccharides ranges from the simple detection of the derivatization process to the development of accurate analytical protocols for the identification of the degree and location of derivatizing pendants on each repeating unit.

As an example, for the development of a new chitosan–atorvastatin (Ch–AT, **1**) conjugate, a coupling reaction was exploited, by formation of amide linkages, the presence of which was very simply checked in the ^{1}H NMR spectra by means of the

appearance of a new amide signal at 9.89 ppm (Anwar et al. 2011). In this case NMR spectroscopy was also employed to prove that no chemical changes occurred during nanonization processes since the ^{1}H NMR spectrum of Ch–AT nanoconjugate was superimposable to the corresponding spectrum of Ch–AT conjugate.

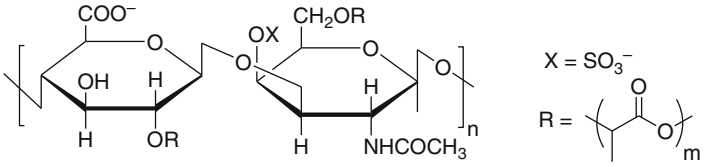


Thiolation of polysaccharides by means of esterification with thioglycolic acid represents a common way to enhance bioadhesive potential of polysaccharides, as described (Mahajan et al. 2013) for a xyloglucan. In this case the modification was confirmed on the basis of the comparison of the ^{1}H NMR spectra of parent xyloglucan (**2**) and thiolated xyloglucan (**3**). NMR spectra were quite superimposable with the exception of an additional signal at 3.27 ppm, which was attributed to the resonance of SH protons.



In the development of new biodegradable brushlike amphiphilic poly(L-lactide)-grafted chondroitin sulfate copolymers **4**, with specific mucoadhesion or receptor recognition abilities, poly(L-lactide) (PLLA) was grafted onto chondroitin sulfate (CS) (Lee et al. 2007). The degree of substitution, the degree of polymerization, and the chondroitin sulfate content (from 1.1 % to 15.4 %) all

were established by ^{1}H NMR. In particular, the degree of substitution was calculated based on the area ratio of signals from methine protons of PLLA segments at 5.2 ppm (one proton) and methyl proton of CS at 1.9 ppm (three protons). This value corresponds to the average number of hydroxyl groups on the disaccharides of CS that have been reacted with L-lactide. The degree of polymerization of L-lactide was calculated based on the area ratio of the terminal methine proton signal of PLLA at 4.1 ppm to the internal methine proton signal of PLLA segments at 5.2 ppm, which gives the average length of every PLLA branch.



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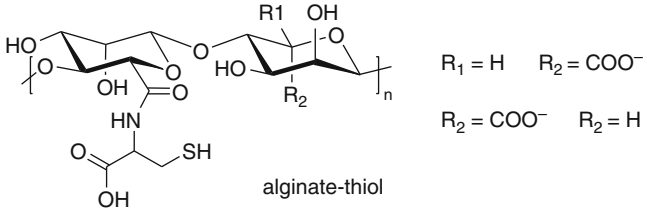
Attempts have been made to improve the mucoadhesive properties via covalent bonds such as disulfide bridges between the polymer to the mucin (Bernkop-Schnürch et al. 2003). Therefore, new mucoadhesive polymers, termed thiomers, have been proposed as drug delivery carriers able to adhere to the mucous layer.

In 2009 Davidovich-Pinhas described the synthesis of a model thiomer, alginate-thiol **5**, which was characterized by NMR (Davidovich-Pinhas et al. 2009). The synthesis was carried out by reacting alginate with L-cysteine. By comparing the NMR spectra of modified alginate and parent alginate, several new signals were detected, supporting molecular structure changes. However, besides expected additional peaks due to cysteine conjugation, eight more resonances were detected. In this case the simple spectral analysis was not sufficient to identify the products, and 2D scalar correlation experiments were carried out, which allowed authors to establish that some units of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, employed to activate the carboxylic acid groups of the polymer, remained attached to the polymer backbone.



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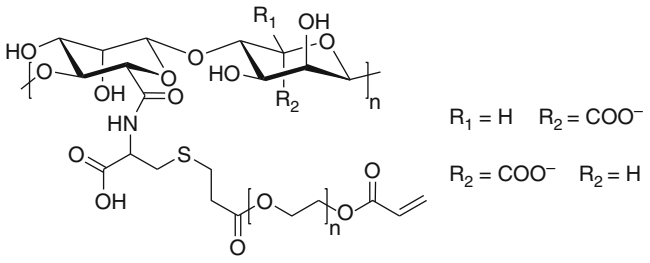
The same author also reported (Davidovich-Pinhas and Bianco-Peled 2011) a novel mucoadhesive alginate–polyethylenglycol acrylate conjugate (alginate–PEGAc, **6**),

which was synthesized from alginate-thiol **5**. The conjugate **6** was characterized by polyethylenglycol chains with one acrylate end group connected to the alginate backbone via the cysteine spacer. This polymer combined the strength, simplicity, and gelation ability of alginate with the mucoadhesion properties of polyethylenglycol. In order to assess the occurrence of derivatization, the native, intermediate (**5**), and final (**6**) products were compared using ^{1}H NMR spectroscopy.



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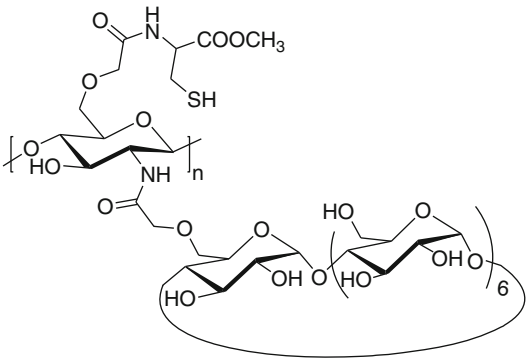
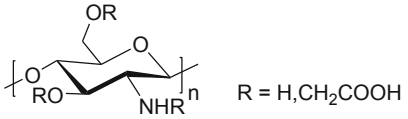
Even though thiomers mimic the natural mechanism of secreted mucous glycoproteins and, hence, show enhanced mucoadhesivity, however their poor interaction with hydrophobic drug molecules often negatively affects their potential applications in pharmaceutical fields. In order to expand potential applications of thiomers as mucoadhesive drug delivery carrier with controlled drug release capability, a thiolated carboxymethyl chitosan-*g*-carboxymethyl-β-cyclodextrin (CMCh-*g*-CM-β-CD, **7**) was proposed (Prabaharan and Gong 2008): firstly carboxymethyl chitosan (CMCh) was prepared from chitosan and chloroacetic acid. From the relative peak intensities between the protons of the carboxymethyl groups (4.4 ppm) at the C6 position of the CMCh and the protons at C2 of monosaccharide residue (3.0 ppm), the degree of substitution of carboxymethyl groups on the primary hydroxyl sites of the modified chitosan CMCh was determined as 35 % using ^{1}H NMR. Carboxymethyl-β-cyclodextrin (CM-β-CD) was obtained from β-CD and chloroacetic acid, with a substitution degree on the primary hydroxyl sites estimated as 14 % using ^{1}H NMR; then it was grafted onto CMCh in the presence of suitable condensing agents and finally conjugated with L-cysteine methyl ester hydrochloride (CMEH). The bases of quantitative determination mainly were the methylene protons of CMEH appearing in the spectrum of thiolated CMCh-*g*-CM-β-CDs. From the relative peak intensities of anomeric protons of CMCh (4.8 ppm), CM-β-CD (5.0 ppm), and methylene protons of CMEH (2.65 ppm), the degree of substitution of CM-β-CD in thiolated CMCh-*g*-CM-β-CDs was estimated as ≈ 77 %. The degree of substitution of CMEH in two different thiolated CMCh-*g*-CM-β-CDs was determined as about 20 %.



Thiolated CMCh-*g*-CM-β-CD

7

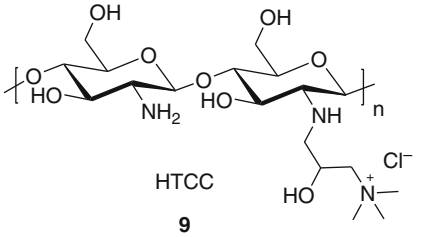
In another preparation of water-soluble 6-*O*-carboxymethyl derivative of chitosan (OCM-Ch, **8**) (Shinde et al. 2013), the ^{13}C NMR spectroscopy was exploited for the characterization of the products; the signals for –COOH substituted on –OH and –NH were detected at 173.4 and 170.1 ppm, respectively. Chemical shifts at 70.9, 69.1, and 48.3 ppm were assigned to methylene carbon of $–CH_2COOH$ groups substituted on O-6, O-3, and N-2, indicating that there were three possible sites for the carboxymethylation of Ch. On account of the signal intensity, it was concluded that the OH-6 was the major site for carboxymethylation of Ch.



OCM-Ch

8

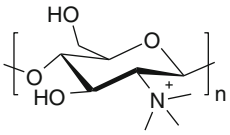
An oral insulin delivery system (*N*-(2-hydroxyl)propyl-3-trimethylammonium-chitosan chloride, HTCC, **9**) was obtained (Sonia and Sharma 2011) by coupling glycidyl trimethylammonium chloride to chitosan in aqueous medium, and the modification of chitosan was assessed by the appearance of intense ^1H NMR peaks at 3.1 ppm, which were attributed to the presence of methyl groups in the quaternary nitrogen atoms.



HTCC

9

In some cases modifying groups may enter in different sites of repeating units and also originate pendants with different lengths, as in the case of ammonium quaternization of chitosan, which represents an efficient way to the improvement of solubility of parent polysaccharide, by affecting strongly its bioavailability and mucoadhesivity. In 2009 Mourya reviewed the synthesis, characterization, and applications of *N*,*N*,*N*-trimethylchitosan (TMCh, **10**) (Mourya and Inamdar 2009). Methylation of amino groups in chitosan can be achieved with methyl iodide in different experimental conditions by obtaining strongly variable degree of quaternization depending on the number of reaction steps or reaction time or using different deacetylation grades of chitosan. At higher degrees of quaternization, *O*-methylation on the 3 and 6 hydroxyl groups of chitosan may occur, which leads to less soluble products. In this regard there is a strong need to have in hand a robust and reliable analytical protocol.



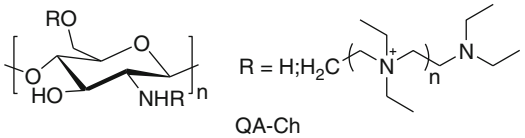
TMCh

10

Mourya (Mourya and Inamdar 2009) reviewed an analytical protocol based on the individuation of well-defined spectral regions of the ^{1}H NMR spectra of TMCh including specific resonances of the glucopyranose rings and derivatizing groups (Sieval et al. 1998; de Britto and Assis 2007). In particular, NMR spectra include signals at 3.4 ppm for quaternized amino group and a peak at 2.5 ppm for dimethylamino group, as well as at 3.36–3.56 ppm for *O*-methylated group. Between 4.5 and 5.0 ppm, anomeric protons are detected, the signal at 3.18 ppm is assigned to H_2 protons, whereas the 3.4–4.0 ppm region includes the other ^{1}H NMR nuclei on the carbon atoms 3, 4, 5, and 6 of the glucopyranose unit; finally acetamido groups give resonances centered at 2 ppm. Therefore, the quaternization degree was established from the ratio of integrated areas of quaternized trimethylamino group and anomeric protons.

It was also reported that mucoadhesion, which is a key element of TMCh polymers for being effective as absorption enhancers at mucosal surfaces, is strongly affected by the increase of molecular weight (Di Colo et al. 2004). Therefore, novel quaternary ammonium chitosan derivatives QACh (**11**) containing different percentages of pendant quaternary ammonium groups were obtained by reacting chitosan with 2-diethylaminoethyl chloride (Zambito et al. 2006). Very complex NMR spectra were obtained with extensively superimposed broad NMR signals, making quite difficult the spectral attribution also in consideration of the fact that some alkylammonium signals are superimposed to ring protons. For this reason the attribution required the combined analysis of scalar and dipolar 2D NMR maps, with a particularly useful contribution from long-range ^{1}H–^{13}C scalar

correlations. Several different kinds of methylene groups were distinguished, i.e., isolated ethyl groups bound to neutral nitrogens (methyls resonating between 0.90 and 1.10 ppm) and ethyl groups bound to quaternary nitrogens (methyls resonating between 1.10 and 1.40 ppm). Other two different clusters of methylene protons were found, which corresponded to methylene groups joining neutral nitrogen atoms to quaternary nitrogen atoms or quaternary nitrogen atoms to the derivatization sites on the glucopyranose ring and, finally, methylene protons on the primary sites of the glucopyranose rings. The presence of strong dipolar correlations due to spatial proximity of H_2 protons with ethyl groups bound to neutral and quaternized nitrogens points to the 2-site on the glucopyranose ring as the preferential substitution site. On the basis of the described spectral characterization, the ratio between quaternary and neutral groups in the pendant chain, which is indicative of chain growth, was calculated. The degree of acetylation was obtained by comparing the proton unit of the acetyl group to the proton unit of the glucopyranose ring. The total degree of substitution can be obtained by comparing the proton unit of the terminal methyl groups linked to the neutral nitrogen to the proton unit of the glucopyranose ring.



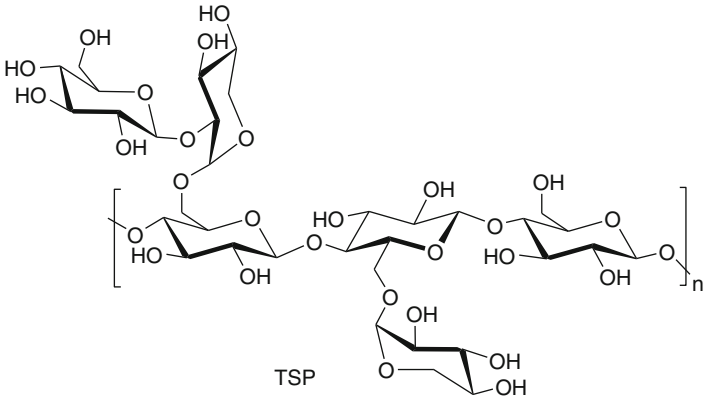
QA-Ch

11

Maestrelli and coworkers (Maestrelli et al. 2006) conjugated mucoadhesive chitosan with cyclodextrins which were composed in nanoparticulate structures in order to develop new mucoadhesive drug release systems. Later different cyclodextrins were incorporated (Trapani et al. 2008), the architectural assembly of which was investigated by NMR. 1D-water-LOGSY experiments (Dalvit et al. 2001) led to detect through space dipole–dipole interactions between the external water phase and other molecules in close contact with it. Through this experiment cyclodextrin component was not detected to indicate that its protons are not in contact with the external water phase (Trapani et al. 2008). By contrast some Ch resonances are observed and hence a fraction of chains of this polymer interacted with the external water phase. Importantly, NMR diffusion experiments (diffusion-ordered spectroscopy, DOSY, Morris 2010; Macchioni et al. 2012) confirmed that Ch is attached to the nanoparticles (NPs) and does not diffuse as free molecules in solution (Trapani et al. 2008). On the basis of the abovesaid results, authors concluded that some chains of the Ch polymer were located on the external side of NPs and exposed to the aqueous medium. On the other hand, the cyclodextrin was entrapped into the NP polymeric network.

Non-covalent modifications by supramolecular aggregations of potentially mucoadhesive materials may be very attractive from an economic and practical point of view and have been exploited for the development of eye drops based on

mixtures of two polysaccharides: tamarind seed polysaccharide (TSP, **12**), a non-ionic, neutral, branched polysaccharide, and hyaluronic acid (HA, **13**), a polyanion alternating 2-acetamide-2-deoxy-β-D-glucopyranose and β-D-glucopyranuronic acid residues (Uccello-Barretta et al. 2010). TSP has a cellulose-like backbone carrying xylose and galactoxylose substituents.



TSP

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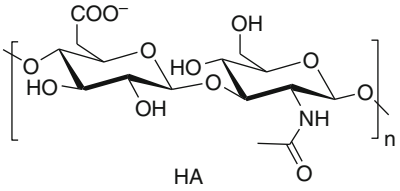

HA

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In such a kind of application, NMR contributed to face two problems: the detection of interpolymer interactions demonstrating their supramolecular aggregation and the evaluation of the mucoadhesivity of pure polysaccharides in comparison to their mixtures, depending on the mixture composition, which will be described in the following paragraph. Taking into account that we are dealing with high molecular weight polymers, which originate very broad signals, it cannot be expected to obtain detectable chemical shift changes, due to interpolymer interactions, in the mixtures relative to single polymers. A possible chance is to measure relaxation parameters, i.e., spin–lattice relaxation rates (R_1), giving the recovery time of the longitudinal magnetization, and spin–spin relaxation rates (R_2), which measure the decay of transverse magnetization. Such a kind of NMR parameters is strongly sensitive (Hills et al. 1991; Cowman et al. 1996) to conformational changes and slowing down of molecular motions due to intermolecular interactions. Regarding TSP/HA interaction processes, spin–spin transverse relaxation rates of anomeric protons of 2-*O*-galactosylxylose (Xyl), terminal xylose (Xyl_t), and all of the glucose and galactose (GluGal)

residues were remarkably sensitive to TSP/HA molar ratios and total concentration (Uccello-Barretta et al. 2010). In particular, at constant TSP concentration, relaxation rate parameters of Xyl and Xyl_t anomeric protons of TSP underwent a fourfold and sevenfold decrease (for Xyl and Xyl_t, respectively) in the 1:4 TSP/HA mixture relative to pure TSP. Importantly, viscosity effects on relaxation parameters could be ruled out as they would have produced opposite changes, i.e., an increase of relaxation rates. By adding further amounts of HA, relaxation rates progressively increased reaching a maximum for the 3:2 TSP/HA ratio, to indicate the formation of stable aggregates at well-defined TSP/HA ratios. Response of glucose backbone and galactopyranosyl side chain (GluGal) units was opposite, as relaxation rates of their anomeric protons underwent an initial sensible increase in the presence of HA, but remarkably decreased with increasing TSP/HA ratio till the limit ratio of 3:2. By contrast, relaxation parameter of GluGal anomeric protons of unmixed TSP was not sensitive to concentration changes. Regarding HA, its acetyl protons constituted the probe of the TSP/HA interaction. The spin–spin and spin–lattice relaxation parameters of acetyl moiety of pure HA reflected the changes of repulsive electrostatic interactions due to changes of HA total concentrations. In the mixtures containing TSP, the effect is significantly lower, to indicate that TSP interacted with HA thus contributing to minimize repulsive electrostatic interactions among the chains of the latter. Another sensitive probe of interpolymer interactions is transverse relaxation rate of solvent molecules, which is strongly responsive both to conformational changes and aggregation processes (Hills et al. 1991) as confirmed for TSP/HA mixtures where the R_2 values of the solvent (water) at the TSP/HA 3:2 ratio were remarkably higher than the sum of the values for the single polymers. This fact indicates that the formation of the TSP–HA supramolecular aggregate strongly affects the nature of polysaccharide interactions with water molecules.

3 Mucoadhesivity Determinations by NMR

Going to the problem of using NMR spectroscopy as an in vitro method for assessing mucoadhesivity, mucin–polysaccharide interactions should be detected, which represents a challenging task as both components produce very broad signals. An alternative approach could be using low molecular weight probes in order to detect changes in their mucin affinity imputable to the presence of polysaccharides and, hence, attributable to supramolecular aggregation between the polymeric materials.

Several NMR techniques may be exploited for the investigation of the drug to macromolecule affinities, which have been exhaustively reviewed by several authors (Shapiro 2002; Zartler et al. 2003; Fielding et al. 2005; Fielding 2007; Viegas et al. 2010; Cala et al. 2014). Here we will focus on selected methods, which have been used in polysaccharide applications.

The fundamental needed premise is that a large excess of a low molecular weight component with respect to the macromolecular system must be typically used in

order to obtain its observable signals in solution, where the total concentration is kept to values, which are suitable for obtaining homogeneous solutions without significant viscosity effects. Furthermore, the drug to macromolecule interaction may originate slow- or fast-exchange conditions. In the first case, signals due to bound and free drug are different, and therefore, in principle, affinity effects could be quantified simply by integrating the two signals. Unfortunately, in the operative conditions of very large excess of ligand with respect to macromolecule, it is difficult to detect the signals of very low amount of bound species.

In fast-exchange conditions, only one set of signals is detected and NMR parameters (P_{obs}) are the weighted average of the ones in the free and bound states (Eq. 1):

$$P_{obs} = P_b x_b + P_f x_f \tag{1}$$

where x_b and x_f are the molar fractions in bound and free state, respectively.

The bound state contribution to the observed parameter is very low, and therefore, NMR parameters strongly responsive to drug to macromolecule interactions must be selected. Such a kind of parameters is proton selective relaxation rates (Valensin et al. 1986).

It has been shown that the selective relaxation rate (${R_1}^s$) of the ligand is a more sensitive indicator of binding than the nonselective rate (${R_1}^{ns}$). In fact, methods based on the determination of the selective relaxation rates take advantage of the favorable dependence of ${R_1}^s$ on the reorientational correlation time (τ_c) in the region of slow molecular motions, in which the small molecule is forced by the interaction with the macromolecule. In the fast motion region ($\omega\tau_c \ll 1$, $\omega =$ Larmor frequency), both the selective and nonselective relaxation rates increase progressively with increasing τ_c. When the molecular motion of the ligand is slowed down to the $\omega\tau_c \gg 1$ region as a consequence of the interaction with the macromolecule, ${R_1}^s$ shows a sharp increase, whereas ${R_1}^{ns}$ reaches a maximum for $\omega\tau_c \cong 1$ and then decreases with further increasing $\omega\tau_c$.

Selective relaxation rates are measured by using the standard inversion recovery sequence by selectively inverting one spin and leaving unperturbed all the other spins and measuring the recovery of the magnetization in the time.

Such a kind of sensitive NMR parameters was exploited (Uccello-Barretta et al. 2011) for gaining some insight on the tamarind seed polysaccharide (TSP, **12**) to bovine submaxillary mucin (BSM) interaction in comparison with a water-soluble arabinogalactan (AG), by using ketotifen fumarate (KT) as low molecular weight probe for mucoadhesivity. Preliminarily, the affinity of KT for TSP was evaluated by measuring the proton selective relaxation rates of aromatic protons of pure KT and evaluating the effect of the presence of TSP (Uccello-Barretta et al. 2008). A twofold increase of selective relaxation rates was detected in the mixtures containing TSP, demonstrating the good affinity of the drug for the polysaccharide. The affinity of KT for mucin was even stronger as more than a twentyfold increase of relaxation rates was detected for KT protons (2 mM) in the

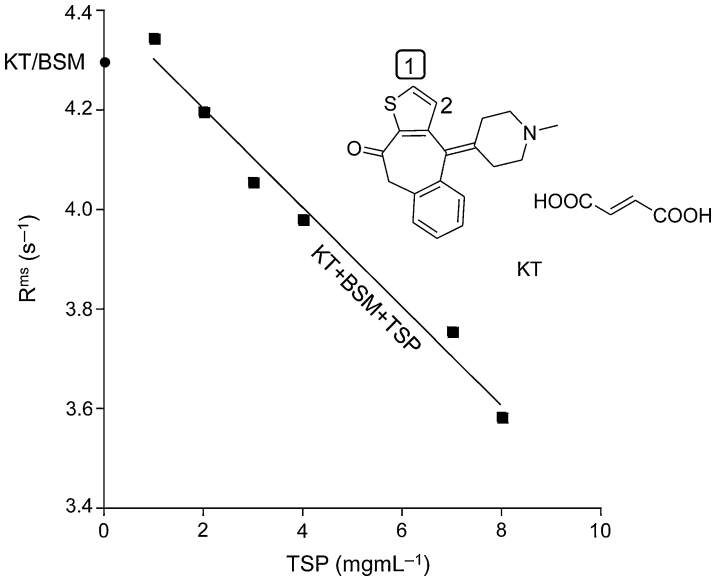


Fig. 1 Plot of mono-selective relaxation rates (R_1^{ms}) of proton H_1 of KT (2 mM) in the presence of BSM (4 mg mL^{-1}) and variable amounts of TSP

presence of 8 mg mL^{-1} mucin, i.e., at concentration levels for which the effect of viscosity changes on the NMR parameters could be ruled out (Uccello-Barretta et al. 2011). By adding increasing amounts of TSP to the binary mixture KT (2 mM)/BSM (4 mg mL^{-1}), concomitant progressive decreases of KT relaxation rates were detected to indicate the mucoadhesive properties of TSP (Fig. 1): the tamarind seed polysaccharide formed stable adducts with mucin and, hence, displaced KT from it. Interestingly, very low changes of relaxation rates of KT protons were detected in the presence of arabinogalactan, which pointed out the lower affinity of the drug for AG. The same polysaccharide did not affect the KT/BSM interaction, as the relaxation rates of KT were nearly unchanged in the binary KT/BSM and ternary KT/BSM/AG mixtures. In this way the minor mucoadhesivity of AG was pointed out in comparison with TSP (Uccello-Barretta et al. 2011). Interestingly, rheological measurements on the mucoadhesive properties of TSP and AG (Burgalassi et al. 2007; Di Colo et al. 2009) gave controversial results.

In another application, both ketotifen fumarate (KT) and diclofenac sodium salt (DS) were employed as mucoadhesivity probes for polysaccharide mixtures containing tamarind seed polysaccharide and hyaluronic acid (Uccello-Barretta et al. 2010). KT, which showed good affinity for TSP (Uccello-Barretta et al. 2008), did not show any propensity to interact with hyaluronic acid as its proton selective relaxation rates remained nearly unchanged in binary mixtures KT/HA in comparison to pure KT. The response of relaxation rates of KT in the

ternary mixtures KT/HA/BSM in comparison to binary mixtures KT/BSM and KT/HA suggested that HA interacted with mucin to a low extent. In the quaternary mixtures KT/TSP/HA/BSM, remarkable decreases of relaxation rates of KT were measured with respect to the values measured in the KT/BSM mixtures, to indicate that TSP/HA mixtures formed stable aggregates with mucin thus displacing KT from it. The abovesaid effect was strongly dependent on the TSP/HA ratio. In particular, TSP/HA mixtures with ratios between 2:3 and 3:2 showed the maximum ability to displace KT from mucin. The formation of stable TSP to HA aggregates with enhanced mucin affinity was confirmed by the values of the cross-relaxation parameters (σ_{ij}) for selected proton pairs *ij* of KT (Uccello-Barretta et al. 2010).

Cross-relaxation term σ_{ij} (Valensin et al. 1986; Uccello-Barretta et al. 1991), which describes the magnetization transfer between the proton pair *ij*, is a function of the reorientational correlation time τ_c of the vector connecting the two spins *i* and *j* and of their distance r_{ij} (Eq. 2):

$$\sigma_{ij} = f\left(r_{ij}^{-6}, \tau_c\right) \tag{2}$$

The cross-relaxation parameters can be determined in a very simple way by measuring the proton mono- (R_i^{ms}) and bi-selective (R_{ij}^{bs}) relaxation rates, which are determined by inverting together the proton pair *ij* and following the recovery of *i* in the time (Fig. 2). On subtracting R_i^{ms} from R_{ij}^{bs}, the cross-relaxation rate σ_{ij} can be calculated (Eq. 3), and hence, the σ_{ij} values for proton pairs at fixed distances can be usefully correlated to the reorientational correlation time, which is a parameter very sensitive to the drug–macromolecule interaction. In particular, in fast motion region, which is typical of low molecular weight molecules, positive values of σ_{ij} are expected (Eq. 4), whereas in the slow motion region, which is typical of macromolecule bound ligands, negative σ_{ij} values are obtained (Eq. 5), and hence, a decrease of σ_{ij} with respect to the free value must be expected, the extent of which depends on the bound molar fraction.

$$\sigma_{ij} = R_{ij}^{bs} - R_i^{ms} \tag{3}$$

$$\sigma_{ij} = 0.5\gamma^4\hbar^2 r_{ij}^{-6}\tau_c \tag{4}$$

$$\sigma_{ij} = -0.1\gamma^4\hbar^2 r_{ij}^{-6}\tau_c \tag{5}$$

In the investigation of mucin affinity of TSP/HA mixtures, the proton pair H_1–H_2 of KT (Fig. 1) was selected being at the fixed distance of 2.94 Å, and its cross-relaxation parameters σ_{12} were calculated on the basis of the corresponding mono-selective relaxation rate (R_1^{ms}) and bi-selective relaxation rate (R_{12}^{bs}). Pure KT showed a low and positive value of σ_{12} (0.04 s^{-1}), which remarkably decreased to -1.83 s^{-1} in the presence of mucin, to indicate that the interaction with mucin forced the small molecule to the slow motion region. In the quaternary mixtures

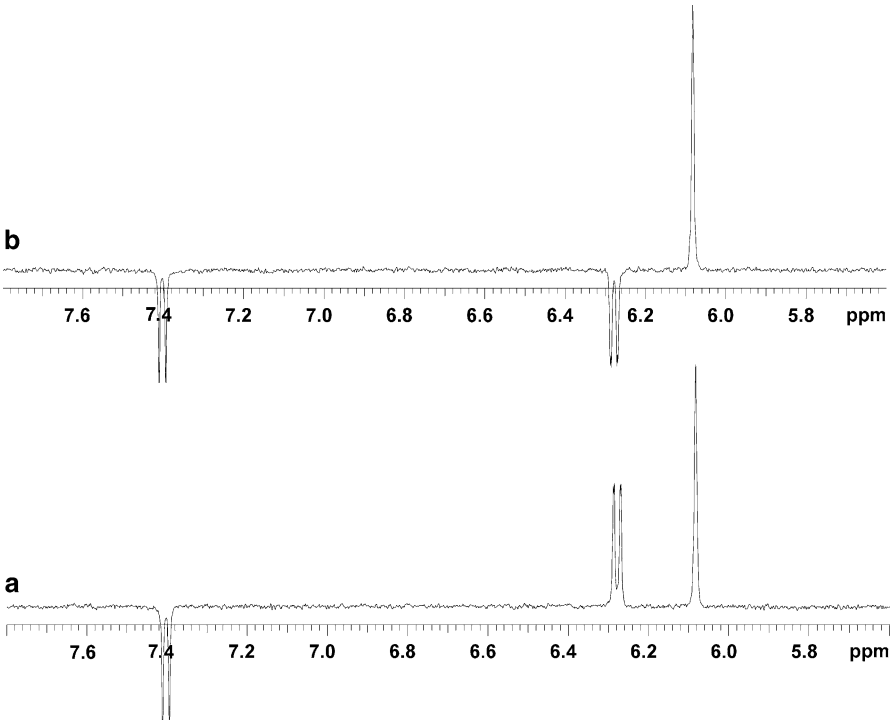


Fig. 2 Example of (**a**) mono- and (**b**) bi-selective proton inversions

KT/TSP/HA/BSM, an increase of the relaxation parameter was detected, the amount of which strongly depended on the TSP to HA ratio.

Diclofenac sodium salt (DS) constituted an alternative low molecular weight interaction probe with negligible propensity to interact both with TSP and HA, whereas its mucin affinity was remarkable (Fig. 3), as about fiftyfold increases of selective relaxation rates of DS were detected in the presence of mucin (Uccello-Barretta et al. 2010, 2013). TSP/HA mixtures produced relevant decreases of DS relaxation rates in quaternary mixtures DS/BSM/TSP/HA with respect to DS/BSM binary mixtures (Fig. 4), confirming the mucoadhesion properties of TSP/HA aggregates.

Such a kind of promising results suggested to extend the NMR approach also to the in vitro evaluation of mucoadhesivity of nanoparticles obtained from quaternary ammonium chitosan conjugates QA-rCh (analogues of QACh, but prepared from chitosan with reduced molecular weight) and QA-rCh-SH (**14**) (Uccello-Barretta et al. 2014). Particularly interesting was the comparison between the polymers and their nanoparticulate aggregates, by using dexamethasone 21-phosphate (DP) as mucoadhesivity probe. The enhanced affinity of the drug for the nanoparticles was assessed with respect to the parent polymer on the basis of responses of relaxation rates of DP in binary mixtures containing the polymer or the nanoparticles; more

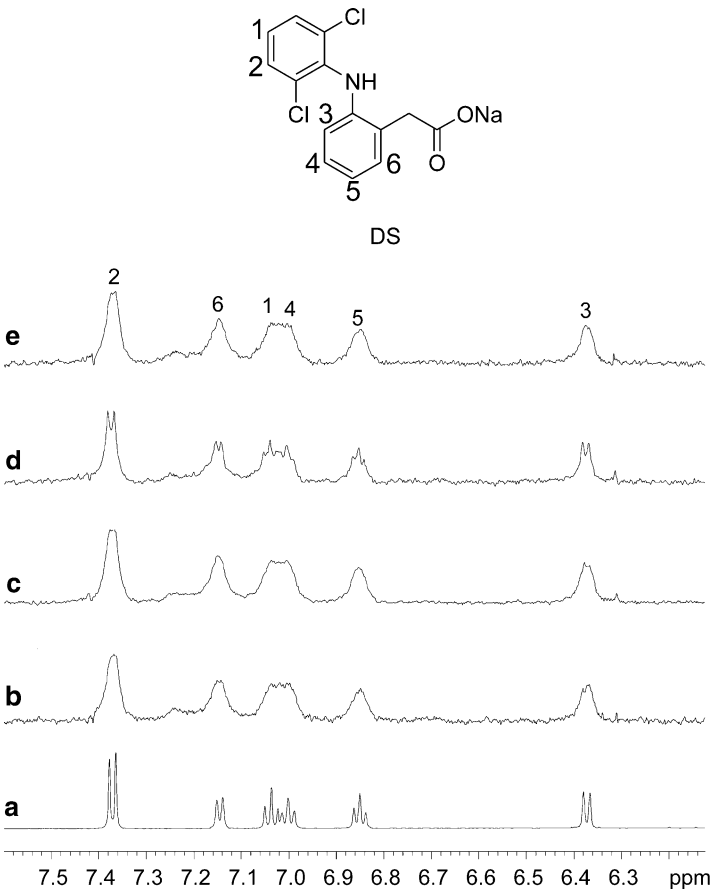
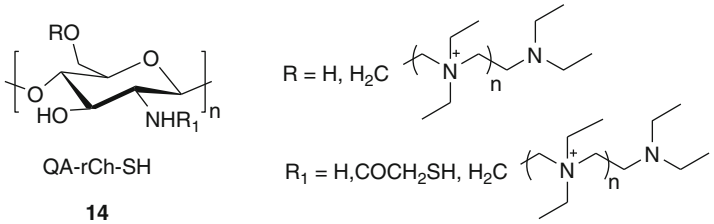


Fig. 3 ^{1}H NMR (600 MHz, D_2O, 25 °C) spectra of DS (2 mM) (**a**) in the presence of BSM (10 mg mL^{-1}) (**b**) and in the quaternary mixtures DS/BSM/TSP/HA at different TSP/HA molar ratios (total concentration 3 mg mL^{-1}): 5:1 (**c**), 1:1 (**d**), 1:5 (**e**)

importantly, the relaxation rates of the drug in the ternary mixtures dexamethasone/nanoparticles/mucin were not additive with respect to the binary mixtures drug/mucin and drug/nanoparticles, to indicate that nanoparticles formed stable aggregates with mucin.



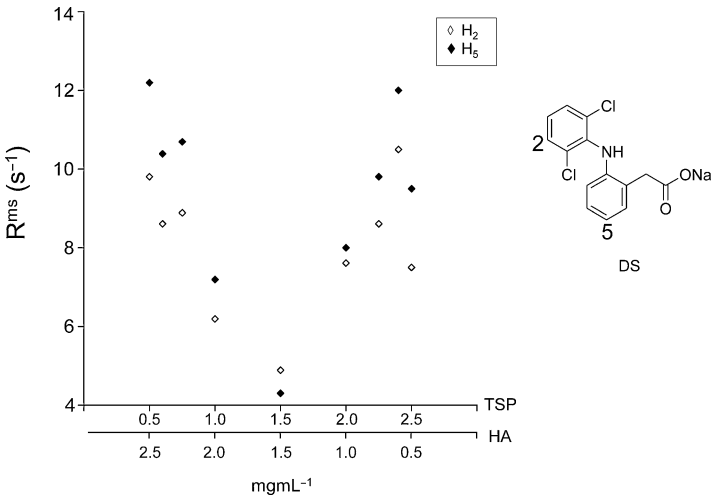


Fig. 4 Dependence of mono-selective relaxation rates of protons H_2 and H_5 of DS (2 mM) in quaternary mixtures with BSM (10 mg mL^{-1}) and TSP/HA at different molar ratios (total concentration 3 mg mL^{-1})

4 Conclusion

NMR spectroscopy has been largely exploited in the field of structural characterization of polysaccharides with proven mucoadhesivity, thus giving a fundamental support to synthetic procedures. On the contrary, in vitro evaluation of their mucoadhesion properties by NMR, although very attractive in terms of noninvasiveness, is still rather limited, but large room is left to future improvements also in view of possible simultaneous applications to the problem of the controlled release properties of mucoadhesive polysaccharide materials in the form of nanostructured aggregates.

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Transition-Metal-Catalyzed Transformation of Monosaccharides and Polysaccharides

45

Zuzana Hricovíniová

Contents

Abstract

The exploitation of transition metals in carbohydrate chemistry has achieved considerable developments in the last decades. This contribution gives an overview of transition-metal-catalyzed transformations of various mono- and polysaccharides into enantiopure compounds, fine chemicals, and building blocks suitable for industrial application. These recent applications in this area have made this field of chemistry very attractive. The utilization of carbohydrate–transition-metal complexes have become a powerful tool for forming and breaking of the carbon–carbon bonds. Particular attention is given to recent developments and advances in the use of Mo(VI) ions as highly stereospecific catalyst in carbohydrate synthesis, highlighting the representative examples from the last decade. In the second part, the review describes transition-metal catalytic systems that are involved in the of transformation natural polysaccharides (cellulose, starch, xylans) into important organic intermediates (5-hydroxymethylfurfural, furfural). It is shown that particularly the application

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of Mo(VI)-catalytic system, in combination with microwave irradiation, in transformation of starch and xylans offers interesting alternative for the preparation of valuable of monosaccharides and corresponding alditols (mannose, xylose, lyxose, mannitol, xylitol, lyxitol). The emphasis is primarily placed on high stereoselectivity in catalytic reactions and transformation of renewable carbohydrate biomass as promising source of organic raw materials for production of useful platform chemicals, rare sugars, and fuels.

Keywords
Carbohydrates • Catalysis • Transition metals • Mo(VI)-catalyzed rearrangement • Isomerization • Microwave irradiation • HMF • Furfural • Mannose • Mannitol • Xylitol

1 Introduction

Carbohydrates are the most abundant biomolecules in Nature and important structural components of biological systems. They play essential roles in living organisms as energy sources and building blocks and are involved in a number of fundamental biological processes (such as cell–cell communication, cell growth and differentiation, bacterial infections, autoimmune disorders, etc.) (Feizi 1993; Dwek 1996; Rudd et al. 2001). In addition, naturally occurring polysaccharides are bio-resources and renewable feedstock that can be used in nutrition and as raw materials. Carbohydrates occur as monosaccharides, oligosaccharides, polysaccharides, as well as components of glycoconjugates. From the chemical point of view, carbohydrates are unique due to their structural diversity having multiple stereocenters and numerous functional groups. Each monosaccharide unit possesses multiple attachment sites and can be linked in a variety of linear or branched forms. These structural features make carbohydrates one of the most complex classes of organic compounds. The study of carbohydrates in biological systems has focused mostly on glycoconjugates, such as glycoproteins or glycolipids. Carbohydrate-containing biopolymers are among the major components of the living cell where they provide the normal course of highly specific biological processes. Some glycosylated natural products have been used as antimicrobial agents, as biological probes for clinical diagnosis, and in recent years as promising anticancer drugs (Kim and Varki 1997; Freeze and Aebi 2005; Geyer and Geyer 2008). However, many functions of carbohydrates and glycoconjugates in living organisms remain still unknown. In order to understand all details of carbohydrate roles and preparation of drug candidates, chemists and biochemists focus their efforts not only on analysis of their functions (e.g., chiral molecules as single-enantiomer chemical intermediates serve as tools for biological studies) but on preparation and testing new carbohydrates and their derivatives as well. To achieve this goal, there is a need for development of new chemical methods for targeted synthesis of complex carbohydrates and glycoconjugates. Particularly, chemist's efforts have been aimed at utilization of unconventional energy sources, novel activating reagents, or

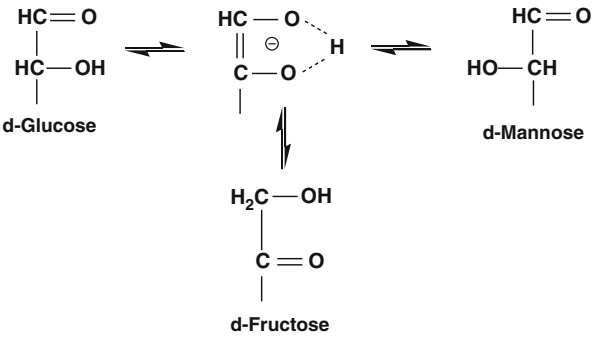
introduction of new effective catalysts. Several reviews have been published in this field (Pratt and Bertozzi 2005; Lichtenthaler 2010).

Catalysis plays central role in many chemical and biochemical processes and has been subject of numerous studies. Crucial role of catalytic functions of enzymes has been recognized long time ago and, together with transition-metal complexes, belongs to the most efficient asymmetric catalysts. However, the inorganic catalysts are usually more stable, readily available, and less expensive and can be applied in demanding reaction conditions. Consequently, catalytic role of metallic ions in saccharide transformations can significantly simplify complex synthetic procedures, and this approach belongs to the most promising synthetic methods at present. Transition-metal complexes of carbohydrates exhibit unique and outstanding properties, which have been discussed in several reviews (Yano 1988; Angyal 1989; Alekseev et al. 1998). The complex formation between sugar molecule and metal ion contributes to superior atom efficiency, usually avoiding several protection and deprotection steps. This strategy allows direct synthesis of structurally complex molecules in a relatively simple way, thus transition metal-catalyzed carbon–carbon bond-forming reactions have become widely used in organic synthesis.

The aim of this review is to describe reactions based on Mo(VI)-catalyzed skeletal rearrangement of carbohydrate backbone. Particular attention will be given to recent advances in the use of Mo(VI) ions as highly stereospecific catalyst in carbohydrate synthesis, highlighting the representative examples from the last decade, especially, to the recent applications of this interesting transformation. In the second part, discussion will be presented on recent progress in the direct transition-metal-catalyzed transformations of polysaccharides which has facilitated access to rare sugars and useful platform chemicals.

2 Isomerization Reactions of Reducing Saccharides

The isomerization of sugars is an important reaction in various essential metabolic pathways and industrial processes. The isomerization of the most common sugar, D-glucose to D-fructose, can be performed under mild conditions using either biological or chemical catalysts. In vivo, these rearrangements are catalyzed by enzymes with high regio- and stereoselectivity. The glucose is converted to fructose, the sweetest one of the natural sugars, through isomerization reaction. Fructose syrups from this process compete with saccharose (beet or cane sugar) in many food applications because they are less expensive than saccharose. In industrial production of these natural sweeteners, starch is hydrolyzed with hydrolase enzymes that convert it into glucose syrup. In order to produce fructose syrups, the glucose is further processed in the presence of isomerase enzymes, which convert D-glucose into its isomer, D-fructose (Schomburg and Salzmann 1990). However, as all biological catalysts, isomerases require very specific reaction conditions (precise control of process variables, such as pH, temperature, etc.) that make biochemical processes relatively costly. Several different ways are presented in the literature for



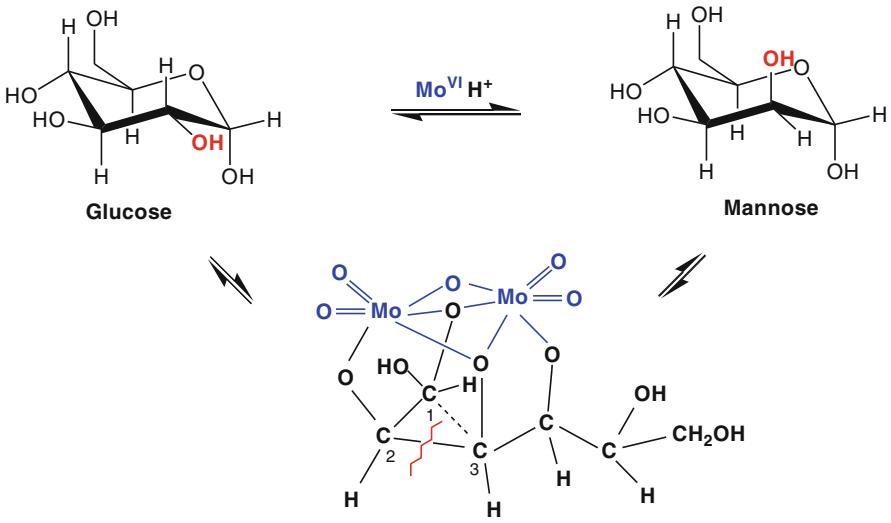
Scheme 1 The Lobry de Bruyn–Alberda van Ekenstein reaction. Equilibrium mixture of glucose–mannose–fructose with 1,2-enediol. *Cis*-form is much more stable, because it is stabilized by an intramolecular hydrogen bond

chemical conversion of saccharides to their isomers besides the enzyme technology. Reducing sugars in open-chain forms can undergo enolization to form different isomers. The reaction is favored in alkaline solutions but can also take place under acidic conditions. This conversion is known as the Lobry de Bruyn–Alberda van Ekenstein rearrangement (Speck 1958; Angyal 2001) and occurs via the base-catalyzed 1,2-enolization of an aldose or ketose to enediol, followed by isomerization. The most investigated case is the glucose–mannose–fructose system, where these three sugars are in equilibrium with 1,2-enediol (Scheme 1).

The Lobry de Bruyn–van Ekenstein reaction has been used for the synthesis of many sugars, and its mechanism has been extensively studied. This reaction can be of preparative use in favorable cases, especially when structural features of starting sugar and products minimize competing reactions (Lichtenthaler and Ronninger 1990). A good example is the preparation of D-psicose from D-allose (Steiger and Reichstein 1936) and preparation of 6-*deoxy*-L-fructose from L-rhamnose (Ennifar and El Khadem 1989) that proceeds in good yields. This reaction was the simplest method for the synthesis of rare monosaccharides for a long period. However, the epimeric aldose is usually obtained in a smaller yield than the ketose, so Lobry de Bruyn–van Ekenstein reaction is not a very practical method for the preparation of epimeric aldoses. The reason for that is that beside the aldose–ketose isomerization a series of side reactions proceed such as aldose–aldose epimerization, inversion of configuration C-3 carbon, or degradation reactions followed by aldol condensation, or another concurrent reactions. In order to minimize these side reactions, the reaction time has to be considerably shortened and, consequently, the equilibrium mixture is seldom reached.

The interactions between saccharides and metal ions have been studied for a long time. The examination of the carbohydrate carbon skeleton upon the formation of metal complexes is an interesting area of the coordination chemistry. It was observed that isomerization reaction proceeds at C-2 carbon without ketose formation when transition metals are used as catalysts. With the development of newer methods, the Lobry de Bruyn–van Ekenstein transformation is of interest only for synthesis of ketoses from aldoses.

One of the best examples of this kind of isomerization reactions was described by Bílik in 1972 when he presented highly stereospecific C-2-epimerization



Scheme 2 Mo(VI)-catalyzed epimerization of aldoses (Bílik reaction)

reaction of aldoses catalyzed by molybdate ions (Bílik 1972a, b, c; Bílik et al. 1972). He found a simple way to use inorganic catalyst in place of the enzymes to generate pure sugar epimers. The Mo(VI)-catalyzed mutual interconversion of epimeric aldoses became a crucial achievement in preparative carbohydrate chemistry. Extensive studies on this catalyzed transformation have been performed using a wide range of epimeric pairs of aldoses to map the scope and limitations of this unique stereospecific carbon-skeletal rearrangement. This rearrangement involves a C-1/C-2 interconversion within the carbon skeleton, wherein the bond between C-2 and C-3 carbon is cleaved and a new bond between C-1 and C-3 is formed. Consequently, the C-2 position has an inverted configuration (Scheme 2).

It was concluded that treatment of an acidic aqueous solution of aldose, in the presence of a catalytic amount of molybdic acid for several hours, gives rise to an equilibrium mixture of two epimeric aldoses without formation of the complementary ketose. The epimeric aldose with lower value of conformational instability is prevailing in the thermodynamic equilibrium mixture, and this equilibrium is the same independently of whether aldose is used as starting material. This stereospecific mutual transformation is applicable for all aldoses in both D- and L-series. Epimerization of aldoses catalyzed by molybdate ions, named after its discoverer (Bílik reaction), become soon the simplest and the most convenient general method for the widespread production of rare monosaccharides. Implementation of the Bílik reaction into practice has made rare aldoses efficiently available. Starting from the less-expensive, more accessible aldoses, it is possible to prepare in one-step reaction rare sugars (e.g., D-talose, D-lyxose, L-ribose, L-glucose, D-mannose, D-altrose, etc.) (Bílik 1972a, b, c; Petruš et al. 2001). Later, other catalytic

systems have been described allowing similar interconversions of aldoses. However, all these catalytic systems have not been as widely exploited as the reaction catalyzed by Mo(VI) ions.

The formation and catalytic effect of transition-metal complexes (Ni^{2+}, Co^{2+}, Ca^{2+}, and Sr^{2+}) on the epimerization reaction depends mainly on their structure (Ishida et al. 1989; Osanai 2001; Tanase et al. 2001). Though glucose–mannose epimerization can be achieved by Ni(II)–tetramethyl-ethylenediamine complex, the yields are lower than those obtained by Mo(VI) catalyst. The mechanism of Ni(II)-catalyzed transformation was extensively studied (Yamauchi et al. 1990; Osanai et al. 1991). The epimerization reaction catalyzed with Ca(II) complexes proceeds via a different mechanism. The results obtained with various aldoses clearly showed that the "mannose-type" aldoses are not isomerized in Ca(II)-tetramethyl-ethylenediamine system (Yamauchi et al. 1990), but Ca^{2+} ions in methanol alone (Yanagihara et al. 1997) lead to epimerization without the ketose formation. According to Angyal (Angyal 1980), these results can be explained by the stability of the various complexes formed between aldoses and Ca^{2+}ions. Reaction between D-glucose and several metal hydroxides in water demonstrate that isomerization proceed only with calcium hydroxide with the formation of all three isomers (Glc/Man/Fru) (Sowden and Schaffer 1952; Angyal and Mills 1979).

3 Microwave-Assisted Reactions in Carbohydrate Synthesis

As mentioned in Introduction, chemists' efforts have been focused also on utilization of unconventional energy sources to improve the conventional methods of organic synthesis. In this respect, microwave-assisted chemistry has gained much popularity as it is very efficient and environmentally friendly technique.

Microwave heating (MW) is based on the interaction of electromagnetic field (wavelengths in the microwave region) with the matter. Molecules having permanent dipole moment tend to align to the applied electromagnetic field. As the field alternates, molecules with dipole moments rotate in order to align with the field. These molecular motions (rotations and collisions) lead to a heat generation (called also dielectric heating) (Caddick 1995; Lidström et al. 2001). The dielectric heating depends mainly on the polarity of molecules (electrical dipole moment). Typically, water and carbohydrates are molecules exhibiting dipole moments. As water is fairly polar molecule, it efficiently "absorbs" MW energy. Consequently, the energy transfer is quite efficient to the absorbing reactants during the MW heating in aqueous solutions. Microwave heating offers several advantages over conventional heating, such as noncontact heating, energy transfer instead of heat transfer (radiation), material effective heating, and fast start-up and stopping.

Dielectric heating generally shows large difference on reaction times, higher yields, and purities of products compared to conventional conditions. Moreover, in many cases the MW synthesis is considered as environmental friendly since the reactions are more efficient and less wasteful than traditional methods. Since microwaves can transfer energy directly to the reactive species, they can promote

transformations that are currently not possible. This is especially in cases when conventional methods require demanding chemical conditions, sensitive reagents, or prolonged reaction times. The advantages of using MW dielectric heating were soon realized and resulted in the increased number of published scientific papers. Microwaves were effectively applied to diverse fields of chemistry, such as organic synthesis (Loupy 2002; Kappe and Stadler 2005), nanotechnology (Tompsett et al. 2006; Taylor et al. 2009), biochemistry (Lill et al. 2007; Nesatyy et al. 2007), and medicinal chemistry/drug discovery (Kappe and Dallinger 2006; Collins and Leadbeater 2007).

Carbohydrate chemistry is special area that can benefit from MW radiation. Carbohydrates because of their complexity, arising from their stereochemistry and multifunctionality, are difficult synthetic targets. Furthermore, as many complex carbohydrate molecules are heat sensitive, chemical reactions preparing or modifying carbohydrates are particularly suitable for application of MW heating. For example, rearrangement reactions represent very useful approach for the stereospecific production of isomeric structures leading to diverse natural products. In this case, high demands in activation energy in the reactions, especially isomerization processes, can be completed in a short time applying MW irradiation. The beneficial effect of MW irradiation, which is direct, fast, selective, and controllable, has been employed to accelerate various organic reactions, including alkylations, condensations, nucleophilic substitutions, cycloadditions, protection, and deprotection reactions with the optimal use of material and energy. The intrinsic advantages of MW irradiation in chemical transformations, the impact, and future potential are illustrated in a number of articles and reviews (Das 2004; Richel and Paquot 2012; Corsaro et al. 2013).

4 Mo(VI)-Catalyzed Transformations of Saccharides

Monosaccharides form various types of complexes with molybdate ions in aqueous solutions. Types of the complexes depend on the solution pH and steric arrangement of the hydroxyl groups (Porai-Koshits and Atovmyan 1974). Structures of molybdate complexes of reducing sugars have been extensively studied in aqueous solution mainly by ^{1}H, ^{13}C, and ^{95}Mo NMR spectroscopy. Data showed that saccharides can create bidentate, tridentate, or tetradentate complexes (Matulová and Bílik 1990a, b; Sauvage et al. 1992; Matulová and Bílik 1993; Sauvage et al. 1996; Matulová and Hricovíniová 2002). A few structures have also been determined by X-ray crystallography (Hedman 1977; Taylor and Waters 1981; Ma et al. 1989). Epimerization of aldoses is based on the ability of molybdate ions to catalyze stereospecific rearrangement that proceeds via acyclic dimolybdate–saccharide complexes. The acyclic hydrated aldehyde forms of aldoses are linked in the binuclear molybdate core as tetradentate ligands via their hydrated carbonyl group and three adjacent hydroxyls (bound on carbons C-2, C-3, and C-4 (Fig. 1)). Only these complexes are considered as catalytically active and can mediate the stereospecific rearrangement. The formation of the molybdate complex influences the

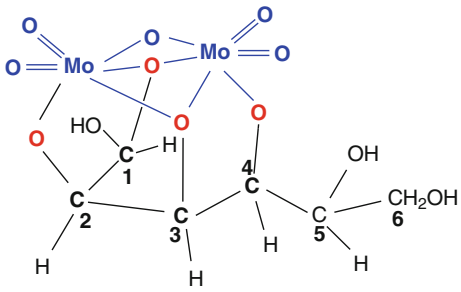


Fig. 1 The acyclic form of aldose linked in the binuclear molybdate core as tetradentate ligand via hydrated carbonyl group C-1 and three hydroxyl groups attached to carbons C-2, C-3, and C-4

development of the thermodynamic equilibrium for epimeric pairs of corresponding aldoses that can be reached in very different periods of time. The most advantageous pH range for epimerization reaction is pH 2.5–3.0.

Recent reports showed that the epimerization of aldoses can be facilitated by combined action of Mo(VI) catalyst and MW irradiation (Hricovíniová 2006). Such approach has much greater potential than would correspond to two independent processes and leads to the considerable improvement in activity and selectivity of the isomerization reaction. This remarkable transformation proceeds by unusual stereospecific rearrangement of carbohydrate skeleton and thus opens up exciting opportunities for the production of various rare sugars using Mo(VI) catalyst. In addition, molybdate ions were further examined as stereospecific catalyst associated with C–C bond-forming reactions. New methodology was successfully applied for the preparation of various sugar derivatives and led to the efficient synthesis of epimeric aldoses (Hricovíniová 2006), ketoses (Hricovíniová 2008a), *deoxy*-aldoses (Hricovíniová 2009), 6-*O*-aryl-aldoses (Hricovíniová et al. 2010), 2-*C*-branched-chain aldoses (Hricovíniová-Bíliková et al. 1999), and 3-*C*-branched-chain aldoses (Hricovíniová and Hricovíni 2013). This approach was also effective in the case of aldoses bearing nitrogen in the branch (Hricovíniová 2010). Transformation proceeds very efficiently, and investigation of Mo(VI)-catalyzed interconversions was therefore further extended to several disaccharides (Hricovíniová 2008b) and polysaccharides (Hricovíniová 2011, 2013).

4.1 Mo(VI)-Catalyzed Isomerization of 2-*C*- and 3-*C*-Branched Aldoses

Branched saccharides occur in nature and have been the subject of intense biochemical studies. Despite their low natural abundance, they have enormous potential in several important applications. They often serve as essential biosynthetic precursors, structural elements, biological probes, pharmaceuticals, etc. Higher-carbon saccharides have been particularly interesting because of the fact that they can be used as non-metabolized analogues of oligosaccharides or components of antibiotics (Grisebach and Schmid 1972; Weizman and Tor 2003). Several enzymatic methods toward preparation of higher-carbon sugars have been developed

(Izumori 2002; Beerens et al. 2012), but the complex nature of biochemical processes makes them very costly.

Epimerization of aldoses catalyzed by molybdate ions has become an effective method for preparation of C-2 epimers (Bílik et al. 1975; Hayes et al. 1982; Petruš et al. 2001). Based on the current knowledge of Mo(VI) complexes of monosaccharides, one could expect the similar mutual interconversion between 2-ketoses and 2-*C*-(hydroxymethyl)-aldoses. The possibility of extension of this approach for the synthesis of other rare, biologically active carbohydrates has been verified on Mo(VI)-catalyzed mutual interconversions of 2-ketoses and 2-*C*-(hydroxymethyl)-aldoses. It was shown that treatment of 2-*C*-(hydroxymethyl)-D-glucose and 2-*C*-(hydroxymethyl)-D-mannose (Hricovíniová et al. 1998a) and D-fructose (Hricovíniová et al. 1998b) with Mo(VI) ions results in dramatic structural changes. Mechanistic studies with the isotopically substituted saccharides have shown that the interconversion of 2-ketoses and 2-*C*-(hydroxymethyl)-aldoses, with mutually inverted positions of hydroxyl groups at their carbon atoms C-2 and C-3, is a consequence of a highly stereospecific carbon skeleton rearrangement. In this respect Mo(VI)-catalyzed isomerization of 2-ketoses and 2-*C*-(hydroxymethyl)-aldoses is very similar process to the mutual interconversion of epimeric aldoses. Two 2-*C*-(hydroxymethyl)-branched-chain aldohexoses are rearranged stereospecifically and reversibly to D-*manno*-heptulose and D-*gluco*-heptulose, respectively. Accordingly, D-fructose provided an equilibrium reaction mixture with its isomer 2-*C*-(hydroxymethyl)-D-ribose (D-hamamelose) under identical reaction conditions. The carbon skeleton rearrangement during this transformation was confirmed by ^{13}C and ^{1}H NMR analysis of D-(2-^{13}C) fructose conversion to D-(2-^{13}C) hamamelose (Hricovíniová-Bíliková et al. 1999). This new, one-step approach was examined on several other pairs of isomers, starting from 2-pentuloses to 2-nonuloses. The equilibria in all cases strongly favored 2-ketoses, and the isomeric ratios in thermodynamic equilibrium mixtures were established by NMR spectroscopy (Table 1).

Table 1 Equilibrium mixtures of Mo(VI)-catalyzed mutual isomerization of 2-ketoses and 2-*C*-(hydroxymethyl)-branched aldoses obtained under conventional conditions

Equilibrium mixture of 2-ketose and corresponding 2-*C*-(hydroxymethyl)-aldose	
D-Ribulose/2-*C*-(hydroxymethyl)-D-threose	11:1
D-Xylulose/2-*C*-(hydroxymethyl)-D-erythrose	10:1
D-Fructose/2-*C*-(hydroxymethyl)-D-ribose	14:1
6-*Deoxy*-L-fructose/2-*C*-(hydroxymethyl)-5-*deoxy*-L-ribose	10:1
L-Sorbose/2-*C*-(hydroxymethyl)-L-lyxose	32:1
D-Tagatose/2-*C*-(hydroxymethyl)-D-xylose	19:1
D-*Gluco*-heptulose/2-*C*-(hydroxymethyl)-D-mannose	12:1
D-*Manno*-heptulose/2-*C*-(hydroxymethyl)-D-glucose	26:1
D-*Altro*-heptulose/2-*C*-(hydroxymethyl)-D-allose	12:1
D-*Ido*-heptulose/2-*C*-(hydroxymethyl)-D-gulose	36:1
7-*Deoxy*-L-*gluco*-heptulose/2-*C*-(hydroxymethyl)-L-rhamnose	9:1

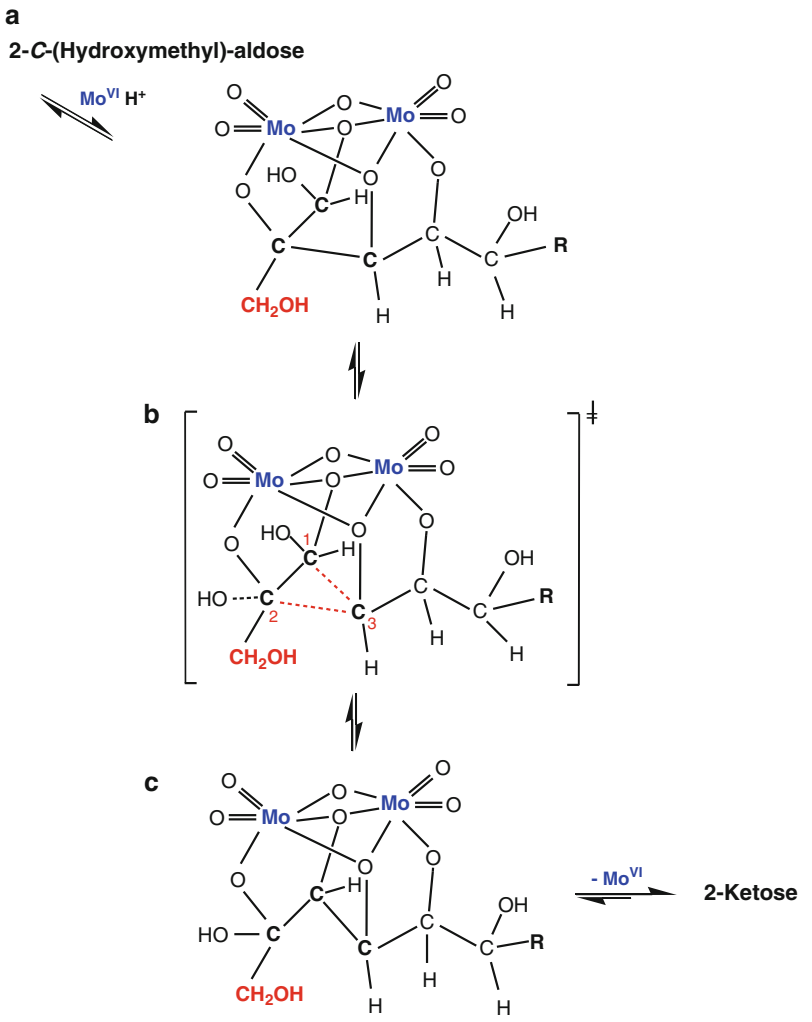
The transformation is initiated by the formation of catalytically active molybdate complexes that promote this unique stereospecific rearrangement. A possible mechanism of the isomerization of 2-*C*-(hydroxymethyl)-branched aldose to 2-ketose has been suggested on the basis of the known capability of molybdate ions to form complexes with monosaccharides. Effective, stereospecific transformation occurs in the case of 2-*C*-(hydroxymethyl)-branched aldoses with C-2–C-3 bond cleavage and transposition. The transformation proceeds via acyclic dimolybdate–saccharide complexes (Scheme 3). The formation of the dimolybdate complex with the carbonyl-oxygen atom C-1 and the adjacent three hydroxylic oxygen atoms at C-2, C-3, and C-4 of the 2-*C*-(hydroxymethyl)-branched aldose (Scheme 3a) leads to the transition state (Scheme 3b) in which branched saccharide function as bidentate ligand bound to the metal center. The design of ligand is the key issue for the catalytic process. The critical C-2–C-3 and new C-1–C-3 bond is formed stereospecifically. Dissociation of the complex produces either the starting 2-*C*-(hydroxymethyl)-branched aldose or the isomeric 2-ketose (Scheme 3c) generated by the stereospecific rearrangement. The thermodynamic equilibrium favors in this case the 2-ketoses (Hricovíniová-Bíliková et al. 1999).

4.2 Mo(VI)-Catalysis in Preparation of Rare Ketoses

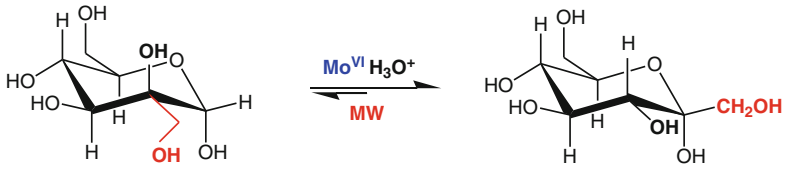
2-*C*-(Hydroxymethyl)-branched aldoses with the C-2, C-3 *erythro* configuration are easily accessible via the base-catalyzed aldolization of their 2,3-*O*-isopropylidene derivatives with formaldehyde (Ho 1978). Treatment of 2-*C*-(hydroxymethyl)-branched aldose with the catalytic amount of molybdic acid in microwave field afforded the thermodynamic equilibrium mixture of the starting branched-chain aldose and the corresponding 2-ketose in several minutes (Scheme 4).

It was observed that the reaction proceeded efficiently with excellent yields and the reaction rates were dramatically enhanced. The same reaction under conventional conditions took 5–8 h to afford comparable yields. Due to easily accessible 2-*C*-(hydroxymethyl)-aldoses, the method is especially advantageous for the one-step preparation of rare higher ketoses. The scope and generality of this stereospecific transformation was illustrated on various 2-*C*-(hydroxymethyl)-branched aldoses (Table 2).

The comparison of these results clearly shows that MW irradiation markedly accelerates the isomerization process and also causes the differences in equilibration of reaction mixtures. As indicated from the data in Table 2, the effect of MW irradiation increases the yields of 2-ketoses up to 10 %, but the beneficial effect of microwaves is significant mainly from the point of view of the reaction kinetic of studied transformation. The reaction time decreased from hours to minutes, that is, up to 160-fold shorter than in the case of conventional oil-bath heating and, at the same time, obtaining the better yields of cleaner products without the formation of



Scheme 3 The Mo(VI)-catalyzed isomerization of 2-*C*-(hydroxymethyl)-branched aldose (**a**), transition state (**b**), and corresponding 2-ketose (**c**)



2-*C*-(hydroxymethyl)-d-mannose

d-*Gluco*-Heptulose

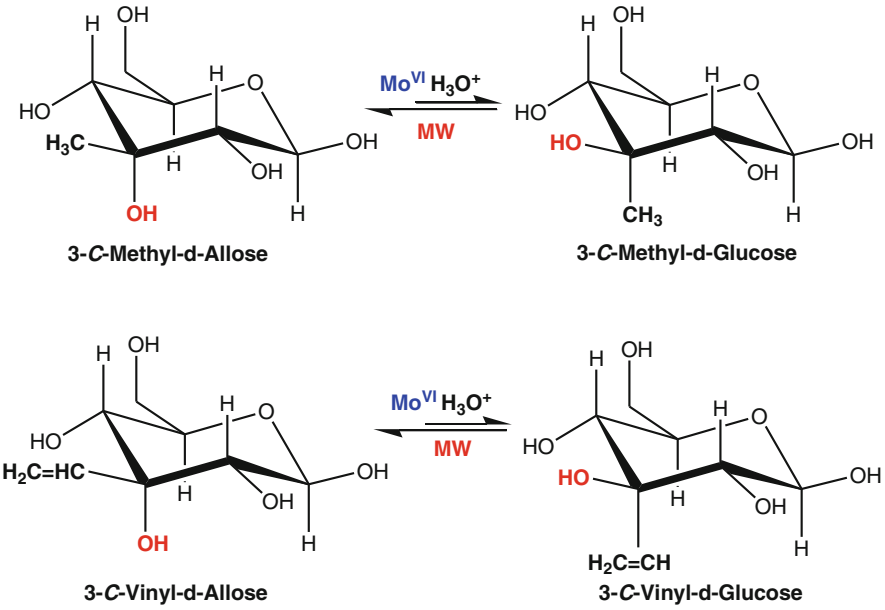
Scheme 4 The Mo(VI)-catalyzed isomerization of 2-*C*-(hydroxymethyl)-branched D-mannose and D-*gluco*-heptulose

Table 2 The comparison of the Mo(VI)-catalyzed isomerization of 2-*C*-(hydroxymethyl)-branched aldoses to 2-ketoses in microwave field and under conventional conditions

		MW filed		Conventional heating	
2-*C*-(hydroxymethyl)-branched aldose	Product 2-ketose	Time (min)	Yield (%)	Time (min)	Yield (%)
2-*C*-(hydroxymethyl)-D-glucose	*Ido*-heptulose	3	86	420	83
2-*C*-(hydroxymethyl)-L-rhamnose	7-*Deoxy*-*gluco*-heptulose	3	85	360	77
2-*C*-(hydroxymethyl)-D-mannose	*Gluco*-heptulose	3	85	120	80
2-*C*-(hydroxymethyl)-D-*gulo*-heptose	*Ido*-octulose	3	46	480	40
2-*C*-(hydroxymethyl)-D-*talo*-heptose	*Galacto*-octulose	3	65	360	59
2-*C*-(hydroxymethyl)-D-*manno*-octose	*Gluco*-nonulose	3	76	300	61

side products. Performing this reaction under MW conditions leads to respectable yields (46–86 %) in several minutes (Hricovíniová 2008a).

The rate enhancement under MW irradiation may be attributed to the absorption of more energy by the polar media, which generates sufficient heat energy to promote this demanding isomerization process. As these reactions occur with high stereoselectivity, there must be high enough difference in activation energy for processes leading to two stereoisomers. The method was tested on preparation of D-*ido*-heptulose (Hricovíniová 2001), 7-*deoxy*-L-*gluco*-heptulose (Hricovíniová 2009), D-*gluco*-heptulose (Hricovíniová et al. 1998a), D-*glycero*-D-*ido*-octulose (Hricovíniová et al. 2001), D-*glycero*-L-*galacto*-octulose (Hricovíniová 2002), and D-*erythro*-L-*gluco*-nonulose (Hricovíniová 2007). The method is universal and leads to rare ketoses in a single step. This approach is suitable also for preparative purposes because both isomers can be very effectively separated by chromatography. In addition, it provides useful alternative in chemical synthesis of rare or biologically active carbohydrates with scarce natural occurrence.



Scheme 5 The Mo(VI)-catalyzed mutual isomerization of 3-*C*-branched alloses and glucoses in MW field

Apart from the C-2 isomerization, which yields C-2 isomeric products, a new type of rearrangement has also been described very recently (Hricovíniová and Hricovíni 2013). In this case, C-3 isomers of 3-*C*-branched aldoses were prepared in a simple way without formation of side products. This type of reaction was demonstrated on selected model 3-*C*-branched-chain aldoses. For example, 3-*C*-methyl-D-allose and 3-*C*-vinyl-D-allose were rapidly interconverted to their isomers, 3-*C*-methyl-D-glucose and 3-*C*-vinyl-D-glucose (Scheme 5).

The structure of 3-*C*-methyl-D-glucose was confirmed by NMR chemical shifts, proton–proton coupling constant, and NOE spectra as well as theoretical analysis. ^{1}H NMR spectrum (Fig. 2) of the reaction mixture contained new doublets at 5.18 and 4.66 ppm, respectively, which were assigned to the pyranose anomeric protons H-1 of the 3-*C*-methyl-D-glucose.

Theoretical analysis (using DFT calculations) agreed with experimental data and supported $^4C_1$ β-pyranose form of glucose derivative. The presence of the methyl group at C-3 was confirmed not only by the characteristic ^{1}H resonance (originating from the CH_3 resonance linked to C-3) but by two-dimensional NOE spectra as well.

As it was explored, a specific catalytically active molybdate complex is formed producing isomeric 3-*C*-methyl-branched aldose. According to the data, the structure of the ligand seems to be a key issue for the catalytic process: Aldoses epimerize at C-2 carbon producing *epi*-aldoses (Scheme 6a). Aldoses bearing hydroxymethyl-substituent at C-2 carbon isomerize under the formation of 2-ketoses (Scheme 6b).

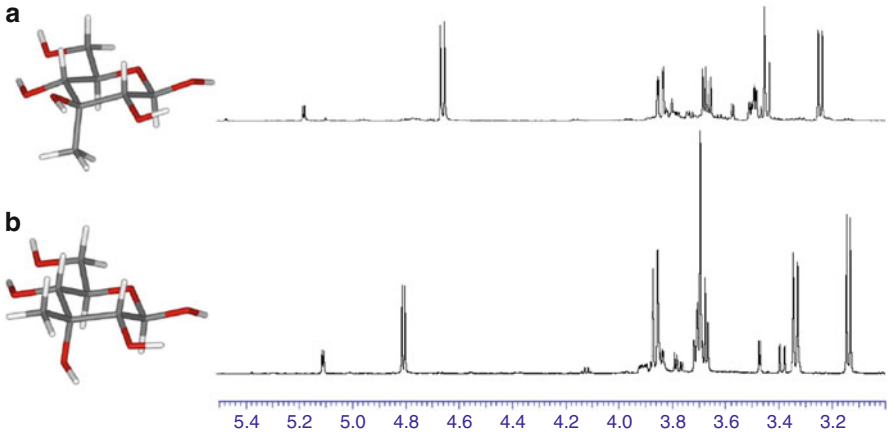


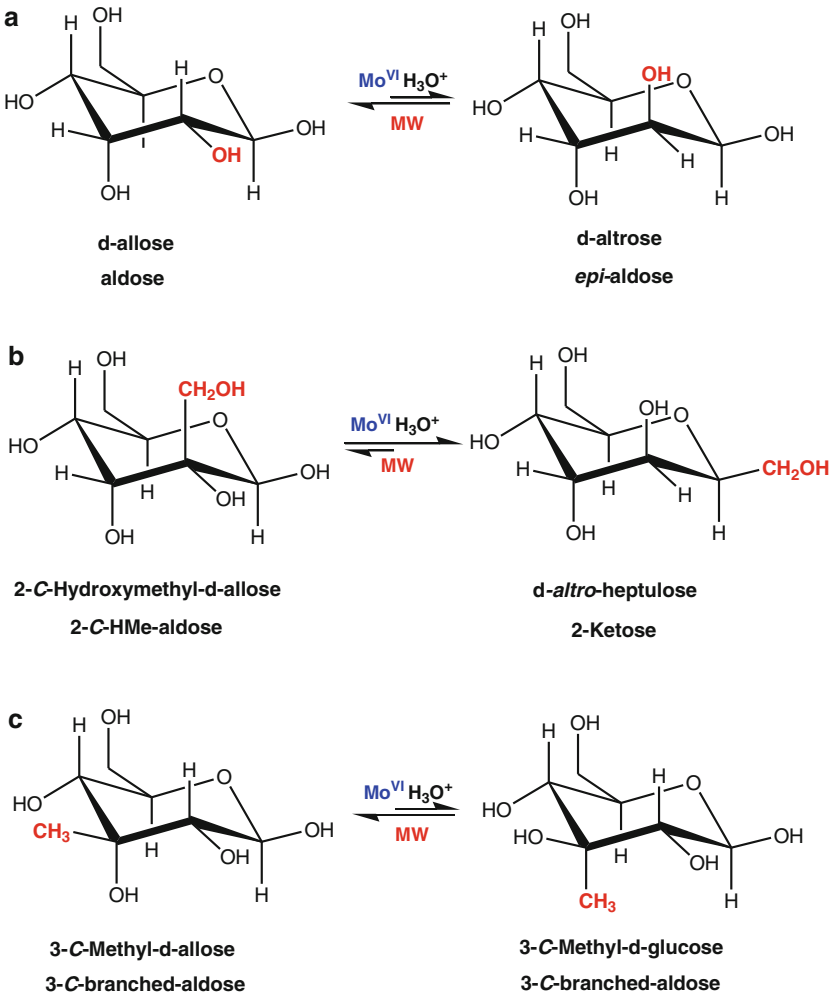
Fig. 2 High-resolution 600 MHz ^{1}H NMR spectra of 3-*C*-methyl-D-allose (**a**) and 3-*C*-methyl-D-glucose (**b**) at 25 °C in D_2O. Insets show DFT-optimized structures of these branched aldoses

3-*C*-branched aldoses with sterically accessible substituents (such as methyl or vinyl) isomerize at C-3 carbon (Scheme 6c), whereas bulky substituents (e.g., phenyl, nitromethyl) linked to C-3 carbon prevent any isomerization.

This new type of rearrangement allows preparation of isomeric products of 3-*C*-methyl- and 3-*C*-vinyl-branched-chain aldoses via C-3 isomerization utilizing the synergic effect of Mo(VI) ions and MW field. The reaction leads to rare saccharides in a single step and provides excellent alternative to conventional methods in chemical synthesis of rare carbohydrates. It also provides insight into the development of other stereoselective transformations used in the synthesis of natural products.

4.3 Mo(VI)-Catalysis in the Preparation of Amadori Ketoses

Amino sugars are another important class of bioactive compounds having broad spectrum of applications in biochemical and pharmaceutical fields (Collins and Ferrier 1995). The substitution of a hydroxyl function by an amino group may alter the properties of the sugar molecule significantly in terms of its solubility, hydrogen-bonding properties, and electronic distribution. The most abundant example is D-glucosamine (2-*amino*-2-*deoxy*-D-glucose), but various amino sugars are frequently occurring components of biological systems. As a consequence, amino sugars play important physiological roles in many glycoconjugates and are of interest for the development of new drugs, biological substrates, and reference standards (Pelyvas et al. 1988; Stütz 1999). Particularly carbohydrates bearing azido functions have emerged as valuable intermediates. Their chemical stability and easy reduction make them attractive compounds for obtaining the corresponding amino derivatives. There are numerous approaches for the synthesis



Scheme 6 The comparison of Mo(VI)-catalyzed epimerization/isomerization reactions of aldoses (**a**) 2-*C*-(hydroxymethyl)-branched aldoses (**b**) and 3-*C*-(methyl)-branched aldoses (**c**)

of amino sugars. Most of them are based on naturally occurring carbohydrates and involve several protecting group manipulations as well as other synthetic steps (Nicotra et al. 2008). An important concept of preparation of amino sugars as synthetic intermediates for glycoarray synthesis is Amadori rearrangement (Amadori 1931). Reaction between α-hydroxy aldehydes and suitable amines leads to α-amino ketones. When applied to aldoses, it allows the introduction of the amino group at position C-1 with concomitant isomerization leading to 1-*amino*-1-*deoxy*-ketoses (Amadori ketoses). The rearrangement is known as an initial step of the Maillard reaction cascade. The dark-colored rearrangement products formed in this reaction are responsible for the nonenzymatic browning

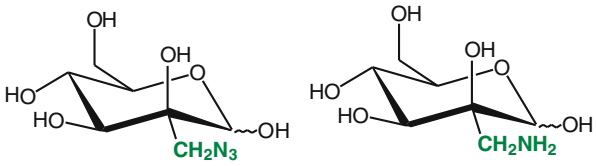
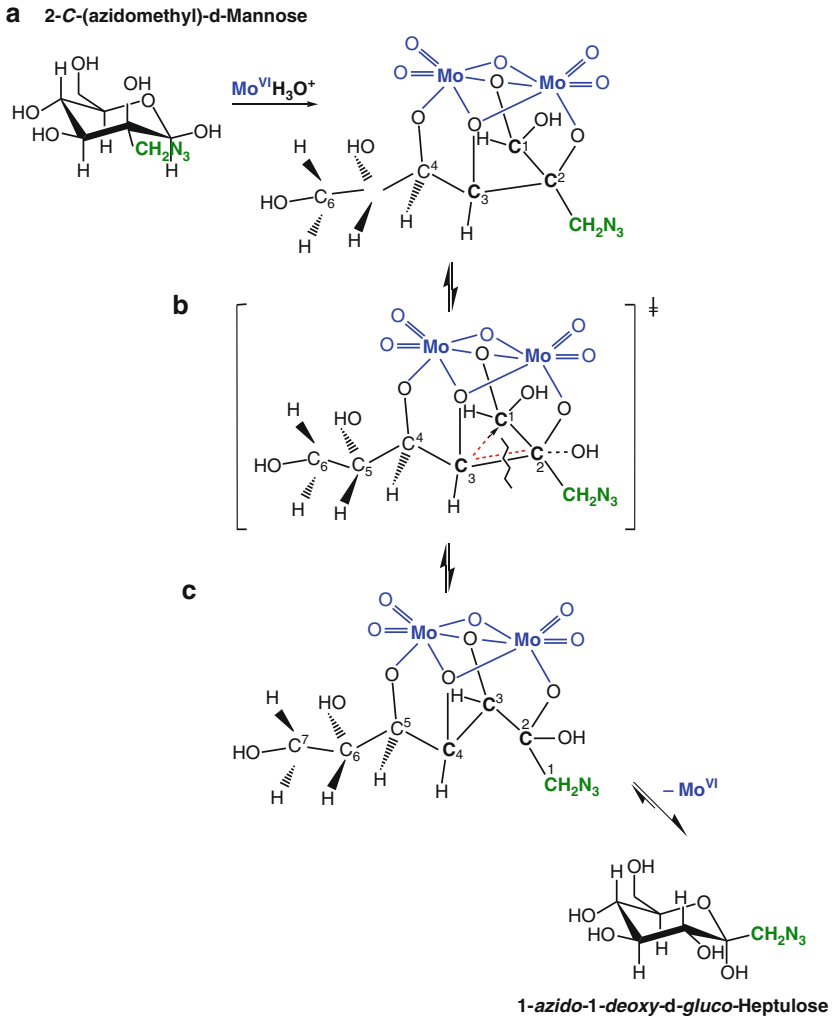


Fig. 3 2-*C*-Azidomethyl-D-mannose and 2-*C*-aminomethyl-D-mannose

of food (Maillard 1912; Yaylayan et al. 1994). The Amadori rearrangement allows a simple introduction of the amino moiety to C-1 position of a carbohydrate. However, the preparative use of this rearrangement is rather limited as several reversible steps occur during the isomerization as well as a number of side and degradation products. Only a few preparatively useful examples are known owing to their difficult isolation from the obtained reaction mixture. Especially aliphatic glycosylamines are very sensitive to hydrolysis and give the rearrangement products only under very specific conditions. According to the relevance of Amadori ketoses in biological processes, several investigations of their roles in the pathological effects of diabetes and ageing processes were conducted (Raabe et al. 1996; Posthuma et al. 2001). Some authors have suggested that blocking of the ketone group in Amadori compounds and their autoxidation might prevent the formation of glycation end products and thus avoid their pathological effects as a result (Asif et al. 2000; Adrover et al. 2008). Additionally, synthesis and evaluation of biological activities of such compounds are of growing interest.

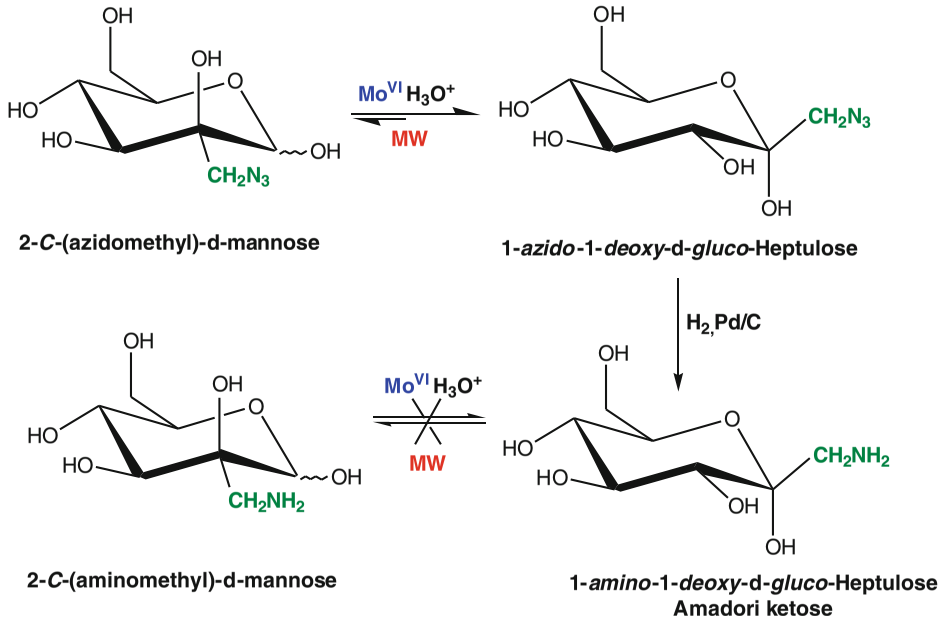
Based on the concept that utilizes the catalytic effect of molybdate ions, an efficient and stereodivergent synthesis of Amadori ketoses was developed (Hricovíniová 2010). The branched-chain aldose bearing azido group at position C-2 provides access to the corresponding 1-*azido*-1-*deoxy*-ketoses and 1-*amino*-1-*deoxy*-ketoses in a single step through a stereospecific isomerization reaction. The transformation was studied using nitrogen-bearing branched-chain saccharides. 2-*C*-azidomethyl-D-mannose and 2-*C*-aminomethyl-D-mannose were selected as the model compounds for examination of the reaction conditions for Mo(VI)-catalyzed rearrangement of carbon skeleton (Fig. 3).

The stereospecific transformation involves the formation of molybdate complexes of branched saccharides bearing azido function. Branched-chain aldose 2-*C*-azidomethyl-D-mannose was subjected to react with a catalytic amount of molybdic acid in acidic aqueous solution. A smooth isomerization reaction took place in MW field and gave the desired product in excellent yield. The equilibrium mixture was reached very fast, and the starting branched aldose was converted to 1-*azido*-ketose in almost quantitative yield (98 %). The same equilibrium mixture can be obtained using conventional heating (oil bath at 80 °C). The mechanism for the isomerization of 2-*C*-(azidomethyl)-D-mannose into 1-*deoxy*-1-*azido*-D-*gluco*-heptulose was suggested (Hricovíniová 2010). The transformation, as in previous cases, proceeds via acyclic dimolybdate–saccharide complexes. Based on studies with isotopically labeled sugars and stereochemical rules of molybdate complexes, the mechanism of the molybdic acid-catalyzed mutual interconversion of the 2-*C*-(azidomethyl)-D-mannose and 1-*azido*-1-*deoxy*-D-*gluco*-heptulose is shown in Scheme 7.



Scheme 7 The Mo(VI)-catalyzed isomerization of 2-*C*-(azidomethyl)-branched aldose (**a**), transition state (**b**), and 1-*azido*-1-*deoxy*-ketose (**c**)

As the isomerization reaction with the 2-*C*-azidomethyl-branched D-mannose progressed flatly in MW field, 2-*C*-aminomethyl-D-mannose acted quite differently (Scheme 8). Intramolecular isomerization did not take place in the case of second model compound. 2-*C*-aminomethyl-D-mannose did not isomerize to corresponding 1-*amino*-1-*deoxy*-D-*gluco*-heptulose even under much stronger reaction conditions. Possible explanation of this fact was based on the theoretical DFT analysis of the α-anomer of 2-*C*-aminomethyl-D-mannose structure. The obtained data showed that the pyranose ring adopts the chair $^4C_1$ form having hydroxymethyl and aminomethyl groups in equatorial positions. Calculations also revealed the



Scheme 8 Mo(VI)-catalyzed transformation of 2-*C*-azidomethyl- and 2-*C*-aminomethyl-branched D-mannose in MW field

strong intramolecular hydrogen bond between OH-2 proton and amine nitrogen. Such types of hydrogen bonds are rather frequent in glycoconjugates (Luisi et al. 1998; Mukherjee et al. 2005). Other two intramolecular hydrogen bonds are located between OH-4...O-6 and OH-3...O-2. These strong hydrogen bonds stabilize structure in the form that prevents formation of catalytically active complex with Mo(VI).

Experimental results demonstrate that molybdate ions can form catalytically active complexes with 2-*C*-azidomethyl-branched sugars and promote the isomerization process leading to 1-*azido*-1-*deoxy*-ketoses in a single step. Although 2-*C*-aminomethyl-D-mannose did not directly isomerize to 1-*amino*-1-*deoxy*-D-*gluco*-heptulose, desired Amadori ketose is easily accessible via catalytic hydrogenation from stable azido derivative. Thus, catalytic hydrogenation of 1-*azido*-1-*deoxy*-D-*gluco*-heptulose afforded quantitatively the corresponding 1-*amino*-1-*deoxy*-D-*gluco*-heptulose (Scheme 8). Preparative use of this approach leading to Amadori ketoses is not limited as it is in the case of Amadori rearrangement. There are no difficulties with reversible steps, formation of side products, and isolation of desired product in excellent yields.

5 Chemical Transformations of Polysaccharides

Carbohydrates, mostly present in polymeric forms, are classified among the most intensively studied precursors with a wide range of properties and applications. The use of environmentally friendly processes for the preparation of various chemical compounds from annually renewable biomass is very important today (Anastas and Warner 2000). Decreasing fossil fuel reserves calls for the development of new, long-term, and sustainable chemical sources. Fossil-based carbon resources can be replaced by carbohydrates that are renewable in nature. Carbohydrates are abundant all over the world as a product of the light-induced fixation of carbon dioxide in plants via photosynthesis. The product, D-glucose, as energy-rich organic molecule can be further transformed into other more complex organic compounds. In this regard, polysaccharides like starch, cellulose, lignocelluloses, or xylans could be the primary target resources for the sustainable chemical industry.

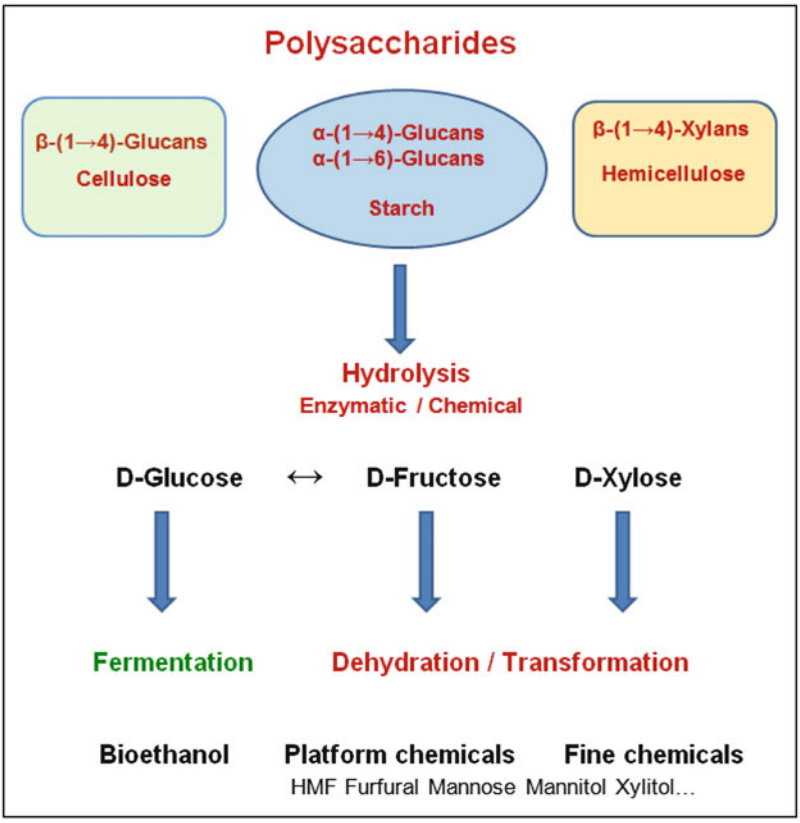
Carbohydrates in their oligomeric and polymeric forms can be easily converted to a wide range of useful intermediates by enzymatic or chemical processes (Belgacem and Gandini 2008). The rate of hydrolysis of a polysaccharide is a function of its structure. In general, α-glycosidic linkages are hydrolyzed more readily than β-glycosidic linkages. Polysaccharides, such as arabans and fructans, whose monosaccharide units occur as furanosides, can be rapidly and completely hydrolyzed under mild acidic conditions. Polysaccharides consisting of pentose units in pyranose form are more resistant. More concentrated mineral acids, especially hydrochloric acid, decompose the pentoses to furfural. Stronger hydrolytic conditions are necessary for polysaccharides composed of hexopyranoside units. The treatment with sulfuric or hydrochloric acid at 100 °C is usually sufficient, but more drastic conditions are necessary in some cases.

Considerable efforts have been done in order to achieve efficient polysaccharide depolymerization and utilization of the obtained monosaccharides (e.g., glucose, fructose, xylose) as a source for synthesis of many industrial chemicals. Dehydration reactions of carbohydrates are important in pulping, in food industry (Maillard reaction) (Saltmarch and Labuza 1982; Ledl and Schleicher 1990), and in the production of bulk chemicals. Extensive research has been initiated worldwide to convert carbohydrates into valuable products, such as ethanol, furfural, 5-hydroxymethylfurfural, and levulinic acid (Huber et al. 2006; Corma and Iborra 2007; El-Zawawy et al. 2011). It is clear that many methods are now available for converting biomass to biofuels and that biomass conversion processes will continue to have industrial interest. In recent years, an increasing effort has been devoted to find cheap and effective methods for the production of furan derivatives which are readily usable for industrial scale-up. Two basic nonpetroleum chemicals are accessible from renewable resources: furfural (2-furaldehyde) arising from the acid-catalyzed dehydration of pentoses and hydroxymethylfurfural (5-hydroxymethyl-2-furaldehyde, HMF) arising from the acid-catalyzed dehydration of hexoses. These compounds are suitable starting materials for the preparation of further monomers required for polymer applications, such as polyamides, polyesters, and polyurethanes. From the commercial point of view, furfural is currently produced on an industrial scale using mineral acids as catalysts, but HMF is not yet a high-volume chemical, partly due to the currently high production costs (Lewkowski 2001; Chheda et al. 2007; Binder et al. 2010). Both HMF and furfural are strategic compounds, which can be derived from agricultural waste products composed from polysaccharides. Thus these versatile and multifunctional compounds are good starting material for the production of useful organic intermediates, fine chemicals, pharmaceutical precursors, polymers, solvents, surface-active agents, and fuels (Scheme 9).

5.1 Transition-Metal-Catalyzed Transformation of Cellulose

Cellulose, hemicelluloses, and starch are the major natural polymers in the world. This widely abundant and renewable carbohydrate biomass is a valuable alternative resource. It mainly consists of oligo- and polysaccharides that can be hydrolyzed to monosaccharides (e.g., glucose, xylose, mannose, galactose, arabinose) by various methods. Monomeric carbohydrates are the most important materials that can be directly used as feedstock for the production of many bio-based chemicals.

Cellulose is a natural polysaccharide that is insoluble in water. The molecules of this straight-chain polymer are composed from two D-glucoses linked uniformly by β-1→4-glycosidic bonds. Breakage of these glycosidic linkages by acids leads to the hydrolysis of cellulose polymers. The first step for cellulose utilization is to depolymerize it into soluble oligosaccharides and monomeric glucose. Unfortunately, the natural polymer forms a robust crystal structure with high chemical stability, thus making the depolymerization processes difficult. The crystallinity of cellulose results from a complex network of intra-strand and



Scheme 9 Transformation of polysaccharides

inter-strand hydrogen bonds (Nishiyama et al. 2003). In order to dissolve cellulose, a solvent must compete for these intermolecular hydrogen bonds. Consequently, acid-catalyzed cellulose hydrolysis is a complex heterogeneous reaction that involves physical factors as well as the hydrolytic chemical reaction (Ott et al. 1965; Zugenmajer 2008).

Multiple conditions are now established to facilitate cellulose hydrolysis. It can be hydrolyzed enzymatically by cellulases into fermentable products. However, there are many factors affecting cellulase accessibility to cellulose, such as high microcrystallinity of cellulose, extensive intermolecular hydrogen bonding, and van der Waals forces (Zhang et al. 2010; Hosseini and Shah 2011).

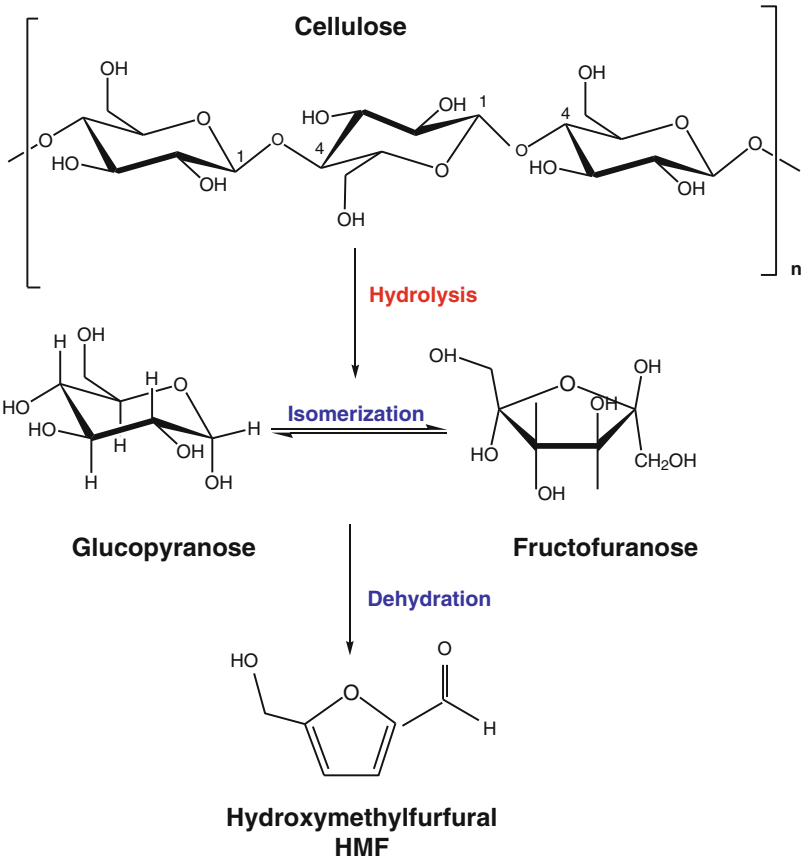
Much work has been devoted on the hydrolysis of cellulose to glucose with mineral acids, such as sulfuric acid (H_2SO_4) or hydrochloric acid (HCl). However, the large-scale use of acid suffers from several problems such as reactor corrosion and catalyst recovery and requires treatment of the acid residue, producing lots of waste (Camacho et al. 1996; Kim et al. 2001). Recently, the increasing attention has gained the application of solid acids for cellulose hydrolysis (Vigier and Jérôme 2010).

The main advantage of solid acid catalysts is that they reduce the pollutants, the product separation is easier, they are recyclable, and they cause less damage to the reactor. Although cellulose hydrolysis has been developed with solid acids toward the greener synthesis of sugars, the reaction efficiency and selectivity still need to be improved. The introduction of ionic liquids was the further step that improved cellulose dissolution and allowed the reaction to proceed more easily under milder conditions. The mechanism of dissolution by ionic liquids is accomplished by disruption of the hydrogen-bonding network forming the electron donor–electron acceptor complexes with the hydroxyl groups of cellulose. As ionic liquids are charged species, they efficiently de-aggregate the polymer strands allowing effective dissolution (Pinkert et al. 2009; Zakrzewska et al. 2010).

As mentioned above, cellulose can be hydrolyzed to glucose and then further transformed into the platform chemicals. HMF is probably one of the most important biomass-derived platform molecules. Actually, it can be prepared through the catalyzed dehydration of glucose or fructose and transformed into highly valuable intermediates. HMF is suitable for the preparation of liquid biofuels, alternative polymers, and macrocyclic compounds or for precursors of pharmaceuticals (Vigier and Jérôme 2010; Rosatella et al. 2011). While HMF can currently be made from fructose and glucose, the ability to synthesize HMF directly from natural cellulose would remove a major barrier to the development of a sustainable HMF platform (Scheme 10).

Thirty years ago, van Dam (van Dam et al. 1986) and Cottier (Cottier et al. 1989) showed that both aqueous and nonaqueous dehydration processes lead to around 37 % yield of HMF. They found that the reactions performed in the aqueous solution increase the degradation of HMF. Furthermore, the polymerization of HMF occurs in both aqueous and nonaqueous media. The acid-catalyzed dehydration of hexoses (D-glucose and D-fructose) to HMF in aqueous media has received considerable attention, but there are still many problems associated with the selectivity and yields of this transformation. The dehydration rate of glucose is about 40 times lower than that of fructose, and much lower is also the product yield. Kinetics studies show that the initial glucose or fructose concentration, the acidity of the aqueous medium, and the presence of various catalysts are factors that influence the conversion of the sugars to HMF (Kuster and van der Baan 1977; Kuster 1990). Fructose has been selected by many researches as the preferred feedstock for the production of HMF. Excellent yields of HMF have been readily achieved applying various methods. The dehydration of D-fructose can generally be catalyzed by protonic acids as well as by Lewis acids. Sulfuric, phosphoric, and hydrochloric mineral acids are the most commonly used to catalyze the transformations of carbohydrate materials (Feather and Harris 1973).

Kuster and van Bekkum reported an exhaustive study of all parameters influencing the course of dehydration of fructose in water as the most convenient solvent (Van Dam et al. 1986). Some improvements were obtained when using acidic ion-exchange resins in water instead of mineral acids. However, any significant selectivity improvement in production of HMF was achieved in this case (Mercadier et al. 1981). The highest selectivities in HMF have been obtained in



Scheme 10 Catalyzed transformation of cellulose

dimethyl sulfoxide (DMSO) as the solvent. Fructose was selectively and quantitatively converted into HMF in the absence of catalyst as well as in the presence of ion-exchange resins. It has been suggested that DMSO preferentially stabilizes the furanoid structure of fructose (five-membered ring) to the pyranoid form (six-membered ring), indicating that the dehydration of fructofuranose is more HMF selective. The advantage of DMSO as the solvent is that it is the dipolar aprotic solvent and prevents the formation of levulinic acid and humins. Its disadvantage is the separation of DMSO, HMF, and water formed and the formation of toxic by-products arising from the decomposition of the solvent (Musau and Munavu 1987).

However, it should be noted that D-fructose is not abundant in nature because it mainly exists as a part of oligosaccharides, in fruits and honey. Moreover, fructose is very expensive because its manufacturing process is very complicated. Glucose which is the monomeric unit of various polysaccharides (mainly cellulose and starch) is a better candidate for this purpose. With its low cost and vast availability,

the conversion of D-glucose to HMF has attracted increasing interest in recent years. However, it is difficult to obtain high yields of HMF from glucose in water (Feather and Harris 1973), organic solvents (Rasrendra et al. 2012), or biphasic systems (Yang et al. 2012), because glucose tends to form a stable six-membered pyranoside structure that is unfavorable to HMF formation. Thus, the effective transformation of D-glucose into HMF remains a challenge. Extensive research efforts are currently performed to develop efficient and sustainable methods to prepare HMF. However, there is still no intensive commercial production of HMF from inexpensive carbohydrates up to now.

To overcome this disadvantage, the use of organic and inorganic salts in the synthesis of HMF in aqueous solution was the subject of numerous works. An interesting catalytic pathway was revealed for the rapid conversion of cellulose to sugars and further to HMF in a single-step process. Recent studies showed that D-glucose can be converted to HMF in good yields in nonaqueous systems using transition-metal elements as the catalysts. The reaction from D-glucose to HMF is generally assumed to involve isomerization of D-glucose to D-fructose followed by dehydration to HMF (Scheme 9). Recently, some significant progress for the metal-catalyzed dehydration of hexoses has been reported (Zhao et al. 2007; Yong et al. 2008).

Transition-metal oxides have catalytic activity for cellulose hydrolysis, and when used as solid acid catalysts, they are reusable and easily separated from reaction products. Mesoporous transition-metal oxides, such as Zr-TMS (transition-metal oxide mesoporous molecular sieves) (Chidambaram et al. 2003) Nb_2O_5, WO_3 (Tagusagawa et al. 2010a), TiO_2 (Kondo et al. 2005), and Ta_2O_5-WO_3 (Tagusagawa et al. 2010b), are widely studied as heterogeneous acid catalysts (Takagaki et al. 2008). Metal oxides are widely used as catalyst supports because of their thermal and mechanical stability, high specific surface area, and large pore size. Sulfonated metal oxides, such as SO_4^{2-}/Al_2O_3, SO_4^{2-}/TiO_2, SO_4^{2-}/ZrO_2, SO_4^{2-}/SnO_2, and SO_4^{2-}/V_2O_5, can supply many acidic species because they operate the same as H^+ for cellulose hydrolysis (Yang et al. 2011; Delaney et al. 2011). The catalysts are active in heterogeneous liquid–solid systems and are recoverable and reusable. However, one limitation of these types of solid catalysts is that the acidic sites become leached from silica surfaces under hydrolytic conditions.

Metallic chlorides in ionic liquids were reported to be an exceptional system capable to convert glucose into HMF in high yield. Zhang and coworkers (Zhao et al. 2007) developed the application of metal salts in 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) as the solvent. The yields of 60–70 % for HMF prepared from glucose were reported when applying $CrCl_2$/EMIMCl as catalytic system (Pidko et al. 2010). The efficiency of the catalytic system is attributed to its ability to isomerize glucose to fructose, which is more easily dehydrated. Similar results were obtained by Hu et al. using $SnCl_4$ as the catalyst in a series of ionic liquids (Hu et al. 2009). It was found that $SnCl_4$ could efficiently convert glucose to HMF in 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF_4). The authors proposed that the formation of the five-membered ring chelate complex of the Sn

atom and glucose plays a key role for the HMF formation. The highest yield of HMF in $SnCl_4$/[EMIm]BF_4 system was about 60 %.

Chou and coworkers used $MnCl_2$ in the acidic ionic liquid 1-(4-sulfonic acid) butyl-3-methylimidazolium hydrogen sulfate ([SA-BMIM]HSO_4) to transform cellulose to HMF (Tao et al. 2011). Ionic liquids combined with lanthanide-based catalysts were also applied for the direct conversion of glucose into HMF. Stahlberg et al. reported only moderate yields (up to 32 %) when a lanthanide salt ($YbCl_3$) was used as catalyst (Stahlberg et al. 2010). Zhao and coworkers used germanium salt ($GeCl_4$) as catalyst in ionic liquid [BMIM]Cl with HMF yield of about 38 % (Zhang et al. 2011). Very recently, the addition of other chlorides, $NbCl_5$, $TaCl_5$, and VCl_3, were applied in ionic liquids to dehydrate D-fructose in HMF (Mittal et al. 2012). The highest yield in HMF production was reported over $NbCl_5$ catalyst. The authors suggested that the remarkable HMF selectivity obtained with these chlorides was due to their weak Lewis acidity, while strongest Lewis acids would favor side reactions.

It was observed that paired ($CuCl_2$/$CrCl_2$) catalysts in [EMIM]Cl exhibited remarkably high activity for hydrolytic cellulose depolymerization. Cellulose depolymerization occurs at a rate that is about one order of magnitude faster than conventional acid-catalyzed hydrolysis. In contrast, single metal chlorides showed considerably less activity under similar conditions. Thus a single-step conversion of cellulose to HMF has been successfully performed with a pair of metal chlorides $CuCl_2$/$CrCl_2$ in [EMIM]Cl solvent, and a 55–59 % yield of HMF was obtained (Su et al. 2009).

Direct transformation of cellulose and sugarcane bagasse into HMF was carried out using single or combined metal chloride catalysts in *N*,*N*-dimethylformamide (DMF) as a solvent. Binder and Raines used $CrCl_2$ and $CrCl_3$ in a solvent mixture of *N*,*N*-dimethylacetamide (DMA)–LiCl and [EMIM]Cl (Binder and Raines 2009). In this solvent system, glucose was transformed into HMF in comparable yields (62 % using $CrCl_2$ and 67 % using $CrCl_3$). Moreover, the system was suitable for cellulose dissolution, and by using $CrCl_3$ a moderate yield of HMF (48 %) was obtained from the corn stover biomass material, along with a 37 % yield of furfural. Though promising, the use of metal salts in ionic liquids has limitations due to the relatively high price of ionic liquids, requiring very efficient recycling strategies. Zhang and coworkers successfully used a pairing of $CrCl_3$ with ammonium halides to transform sucrose, glucose, and fructose into HMF. Application of NH_4Br in DMA afforded conversion to HMF in 87 %, 74 %, and 92 % yields (Wang et al. 2012). The lanthanide metals have been found to serve as Lewis acids in their ionic forms to catalyze the conversion of various saccharides into HMF. The high yields of HMF (>90 %) could be obtained from fructose using the lanthanide chlorides in DMSO (Seri et al. 2000). Another study has shown that the lanthanide (III) ions could dehydrate hexoses in water without rehydration to levulinic and formic acids (Seri et al. 2001).

It was shown recently that both chromium and aluminum salts promote the isomerization of D-glucose to D-fructose in water (with only marginal yields of HMF 10 %). Recently, good yields of HMF were obtained from fructose, glucose,

Table 3 Examples of various transition-metal catalysts used in the transformation of saccharides into HMF in ionic liquids or organic solvents

Saccharide	Catalyst	Solvent	HMF (%)	References
Glucose	$YbCl_3$	[OMIM]Cl	23	Stahlberg et al. 2010
Glucose	$Yb(OTf)_3$	[BMIM]Cl	24	
Cellulose	$MnCl_2$	[SA-BMIM]HSO_4	37	Tao et al. 2011
Fructose	$SnCl_4$	[EMIM]BF_4	62	Hu et al. 2009
Glucose	$SnCl_4$	[EMIM]BF_4	61	
Sucrose	$SnCl_4$	[EMIM]BF_4	65	
Cellobiose	$SnCl_4$	[EMIM]BF_4	57	
Fructose	$CrCl_3$	[EMIM]Cl	70	Zhao et al. 2007
Glucose	$CrCl_2$	[EMIM]Cl	68	
Fructose	NaBr	DMA	93	Binder and Raines 2009
Glucose	$CrCl_2$	DMA/LiCl/[EMIM]Cl	62	
Glucose	$CrCl_3$	DMA/LiCl/[EMIM]Cl	67	
Cellulose	$CrCl_3$/HCl	DMA/LiCl/[EMIM]Cl	54	
Fructose	NHC/$CrCl_2$	[BMIM]Cl	96	Yong et al. 2008
Glucose	NHC/$CrCl_2$	[BMIM]Cl	81	
Fructose	$LaCl_3$	DMSO	95	Seri et al. 2000
Fructose	$NdCl_3$	DMSO	91	
Fructose	$EuCl_3$	DMSO	92	
Fructose	$DyCl_3$	DMSO	93	
Fructose	$LuCl_3$	DMSO	95	
Sucrose	$CrCl_3$	DMA/NH_4Br	87	Wang et al. 2012
Fructose	$CrCl_3$	DMA/NH_4Br	92	
Glucose	$CrCl_3$	DMA/NH_4Br	74	

sucrose, and inulin (60 %, 31 %, 43 %, and 46 %, respectively) using $CrCl_3$ as catalyst (Ilgen et al. 2009). Among several metal chloride catalysts studied, combined catalyst $Zr(O)Cl_2/CrCl_3$ was the most effective enabling conversion to HMF 57 % from cellulose fiber. $Zr(O)Cl_2/CrCl_3$ catalyst was also effective for the conversion of sugarcane bagasse to HMF and 5-ethoxymethyl-2-furfural (EMF) another promising biofuel (Dutta et al. 2012). Indium trichloride was also used as catalyst for the conversion of monosaccharides into HMF and levulinic acid (LA) in aqueous media. It was found that $InCl_3$ could not only catalyze the isomerization of glucose to fructose but also have the positive effects on the dehydration of monosaccharides. Li et al. found that $InCl_3$/acidic ion-exchange resin/ionic liquid catalytic system was effective for the conversion of cellulose to HMF. However, the expensive ionic liquid restricted its extensive application. The application of $InCl_3$ catalyst for the conversion of biomass in aqueous media was also tested (Li et al. 2013; Shen et al. 2014). Several examples of metal catalysts used for the transformation of saccharides into HMF are illustrated in Table 3.

Examples of various transition-metal catalysts used in the transformation of saccharides into HMF in ionic liquids or organic solvents.

5.2 Transition-Metal-Catalyzed Transformation of Starch

Plants store polysaccharides as a food reserve. The most important of these reserves are starches, fructans, mannans, and galactomannans. Starch is the most abundant carbohydrate reservoir in plants and can be found in the grains of all cereal crops including rice, wheat, maize, barley, tapioca, etc. The structure of starch varies widely between plant species and differs in chemical composition as well. Starch granules are largely composed of carbohydrates but also contain minor constituents (fatty acids, minerals, phosphorus), which may influence the properties of the granules. Starch is the second most abundant biopolymer in Nature after cellulose. In particular, because starch is available at relatively lower cost than other sugars (such as fructose and glucose), it could be a cost-effective biomaterial for their mass production. Starch possesses valuable properties like biodegradability, lower environmental contamination, adhesive properties, ability to make layers, etc. High amounts of starch are used in food, textile, and papermaking industries (Steve 2004; Belgacem and Gandini 2008).

Starch varieties contain primarily two structurally different polysaccharides. One is a linear molecule termed amylose, and the other is branched structure termed amylopectin. Both are composed of D-glucopyranose units. Amylose is primarily a linear macromolecule forming predominately single chain. In amylose, 250–300 units are uniformly linked by α-1→4-glucosidic linkages. Such structure tends to induce a spiraling of the molecule in a helix-like fashion. In amylopectin the majority of the units are connected 1→4 with α-links, but there are α-1→6-glycosidic bonds at the branch points. Amylopectin makes up 70–80 % of most starch varieties and so is the major component, strongly influencing the physiochemical characteristics of starch (Bertolini 2010).

The present overview was conducted to examine the effect of metal salts on the acid hydrolysis and other transformations of starch using various reaction conditions. It has already been demonstrated that starches form Werner-type complexes with the non-transition- (Lai et al. 2001) and transition-metal (Ciesielski et al. 2003) cations. Acetates, nitrates, and chlorides of Co(II), Cu(II), Fe(III), Mn(II), and Ni(II) were ligated with starch, and the formation of polycenter Werner complexes was widely studied by Tomasik et al. (Tomasik et al. 1989). The coordination of starch to central metal ions influenced the thermal decomposition of starch and resulted in the slower decomposition compared to the decomposition of non-coordinated starch. Stepwise decomposition of starch potentially provides series of novel dextrins. Thus, properly understood effect of coordination upon the course of thermolysis might lead to custom-made dextrins of a wide range of application (Ciesielski and Tomasik 2003).

Considerable efforts have been done to achieve efficient starch depolymerization and utilization of the obtained monosaccharides for synthesis of many chemicals. Starch can be easily converted by enzymatic hydrolysis or chemical catalytic processes to a wide range of chemical intermediates and useful products used in the food and pharmaceutical industries (Östergård et al. 1988; Quignard et al. 2010). It can be hydrolyzed to glucose and other oligosaccharides by acid

catalysis and further transformed by thermal dehydration. As a starting biomaterial for sustainable synthesis of HMF, starch has a higher viability than other biopolymers, such as cellulose, because the depolymerization process of starch is much easier than that of cellulose.

The effect of MW heating has been widely studied in chemistry of polysaccharides (Corsaro et al. 2013). Microwave reactors produce efficient heating by direct transfer of MW energy to molecules (solvents, reagents, or catalysts) present in the reaction mixtures. Microwave dielectric heating is a very efficient process and results in higher rate of polysaccharide depolymerization. There are several reports describing the use of MW heating in the degradation of starch from different sources, such as wheat, rice, potato, and corn starches (Khan et al. 1979; Lewandowicz et al. 2000). Treatment or degradation of starch using microwaves usually use water or dilute mineral acid (such as HCl or H_2SO_4) as a reaction medium. The reported concentration of starch suspension varied from 1 % up to 50 % in most of studies, but usually 10 % of starch suspension was used. Yu et al. showed that 10 % starch solution in dilute HCl was completely hydrolyzed within several minutes of MW irradiation without the formation of colored by-products (Yu et al. 1996). In contrast, the retrograded starch in similar solutions has not hydrolyzed when conventional methods were used. Thus MW irradiation not only increases the rate of energy transfer but can also change the structure (or conformation) of the reactant accelerating the reaction times.

The addition of inorganic salts is another factor that can enhance the starch hydrolysis. It is known that addition of salts to solvents can increase their conductivity and the solution of substance is heated up more rapidly than by other convective–conductive heating sources, such as oil bath. Furthermore, it was observed that different kinds of salts manifest different effects during starch hydrolysis (Mingos and Baghurst 1991; Kunlan et al. 2001). It was found that starch could be hydrolyzed to D-glucose completely in a short time in the presence of metal halides. The maximum yield of D-glucose was reached using metal halides LiCl, $BaCl_2$, and $FeCl_3$, with 120–180 s of MW irradiation. The difference between metal halides and sulfate salts illustrate that the properties of the salts are very important in affecting the hydrolysis reaction in a MW field. Results showed that Na_2SO_4, $MgSO_4$, and $ZnSO_4$ did not accelerate the acid hydrolysis of starch, and the highest yield of D-glucose was only 12.6 % in the presence of Na_2SO_4. However, when $Ce(SO_4)_2$ was employed, the yield of D-glucose reached 88.9 %, and thus $Ce(SO_4)_2$ facilitates hydrolysis of starch more than other sulfates in a MW field. These results suggest that the catalytic activities of sulfates are lower than metal halides, and the presence of sulfates, such as Na_2SO_4, $MgSO_4$, and $ZnSO_4$, even inhibits the acid hydrolysis of starch. The reason is probably that sulfate buffers the pH and decreases the acidity of HCl because the hydrogen sulfate ion is a weak acid (Kunlan et al. 2001).

The acid-catalyzed dehydration of glucose to HMF in aqueous solution is possible without a catalyst both by conventional and MW-assisted heating. However, the effect of a catalyst results in increase of the dehydration rate significantly. Several recent studies have demonstrated the HMF production in substantial

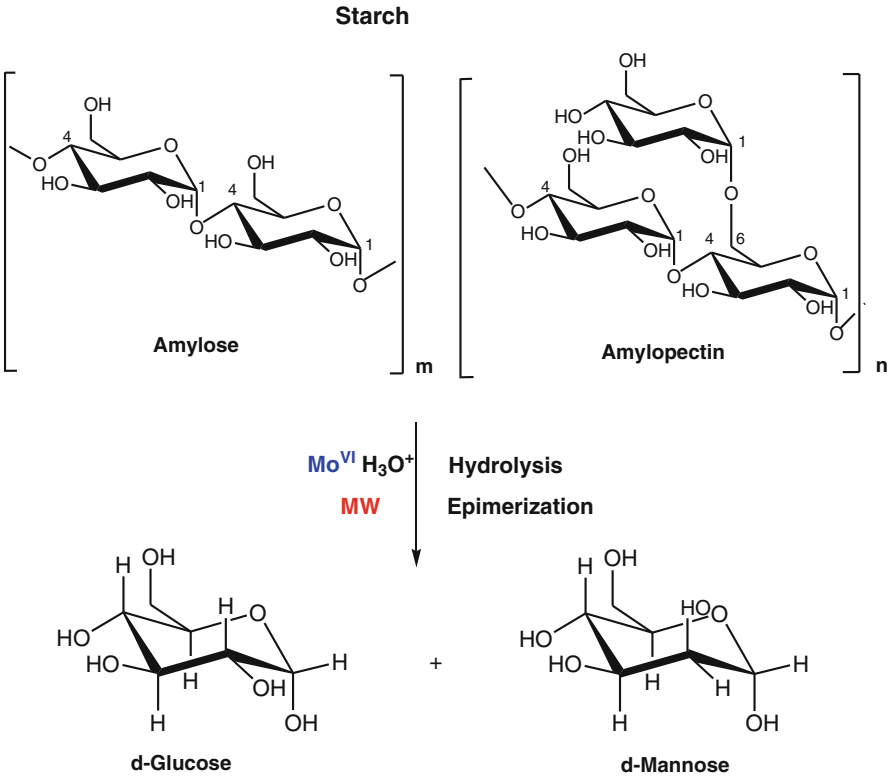
amounts using fructose and glucose by modifying its synthetic processes in the presence of ionic liquids. Recently, Chun et al. published a simple method of producing HMF from various starches using ionic liquid, 1-octyl-3-methylimidazolium chloride ([OMIM]Cl), and $CrCl_2$ as transition-metal catalyst. The addition of $CrCl_2$ to the reaction mixture significantly affected the yields of HMF. The yields of HMF increased approximately twofolds on average in the presence of this catalyst. Among eight starch sources (corn starch, wheat starch, rice starch, potato starch, sweet potato starch, tapioca starch, acorn starch, and kudzu starch) the highest yields of HMF were achieved in tapioca starch (75 %), showing that this starch is most suitable for production of HMF compared with other ones (Chun et al. 2010).

Data from literature consistently pointed out that use of MW irradiation is powerful tool in the field of polysaccharide chemistry. The first goal could be achieved by facilitating reactions under bulk conditions. Reactions can proceed even when the reagents do not absorb the radiation by selecting an appropriately absorbing solvent or by adding ionic liquids. However, since MW equipment has been designed for use in the laboratory, it often limits the scalability of the process and demanding batch reactions using in industrial processes.

5.3 Mo(VI)-Catalyzed Transformation of Starch: Preparation of Mannose and Mannitol

One of the important features of current chemistry methods is the effective preparation of enantiomerically pure compounds from naturally occurring sources. Recent research showed that starch is strongly affected by MW irradiation and has different properties compared to conventionally heated reactions. Several papers have reported the hydrolysis of starch in MW field (Lewandowicz et al. 2000; Palav and Seetharaman 2007; Bhat and Karim 2009). Microwave-assisted transformations are attractive due to short reaction times, high selectivity, and improved yields. As mentioned previously, the presence of ions can also enhance effects of dielectric loss, and MW coupling could produce superheating (Mingos and Baghurst 1991; Kunlan et al. 2001). Molybdate salts were examined in order to increase the efficiency of MW heating together with their catalytic properties. It was observed that in the presence of Mo(VI) ions, the process of starch hydrolysis proceeded much faster and simultaneous epimerization of obtained D-glucose to commercially interesting D-mannose was considerably improved (Hricovíniová 2011).

The effects of starch sources on the yields of D-mannose were compared using three different starches (potato starch, rice starch, and corn starch). In the optimized reaction conditions, a 5 % solution of starch in aqueous solution of HCl with a catalytic amount of sodium molybdate was exposed to MW irradiation. Homogenous blue solutions were obtained indicating completion of the complex formation. Selected reaction conditions lead to complete depolymerization of starch and high conversion by means of D-glucose and D-mannose content (2:1). It was observed



Scheme 11 Reaction scheme of starch hydrolysis and simultaneous Mo(VI)-catalyzed epimerization of obtained D-glucose to D-mannose

that transformation occurs with full selectivity and equilibrium reaction mixture was reached within minutes. The reaction scheme of the combined starch hydrolysis and simultaneous epimerization reaction of obtained D-glucose to D-mannose is illustrated in Scheme 11.

The ratio of sugars present in equilibrium reaction mixture with respect to the production of D-mannose was determined by ^{1}H NMR spectroscopy. The efficiency of the method was demonstrated also in the semi-preparative scale yielding mixture of glucose and mannose (Table 4). Starch hydrolysis is predominantly determined by acid concentration, but the simultaneous mutual interconversion of glucose and mannose is governed by highly stereospecific isomerization reaction catalyzed by molybdate ions.

The microwave-heated samples of starch were compared with the samples prepared by conduction heating. In the latter case, the equilibrium reaction mixtures were obtained after long heating (40 h). The NMR analysis indicated that starch was completely hydrolyzed and converted to the equilibrium mixture (3:1) of glucose and mannose. The formation of D-mannose started after 6 h, and the maximum was reached in about 35–40 h. The final equilibrium mixture is comparable to that found

Table 4 Comparison of MW-assisted and conventional starch hydrolysis and simultaneous epimerization reaction of obtained D-glucose to D-mannose. Both partial and full conversions are shown for both approaches to demonstrate vast differences in reaction kinetics and amounts of D-mannose formations

	MW filed		Conventional		Heating	
Starch	Time (min)	Starch/Glc/Man (%)	Time (h)	Starch/Glc/Man (%)	Time (h)	Starch/Glc/Man (%)
Potato starch	3	0/65/33	10	52/35/7	40	0/73/24
Rice starch	3	0/63/31	10	55/31/6	40	0/73/22
Corn starch	3	0/62/30	10	56/32/6	40	0/74/24

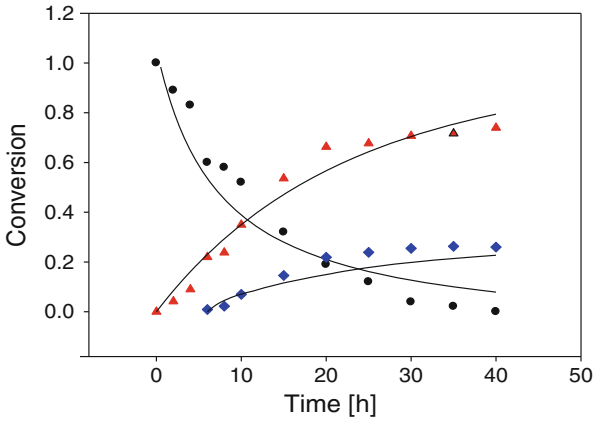


Fig. 4 Conversion of potato starch (●) to D-glucose (▲) and D-mannose (◆) as a function of time under conventional, oil-bath heating. Formation of D-mannose starts about 6 h after starch hydrolysis to D-glucose

in previous studies of Glc/Man epimerization (Bílik 1972a). The composition of the reaction mixture in terms of starch, glucose, and mannose content as a function of time is depicted in Fig. 4. Different reaction kinetics and thermodynamics were observed using MW heating. Starch hydrolysis and the molybdic acid-catalyzed isomerization reached thermodynamic equilibrium already after 3 min under the identical reaction conditions (concentrations, temperature) as used for conventional heating (Fig. 5).

The rearrangement of obtained D-glucose to D-mannose was accomplished after acid hydrolysis of starch in MW field using Mo(VI) ions as catalytically active species. In this respect, D-glucose isomerization is a crucial step in the efficient production of D-mannose. The mechanism of this stereospecific transformation is based on the coordination of glucose with the dimolybdate anion, and, consequently, the creation of catalytically active complexes leads to isomerization of

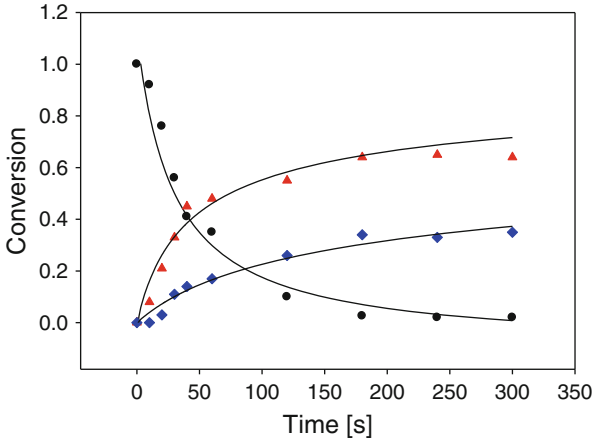


Fig. 5 Conversion of potato starch (●) to D-glucose (▲) and D-mannose (◆) as a function of time in MW field

D-glucose to D-mannose (Bílik 1972a; Petruš et al. 2001; Hricovíniová 2006). The comparison of the results clearly shows that MW irradiation markedly accelerated the isomerization process (by two orders of magnitude compared to conventional heating) and that MW field also caused the differences in the equilibration of the reaction mixtures. The obtained data suggest that the synergistic coordination ability of the catalyst used under the given reaction conditions enables simultaneous efficient starch depolymerization and formation of new compound D-mannose in a single step. Thus, unlike to conventional heating, D-mannose is formed virtually simultaneously with D-glucose using microwaves. The presented approach opens way for the preparation of many interesting sugar derivatives. It should be also noted in this respect that the presence of molybdate ions improves efficiency of energy absorption and thus reaction proceeds faster compared to that without the presence of this catalyst. The Mo(VI) ions thus perform a double role in the presented reaction: the first role is the improvement of the dielectric properties of the solution, leading to high energy transfer to solution from a MW source; secondly, Mo(VI) acts as an efficient catalyst that enables stereospecific conversion of glucose to mannose. These twofold roles of molybdate ions make them a unique catalyst for the isomerization reactions of carbohydrate molecules.

D-Mannose and D-mannitol are widely used in food and pharmaceutical industry (De Guzman 2005; Ghoreishi and Shahrestani 2009). It is only half as sweet as sucrose, which makes mannitol applicable as sweetener in the so-called "light" foods. Mannitol is metabolized independently of insulin, and thus it is also applicable in diabetic food products. Besides applications in the food industry, mannitol is also used in human medicine to decrease cellular edema (excessive accumulation of fluid) and increases the urinary output. Moreover, it is also chemically inert. These properties make mannitol very useful in production of tablets and granulated powders. Furthermore, its sweet cool taste is used to mask the unpleasant taste of

many drugs. The reduction of D-mannose with sodium borohydride leads to D-mannitol in a good yield (78 %); hence the production of pure D-mannose is needed in sufficient amounts. At present, D-mannitol is mainly produced by reduction of D-fructose from less-expensive glucose–fructose syrups which leads to a mixture of D-mannitol and D-sorbitol (1:1). Mixtures of mannitol and sorbitol are also produced enzymatically from fructose with different microorganisms (Peterson and Fred 1920; Soetaert et al. 1999). The main problem is that D-mannitol is difficult to separate from its stereoisomer D-sorbitol. Compared to enzymatic production, chemical catalysis employing inorganic materials for isomerization of reducing sugars could offer attractive advantages, as it is environmentally friendly and inexpensive. Furthermore, the isomerization process can be completed in very short reaction time, which makes transformation more efficient. The application of this methodology provides an attractive way in the field of MW-assisted metal-catalyzed reactions in aqueous media. MW irradiation in combination with Mo(VI) catalyst proved to be an efficient method for the hydrolysis of starch and isomerization of the obtained D-glucose to D-mannose. The short reaction times and good conversions in combination with the easy performance and work-up make this method attractive and applicable also in semi-preparative scale.

5.4 Transition-Metal-Catalyzed Transformation of Hemicellulose

Hemicellulose is, after cellulose and starch, the third most abundant biopolymer. Hemicellulose is a complex branched heteropolysaccharide composed of linear polymers with short branched chains. This plant cell wall polymer is mainly found in woods, grasses, algae, and cereals. The major constituents of hemicellulose chains are xylans, polysaccharides formed by β-(1→4)-linked D-xylopyranoside monomer units. The xylose units of the β-(1→4)-linked backbone chain can be substituted with *O*-acetyl groups at O-2 and/or O-3 or branched with L-arabinose (arabinoxylans) or D-glucuronic acid (glucuronoxylans) (Deutschmann and Dekker 2012). Hemicellulose biomass is widely available renewable low-cost raw material suitable for the production of biofuels and value-added organic products. It is rich in pentosans present in agricultural waste, such as rice husks, oat hulls, cottonseed, hull bran, almond husks, etc. (Akpinar et al. 2009; Amiri et al. 2010; Ren et al. 2012). Conversion of this source of biomass utilizing various chemical methods is an important process mainly due to the development of renewable platform chemicals, mainly furfural (Scheme 9). Furfural applications range from the raw furan-based chemical compounds to solvents. Industrially, furfural is used as a building block for polymers (polyester, polyurethane), thermosetting copolymer resins or high-molecular-weight plastics (polyvinylfurfural). Bulk chemicals such as tetrahydrofuran, tetramethylene chloride, furfuryl alcohol, and pyridine are also obtained from furfural (Moreau et al. 2004).

Furfural is produced through the dehydration of pentoses (mainly from D-xylose). Consequently, the acid-catalyzed dehydration of polysaccharides has been the subject of many studies (Antal et al. 1991; Vásquez et al. 2007).

Pentose dehydration reactions are currently carried out at high temperatures using the acid catalysts. In industrial process, furfural is produced by the homogeneous reaction catalyzed by mineral acids (such as sulfuric acid, hydrochloric acid, acetic acid, or phosphoric acid). These catalysts are effective and give furfural yields of 40–60 %. However, the utilization of mineral acids has serious drawbacks of extreme corrosion, toxicity, and excessive waste disposal. This process causes environmental problems due to the large quantity of wastewater needed to dilute the acid and difficulties arising from separation and recycling (Zeitsch 2000). Furthermore, the process can also produce many secondary reactions that contribute to selectivity losses, including the condensation of furfural and the production of other undesirable by-products. For this reason, it is necessary to develop selective low-cost processes and environmentally friendly methods for the conversion of hemicellulose biomass into furan derivatives.

A number of highly efficient catalysts were developed in the last decade. Among them, inorganic oxides (Corma 1995), heteropolyacids (Dias et al. 2005, 2006), ion-exchange polymer resins (Agirrezabal-Telleria et al. 2011), zirconium phosphate (Weingarten et al. 2011), vanadium phosphate (Sádaba et al. 2011), sulfated metal oxides (Shi et al. 2011; Suzuki et al. 2011), and zeolites (Lima et al. 2008; Lessard et al. 2010) have been reported to be active in the dehydration of xylose to furfural. Indeed, zeolites and related materials can be used as highly selective and active catalysts. The structural properties of these materials, acidic and basic sites, as well as their hydrophilic and hydrophobic properties, make them applicable as efficient solid heterogeneous catalysts. Furthermore, the oxides or pillars could be converted into active sites with different levels of acidity, depending on the characteristics of the cations incorporated. Thus, metal cations such as Al, Zr, Hf, and Ce have been evaluated for a wide range of reactions (Campos et al. 2008). Al–Hf pillared clays were investigated as promising catalysts for the dehydration of pentoses in water, producing furfural with an average conversion up to 78 %. The conversion rates achieved with Al–Hf catalyst using water as a solvent were comparable to those obtained with other catalysts, such as zeolites, which yield conversions ranging from 45 % to 80 % under similar reaction conditions (Lima et al. 2008) Nevertheless, these reactions were carried out in water/organic solvent mixtures, such as water/toluene (Lima et al. 2010) and water/DMSO (Dias et al. 2005). The application of solid heterogeneous catalysts is the promising alternative to strong mineral acids, such as concentrated sulfuric acid, due to their high activity, stability, and simple separation from the reaction products.

Alternative investigations have already been implemented which focus on the conversion of pentoses into furfural by thermochemical processes. Recent processes involve the use of inorganic salts, metal oxides, as well as solid acids instead of mineral acids (Choudhary et al. 2013). The aqueous phase is more favorable from the economic and environmental view for the industrial production. However, the efficiency is very low due to further rehydration of furfural in aqueous solution and the formation of oligomeric species. Organic solvents, such as DMSO and DMF, which favored the formation of furan compounds, have been proven to depress effectively side reactions in the dehydration reactions of D-fructose and

D-xylose (Brown et al. 1982). However, monosaccharide, polysaccharide, and biomass can be only dissolved in a few types of organic solvents to form a homogeneous system. Moreover, the use of organic solvents gives rise to environmental pollution leading to costly recycling procedures.

Ionic liquids with desirable properties of negligible vapor pressures and high thermal stabilities have been proven to be effective solvents in pentose dehydration. The catalyzed conversion of xylan into furfural in ionic liquids in the presence of metal chlorides as catalysts under MW irradiation was studied. Zhang and Zhao demonstrated that $CrCl_3$ in ionic liquids could promote biomass (cornstalk, rice straw, and pine wood) conversion to produce furfural in yields of 23–31 % under MW irradiation (Zhang and Zhao 2010). Zhang et al. studied the catalyzed conversion of xylan in [BMIM]Cl with metal chlorides as catalysts in MW field. Among catalysts, $AlCl_3$ in [BMIM]Cl resulted in the highest furfural yield (84.8 %). The yields of furfural from untreated corncob, grass, and pine wood were in the range of 16–33 % (Zhang et al. 2013). Catalytic system $AlCl_3$/[BMIM]Cl could be recycled for four runs with stable catalytic activity. Moreover, the use of ionic liquid as a reaction medium has not generated any toxic wastewater. However, the high cost of ionic liquids has kept them from being applied in a large-scale furfural production.

One of the promising strategies for furfural production is the continuous extraction of the target product from the aqueous solution utilizing organic solvents. Biphasic systems (water/organic solvent) were introduced as an ecologically viable catalytic pathway for furfural production without the addition of mineral acids. This approach reduces the formation of degradable side products through the consecutive polymerization reactions. Biphasic system using the solid acid catalyst SO_4^{2-}/TiO_2-ZrO_2/La^{3+} in different kinds of aprotic organic solvents (DMSO, DMF) in water phase was used for the production of furfural from xylose. The addition of DMSO, DMF, and 1,3-dimethyl-2-imidazolidinone (DMI) to the aqueous phase of the biphasic system can greatly improve the furfural yield, especially for the DMI solvent (Li et al. 2014).

Very recently, niobium phosphate (NbP) and niobium oxide (Nb_2O_5) were used for the dehydration of xylose to furfural in biphasic solvent system. Xylose conversion and furfural yield and selectivity were tested over different solid acid catalysts (such as zirconium phosphate, sulfated zirconia, H-beta (SiO_2/Al_2O_3=25) and H-Y (SiO_2/Al_2O_3=4) zeolite catalysts) in toluene/water system. Results have shown that among all catalysts tested, NbP exhibited the best catalytic performance. Moreover, NbP showed good stability, and no obvious deactivation of Nb could be observed after six continuous recycles. On the other hand, only moderate yield of furfural (22.5 %) was obtained. The acid-catalyzed xylose conversion and furfural yield over the mesoporous Nb_2O_5 were found up to 90 % and 50 %, respectively (Pholjaroen et al. 2013; García-Sancho et al. 2014).

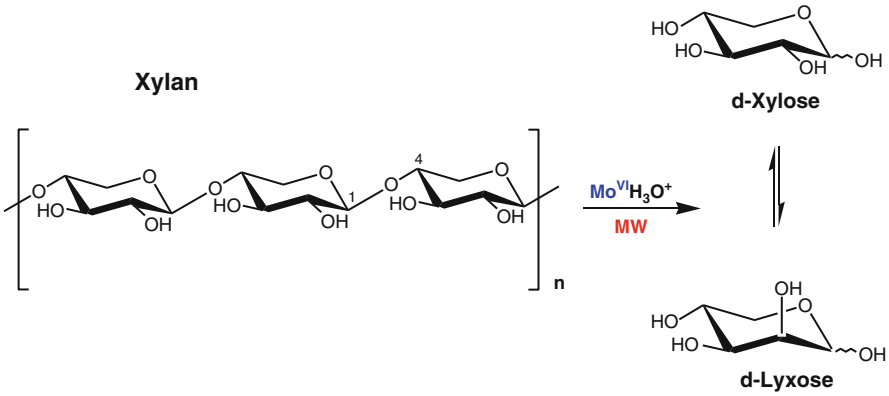
Many research groups are focusing their efforts on finding more economical ways of producing xylan-related chemical intermediates for different applications. The production of chemicals based on xylans is still a challenging task and requires extensive research depending on the source and the desired final products.

Application of new methodologies, careful modification of the reaction conditions, and the accurate and reliable analyses of polysaccharide resources could significantly improve the utilization of hemicellulose biomass.

5.5 Mo(VI)-Catalyzed Transformation of Xylan: Preparation of Xylose, Lyxose, Xylitol, and Furfural

A number of chemical technologies are known and several studies have appeared that describe the transformation of hemicellulose in a MW field. Some polysaccharides require harsh conditions to degrade. While cellulose is crystalline and strongly resistant to hydrolysis, hemicellulose containing predominantly D-xylose units has an amorphous structure with little strength (Binder and Raines 2009). MW irradiation is able to enhance the hydrolytic cleavage of its macromolecular chains. It was shown that the application of microwaves in combination with hexavalent molybdenum catalyst is the efficient method for the production of commercially interesting chemicals from starch. Several experiments were designed for exploitation of this catalytic system to improve xylan hydrolysis with subsequent epimerization of D-xylose monomer. Beechwood xylan was selected as the model compound for determination of the influence of MW-induced Mo(VI)-catalyzed conversion of hemicelluloses to epimeric pentoses. Appropriate reaction conditions led to complete depolymerization of xylan and high conversion to D-xylose and D-lyxose. The analysis of ^{1}H NMR spectra confirmed that xylan was completely hydrolyzed and converted to the equilibrium mixture of D-xylose and D-lyxose (1.6:1) in several minutes. The hydrolysis of xylan and simultaneous epimerization of obtained D-xylose to D-lyxose is illustrated in Scheme 12.

The yields of D-xylose/D-lyxose were compared using three different xylan substrates (beechwood, birch wood xylan, and 4-*O*-methyl glucuronoxylan).



Scheme 12 Reaction scheme of xylan hydrolysis and simultaneous epimerization of obtained D-xylose to D-lyxose

Table 5 Comparison of MW-assisted and conduction-heated xylan samples during combined hydrolysis and simultaneous epimerization reaction of obtained D-xylose to D-lyxose. Vast differences are seen in xylan/xylose/lyxose conversions and reaction kinetic of two heating techniques

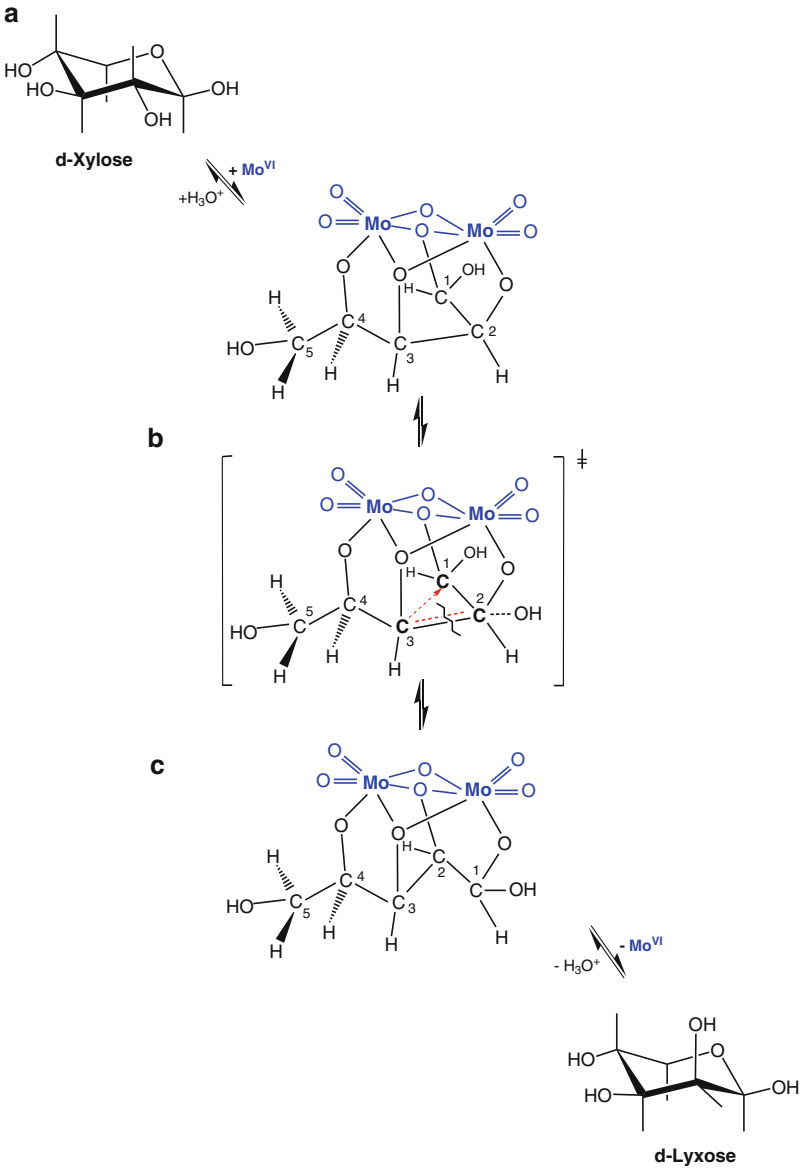
	MW field		Conventional		Heating	
Xylan source	Time (min)	Xylan/Xyl/Lyx (%)	Time (h)	Xylan/Xyl/Lyx (%)	Time (h)	Xylan/Xyl/Lyx (%)
Beechwood	3	0/62/38	10	6/68/25	20	0/70/30
Birch wood	3	0/63/37	10	7/68/24	20	0/69/31
4-*O*-Me-gluX	3	0/61/39	10	7/67/25	20	0/68/32

The ratio of sugar components present in the equilibrium reaction mixture was determined by ^{1}H NMR spectroscopy. The transformation occurs in 3 min affording pure epimers of D-xylose and D-lyxose in good yields. Table 5 summarizes the data obtained for various xylan samples heated in MW field and by conventional heating. It should also be noted that the increasing amount of catalyst did not influence the product composition.

The mechanism of this stereospecific transformation, as in the case of starch, is based on the formation of catalytically active Mo(VI) complexes of D-xylose monomer and through rearrangement of the carbohydrate carbon skeleton which led to the formation of equilibrium reaction mixture of D-xylose and D-lyxose. The accepted mechanism is depicted in Scheme 13. The first step is formation of the binuclear molybdate complex with the acyclic hydrated form of D-xylose (Scheme 13a). This rigid framework holds the acyclic carbohydrate skeleton in required conformation for stereospecific carbon backbone rearrangement. The rearrangement occurs through a transition state (Scheme 13b). Bond formation between C-2 and C-3 regenerates the starting D-xylose, while bond formation between C-1 and C-3 produces the 2-epimer, D-lyxose (Scheme 13c).

The kinetics and composition of the reaction mixture under microwave and conventional conditions are depicted in Figs. 6 and 7. As shown in Fig. 6, more than 80 % of xylan is hydrolyzed to D-xylose monomers in MW field after 25 s. Epimerization of xylose to lyxose starts in 30 s, simultaneously with the hydrolysis of xylan. Results indicate that 3 min is sufficient reaction time for the epimerization of D-xylose to D-lyxose, whereas prolonged duration might lead to the occurrence of side reactions. The traces of furfural were also observed after 3 min. Under conventional conditions about 45 % of xylan is hydrolyzed to D-xylose monomers after 2 h. As seen from Fig. 7, formation of D-lyxose started after 2 h. The ratio of xylose/lyxose was 2.7:1 after 10 h of heating, and the equilibrium mixture of xylose/lyxose 2.2:1 was reached after 20–25 h of heating.

The results obtained by application of two heating techniques show that MW irradiation caused the differences in the equilibration of the reaction mixtures. The different ratios of stereoisomers as a function of time were observed owing to differences in the mode of energy transfer. The reaction equilibrium was shifted in both cases to the aldose with the lower value of conformational instability, thus a larger amount of D-xylose was present in reaction mixture. MW approach allows



Scheme 13 Reaction mechanism of the Mo(VI)-catalyzed epimerization of D-xylose to D-lyxose

faster product formation (min) compared to conventional conditions (20–25 h) (Hricovíniová 2013).

Xylose and lyxose were obtained from various xylan samples in a one-pot transformation. Lyxose occurs rarely in Nature, and its higher amounts can be obtained only by chemical synthesis (Bílik and Caplovic 1973; Giudici and Griffin

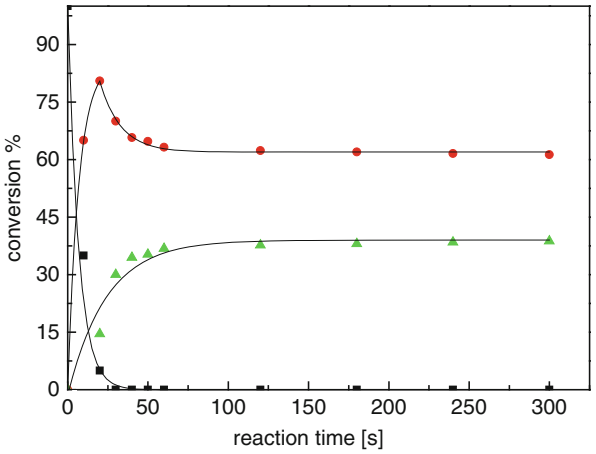


Fig. 6 Conversion of beechwood xylan (■) to D-xylose (●) and D-lyxose (▲) as a function of time in microwave field

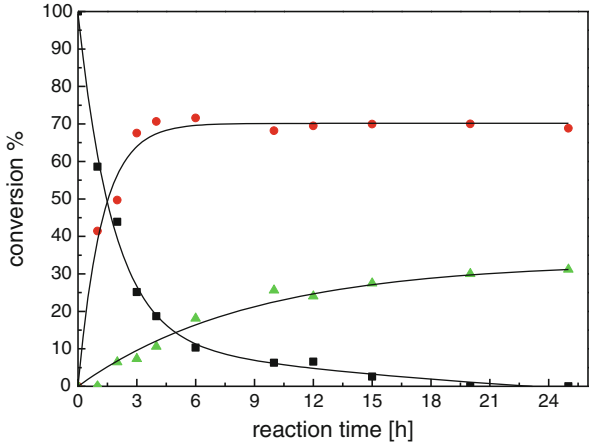


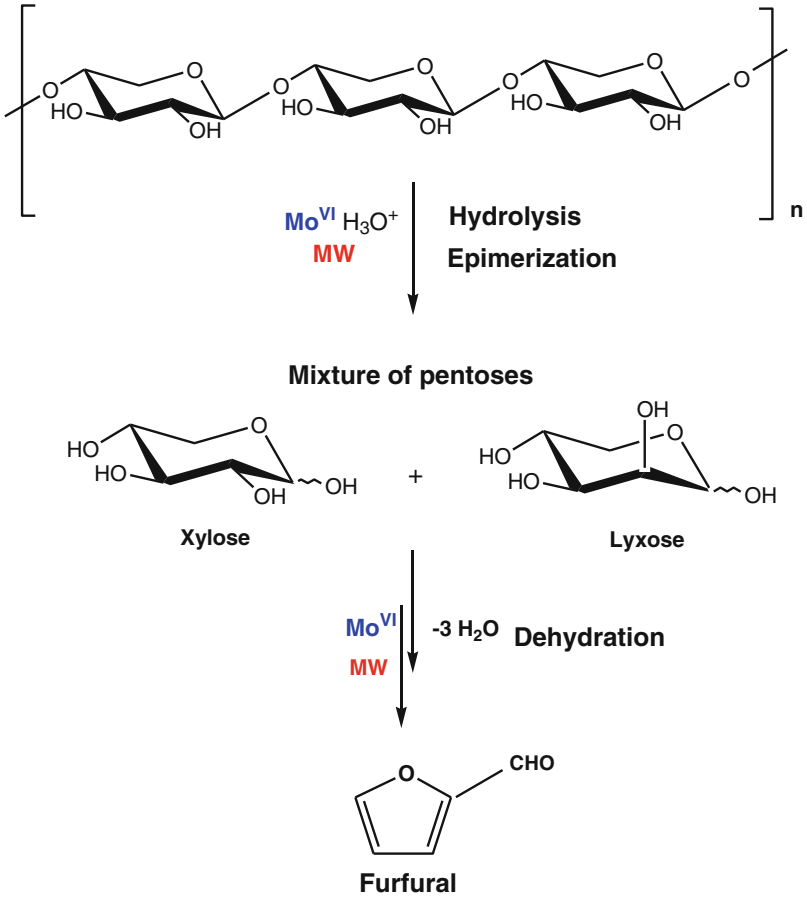
Fig. 7 Conversion of beechwood xylan (■) to D-xylose (●) and D-lyxose (▲) as a function of time under conventional conditions

1974). Xylose is an important compound mainly for its conversion to xylitol with its wide variety of applications. It metabolizes easily in the human body and produces the same amount of energy. Xylitol, with its high sweetening power (matching that of saccharose), is applicable as a sugar substitute in the food processing industry. It can be used as in diabetic foods, beverages, and pharmaceuticals (Kontiokari et al. 1995; 1998; Granström et al. 2007). Industrially, it is produced by catalytic hydrogenation or by enzymatic reduction of xylose. However, industrial bioconversion of detoxified hemicellulosic hydrolysate by yeast strains to xylitol is complicated by inhibitory effects of the often detected phenolic by-products

(Wisniak et al. 1974; Zhang et al. 2012). Compared to enzymatic production Mo (VI)-catalysis could offer attractive alternative. Utilizing an efficient catalytic system and water as the solvent has many advantages. Xylitol and lyxitol can be prepared by the simple reduction of corresponding aldoses.

The direct conversion of xylan to furfural in the presence of molybdate ions has also been studied. The hydrolysis of xylan proceeds much faster than the dehydration reaction, but the use of microwaves in the dehydration of pentoses revealed notable effect on the reaction rate. The combined xylan hydrolysis, epimerization reaction, and dehydration of obtained mixture of pentoses to furfural are illustrated in Scheme 14.

The analysis of furfural formation in the molybdate-catalyzed transformation of xylan in MW field has shown that the single-step process provided higher amounts of furfural (53 %) compared to reaction without Mo(VI) catalyst (42 %).



Scheme 14 Mo(VI)-catalyzed one-step transformation of xylan to furfural in MW field

The interaction of molybdate ions with monomeric D-xylose promotes the stabilization of its acyclic form that is quickly equilibrated with its furanose and pyranose forms. The less stable pyranose ring leads via 2,5-anhydride in dehydration pathway to furfural (Antal et al. 1991). The preparation of furfural from xylan was compared with conventional oil-bath heating, but maximum yield of furfural was only 28 %. Longer reaction times and nonselective heat transfer at the surface of reaction vessels resulted in the loss of furfural due to secondary reactions and undesired by-products that decreased its final yield.

The molybdate salts thus play an important role in the presented reaction. Mo(VI) acts as special catalyst for stereospecific conversion of xylose to lyxose. Moreover, Mo(VI) catalyst is also efficient in the transformation of mixed source of pentoses to furfural, where three simultaneous steps (hydrolysis, epimerization, and dehydration reaction) are combined in the one-pot reaction. Mo(VI)-catalyzed isomerization reactions thus provide an interesting alternative toward preparing platform chemicals from renewable natural lignocellulosic polymers.

6 Methods of Analysis

The structure of synthesized saccharides is analyzed by various methods. Nowadays, fast and nondestructive methods, such as NMR, are essential for the compositional analysis of reaction mixtures. They provide information that is necessary for possible optimization or changes of reaction conditions. Depending on the nature of the sample, the analysis may need several steps of pretreatment.

Mixtures of mono-, oligo-, and polysaccharides are separated by several chromatographic methods and analyzed by various analytical techniques. Monosaccharides and their derivatives can be separated by ligand-exchange liquid chromatography. The formation of a donor–acceptor complex between the cations immobilized on the ion-exchange resins and the carbohydrate hydroxyl groups makes this separation method very effective (Caruel et al. 1991). High-performance liquid chromatography (HPLC) equipped with a refractive index detector and utilizing aqueous mobile phase is widely used in the analysis of mono- and oligosaccharides. Different types of columns may require also other solvents. When mono- and oligosaccharides are present together in one sample, solvent mixtures in gradient mode are usually used. Beside HPLC several other methods for sample analysis are used. The oldest one is a colorimetric method for the determination of total carbohydrate content with phenol-sulfuric acid reagent in combination with paper chromatography (Dubois et al. 1956). The other one is based on the gas chromatography–mass spectrometry (GC-MS) analysis of derivatized monosaccharides. Each sugar constituent of polysaccharide is derivatized to alditol acetate or per-*O*-trimethylsilyl derivative (Sawardeker et al. 1965; Ciucanu and Kerek 1984; Merkle and Poppe 1994). Both methods have several disadvantages (in terms of long-time analysis or time-consuming derivatization steps) compared to HPLC method. In carbohydrate analysis simpler spectroscopic methods might be used. Colored products obtained after

acid-catalyzed degradation of pentoses (e.g., furfural) have characteristic absorption in UV spectroscopic region (Douglas 1981; Martinez et al. 2000). Infrared spectroscopy (IR) could be useful to assess chemical composition of polysaccharides (Kelley et al. 2004).

High-resolution nuclear magnetic spectroscopy (NMR) is the most powerful analytical method used for determination of the structure of monosaccharides and polysaccharides. NMR allows primary and secondary structural analysis of pure saccharides in solution from determination of chemical shifts and coupling constants (Bock and Pedersen 1983; Angyal 1984; 1994; Shin and Cho 2008; Mittal et al. 2009). Conformation and dynamics of saccharides can be studied by relaxation measurements and measuring dipolar interactions (employing NOESY or ROESY experiments). Analysis of mixtures of products obtained by synthesis or by degradation of polysaccharides is provided by diffusion-ordered spectroscopy. NMR techniques thus enable detailed analysis of composition (determination of individual monosaccharide units and the type of linkages) of branched polysaccharides without necessity of their degradation. Furthermore, the anomeric ratio or composition of polysaccharides can be quantitatively determined by integration of proton and carbon resonances in ^{1}H or ^{13}C NMR spectra. Both one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy experiments are mostly used for carbohydrate structural analysis. The proton and carbon chemical shifts in aqueous solutions are usually referenced to internal TSP (trimethylsilyl propionate) or DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) calibration standards. The combination of 2D COSY, HSQC, and HMBC is routinely used for the determination of ^{1}H and ^{13}C chemical shifts. These three experiments are mostly sufficient to determine chemical shifts. In more complex molecules, however, other experiments can also be performed, such as 1D or 2D TOCSY, 2D NOESY, HSQC-TOCSY, etc. Nonuniform sampling type of experiments can also be useful for structural analysis of polysaccharides. Proton–proton coupling constants are usually determined directly from proton spectra; in some cases 1D TOCSY or COSY spectra can also be used. One-bond proton–carbon coupling constants can be retrieved from 2D HSQC-coupled spectra.

Further details on carbohydrate molecular structures can be obtained from molecular modeling (MM). MM is necessary for interpretation of solution NMR spectra when analyzing 3D molecular structures. Molecular mechanics methods are simple methods that can give data for large molecular structures (large oligosaccharides) without demands on computer time and memory. However, this approach may not always give reliable data due to application of force fields that are not optimized for given carbohydrate structures. More general are quantum chemical methods. Density functional theory (DFT) approach is frequently used at present as it represents a good compromise between demands on computer hardware and accuracy of structures and computed energies. Solvent effect can also be modeled by various methods; the most usual is a continuum model. More rigorous is explicit solvent model which is also more time-consuming especially when computing large saccharide molecules.

7 Conclusion and Future Perspectives

An increasing interest of chemists in carbohydrates originates from the fact that these molecules are not only the most abundant biomolecules in Nature, but, perhaps even more importantly, carbohydrates belong to very few renewable energy and material sources. Carbohydrates can be obtained from various natural sources but also from agricultural waste, thus through the recycling processes. In the present, there are strong incentives to convert such carbohydrate resources into valuable products via environmentally friendly and effective processes, ideally by one-pot transformations. Many research groups try to find simple and economical ways to chemically modified saccharides with special properties for the requirements of various areas of life.

Numerous technologically important compounds can be prepared from renewable polysaccharides through the catalytic transformations. Acid-catalyzed hydrolysis of polysaccharides coupled with dehydration reaction afforded important platform chemicals in good yields. Solid catalysts, bearing both Brønsted and Lewis acid sites, have great potential for efficient transformation and can replace many conventional liquid acids. They can be reused several times and operate at low temperatures. Ionic liquids applied as reaction solvents and as catalytic reagents gave also promising results. Dissolution of hardly soluble compounds in highly ionic solvents at lower temperatures improves the selectivity, but the use of ionic liquids for the industrial processing, especially for cellulose, is not optimal solution. On the other hand, water as cheap and environmentally friendly reaction medium is useful only in high-temperature processes to achieve good conversions. In this respect, one-pot transformations of polysaccharides using direct extraction into biphasic system are inspiring examples. Application of biphasic systems can significantly improve the production of furans and avoid their destruction.

Significant interest has been directed toward the use of solid heterogeneous catalysts in combination with transition metals. They have several advantages over other catalytic systems, because they can work at high temperatures, facilitate the separation of product, and can be recycled several times. Combination of metal salts and ionic liquids lead to improved depolymerization of polysaccharides and transformation of obtained monomers to attractive chiral raw materials and fine chemicals. Introduction of MW irradiation to carbohydrate chemistry provides an interesting alternative. It is possible to alter the selectivity in many reactions under controlled MW heating. The effect of MW irradiation on transformation of polysaccharides, using various catalytic systems, leads to improved yields and cleaner reaction profiles in reduced timescale.

Application of transition-metal catalytic systems in combination with microwaves offers interesting improvements in yields and selectivity. Among them, microwave-assisted Mo(VI)-catalyzed isomerization of sugars in water represents a useful synthetic method in synthetic carbohydrate chemistry. The application of Mo (VI) catalyst in MW field offers a remarkable synergic effect with the great potential. The employment of this catalytic system represents the innovative approach with

processing advantages, especially where the extended conventional heating can cause degradation reactions. Numerous carefully controlled experiments were performed to evaluate the scope and limitations of this approach. The usefulness of this method was demonstrated in highly stereospecific, rapid, and efficient transformation of various reducing monosaccharides, oligosaccharides, and polysaccharides.

Each of the mentioned methods has important advantages and disadvantages. Considerable efforts are now directed toward the improvement of methods of selective transformation of saccharides by deeper understanding of their structural properties. In addition, modification of the reaction conditions and introduction of more efficient chemo- and stereoselective routes will allow several reaction steps to be finished in one reactor and thus allow easier access to various valuable compounds. Further progress in synthetic carbohydrate chemistry will be greatly supported by introduction of new methods and techniques. Developing of efficient synthetic methodologies will have in longer term significant impact in diverse areas of natural sciences, medicine, and industry.

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Surface Properties of Polysaccharides

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Yi Wang and Gary A. Dykes

Contents

Abstract

Polysaccharides are used in a wide range of applications across the medical, food, and material science spheres. Their applicability is in part a consequence of their specific surface properties and the nature of their interactions at interfaces. Intensive research into polysaccharide surface properties and methods to investigate these has been undertaken for decades. Due to the complicated structural, chemical, and physical features of polysaccharides, however, fully understanding their surface properties and developing effective methods for studying these properties are still challenging tasks. In this chapter methods frequently used for polysaccharide surface characterization, including sample preparation and surface analysis, are described and critically discussed. In addition to the traditionally used methods, several novel techniques of preparing polysaccharide model surfaces and description of advanced

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instrumentation for this purpose are presented. Several examples of how surface modification of polysaccharides measured using these methods may be practically applied are also discussed.

Keywords

Polysaccharides • Surface properties • Cellulose • Polysaccharide model surfaces • Surface modification

Abbreviations

AB	Lewis acid-based
AFM	Atomic force microscopy
APTT	Activated partial thromboplastin time
BC	Bacterial cellulose
CMC	Carboxymethyl cellulose
FT-IR	Fourier transformation infrared spectroscopy
GPC	Gel permeation chromatography
ICP-MS	Inductively coupled plasma mass spectroscopy
IR	Infrared spectroscopy
LB	Langmuir–Blodgett
LP	Lacquer polysaccharide
LW	Lifshitz–van der Waals
MALDI-TOF	Matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
QCM	Quartz crystal microbalance
SEM	Scanning electron microscopy
TMSC	Trimethylsilyl cellulose
XPS	X-ray photoelectron spectroscopy
ZP	Zeta potential

1 Introduction

Polysaccharides are critical components of a great diversity of fundamental processes and structures in all forms of life including plants, animals, and microorganisms. Cellulose, for example, the most abundant of all polysaccharides, occurs as a structural component of plant cell walls in combination with other biopolymers. It is, however, also produced by some bacteria as an extracellular mat which is thought to act as a floatation platform to increase bacterial access to oxygen. The surface properties of polysaccharides and their mechanism of interactions with water and other macromolecules need to be thoroughly studied in order to understand their roles in food and biological systems and consequently to use them for industrial applications.

Investigations into the structural, morphological, physical, and chemical properties of polysaccharides have been undertaken for many years. Despite the

development of new methods and techniques over the years, the characterization of polysaccharide surfaces is still challenging and time consuming. Difficulties associated with this analysis are often related to sample preparation and the selection of appropriate methods. For example, common methods for obtaining polysaccharides from their original sources such as enzymatic, chemical, and physical extractions (Ahola et al. 2008a; Lu and Hsieh 2010; Man et al. 2011) are often unable to preserve their inherent properties or structure intact. The selection of methods for surface analysis is based on the state and structural features of the sample (e.g., crystalline or amorphous, dry or swollen) which affect the levels of accessibility of solvents and the functionalities of the hydroxyl groups on the polysaccharide chains (Lin et al. 2012). Using only one method to investigate a particular polysaccharide is therefore often not sufficient, and employing a combination of different methods is sometimes necessary to thoroughly describe a surface property. For these reasons, polysaccharide model surfaces with well-defined structural and physicochemical features are often prepared for research purposes. In fact, some frequently used methods for surface characterization can only be applied to model surfaces as opposed to real systems because of the restrictions of the methods (Stana-Kleinschek et al. 2012).

The specific surface properties of polysaccharides which impact on features such as mechanical strength, environmental friendliness, electrostatic properties, and structural features have led to them finding a wide range of applications in the fields of medical, food, and material sciences. The application of polysaccharides is, however, limited by a variety of factors such as their adaptability to different systems (e.g., solubility, compatibility) and sensitivity to environmental factors (e.g., pH, temperature). Research has therefore focused on using their surface properties, with potential physical and chemical modification, to improve their mechanical, interfacial, chemical, and biological performance.

This chapter describes the methods used for surface characterization of polysaccharides in modern research. Specifically, methods often used for sample preparation including preparation methods for polysaccharide model surfaces are presented. In addition, different methods for analysis of surface properties are discussed and compared. Finally, several examples of how surface modification of polysaccharides measured using these methods may be practically applied are also discussed.

2 Methods of Sample Preparation

Over the past 20 years, substantial work has been conducted on the purification and characterization of polysaccharides, and especially cellulose, for studies of surface interactions and properties. A major challenge of this work is the difficulty in the preparation of samples directly from plant/animal sources for these studies. For example, it is very difficult to measure surface properties of cellulose in plant cell walls or to quantify specific interactions between cellulose and other molecules using plant cell wall as the sample. Consequently, model polysaccharide systems

have been used to study these properties and provide knowledge that cannot be obtained directly from plant cell walls. Additionally, model systems provide an excellent way to study the chemical, physicochemical, and morphological changes during various processes, such as interactions with water (swelling) and surface chemical modification, involving polysaccharides. Here several commonly used methods for polysaccharide extraction as well as preparation of model surfaces are described.

2.1 Extraction of Native Polysaccharides

2.1.1 Acid Extraction

Acid is often used to extract cellulose from native samples and produce cellulose nanocrystals. Nanocellulose is important for surface property studies due to its nanoscale dimensions, high surface-to-volume ratio, low density, unique morphology, unique optical and chiral properties, and high mechanical strength.

Nanocellulose is prepared by acid hydrolysis of cellulose fibers such as cotton, algae, and wood. A fiber suspension is treated with a strong acid, followed by a series of cleaning and purification procedures before solvent evaporation to obtain the final product (Lu and Hsieh 2010). During treatment with strong acids, crystalline regions in cellulose structures are hydrolyzed slower than amorphous regions, and the resultant nanoparticles are mainly crystalline. The geometric dimensions and properties of nanocellulose vary depending on the source and hydrolysis conditions (e.g., the type of acid used, the time and temperature of treatment). Commonly used acids are sulfuric acid, hydrochloric acid, and hydrobromic acid. If sulfuric acid is used, the hydroxyl groups on the particle surfaces are esterified with sulfate groups, and consequently the nanoparticles are electrostatically stabilized. If on the other hand hydrochloric acid is used as the hydrolyzing agent, the ability to disperse the resulted nanocellulose is limited (Araki et al. 1998). The shapes of the final particles can be identified by scanning electron microscopy (SEM) and atomic force microscopy (AFM) to be rod-, sphere-, or network-shaped, but they cannot be easily separated from each other (Lu and Hsieh 2010).

Due to the high surface-to-volume ratio of nanocellulose, its functionality as compared to that of other forms of cellulose is greatly enhanced as more hydroxyl groups are accessible for further modification including esterification, etherification, silylation, and polymer grafting. These functions and advantages enhance its potential application in the food and medical industries for use as nanocomposite films and in drug delivery, protein immobilization, and metallic reaction templates (Peng et al. 2011).

A significant challenge of developing cellulose nanocrystals and for further applications is their tendency to form large aggregates during solvent evaporation, which greatly limits their advantage and functionality. During drying a high number of hydrogen bonds form and cause aggregation which poses significant problems in the distribution and re-dispersion of the particles in solvents. To solve this problem, different methods to isolate the nanoparticles need to be developed. One potential

way to overcome this problem is to employ freeze drying (Lu and Hsieh 2010). The hydrolyzed product is quickly frozen with liquid nitrogen to keep the particles separated and fixed in the solidified ice, followed by freeze drying under strong vacuum to dry the sample without formation of hydrogen bonds. Another problem that limits the applicability of acid-extracted cellulose is the toxicity of the concentrated acids, which restricts its use in food industries.

2.1.2 Enzymatic Extraction

Enzymatic treatment of polysaccharides, such as cellulose, is generally designed to degrade them into fermentable sugars. The conversion of lignocellulosic material to monomeric sugars is a more complex process, however, and is difficult to achieve. The process is influenced by the chemistry, heterogeneity, crystallinity, and surface area of the substrate, all of which can strongly affect the kinetics of enzymatic conversion (Ahola et al. 2008a). For this reason these incomplete enzymatic degradations of lignocellulose are often used to extract cellulose from its natural sources.

Commonly used enzymes are endoglucanases, which randomly attack and hydrolyze the amorphous region of polymers; exoglucanases, which attack cellulose polymer chains from either the reducing or nonreducing ends; and cellobiohydrolases, which attack cellulose at the C-1 or C-4 positions (Filson et al. 2009). The enzymatic reactions are accelerated by microwave heating, and the degraded cellulose particles are purified by filtration, solvent evaporation, and freeze drying. This method has been used to collect cellulose from pulp samples (Ahola et al. 2008a; Filson et al. 2009). A problem with this method is the difficulty in controlling the sizes of the cellulose products.

2.1.3 Ionic Liquid Extraction

Ionic liquids, which are organic salts with low melting points that form liquids at temperatures below 100 °C, are good solvents for polysaccharides. For example, 1-butyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium hydrogen sulfate dissolve cellulose (Swatloski et al. 2002; Man et al. 2011), while 1-allyl-3-methylimidazolium bromide at a high concentrations dissolves chitin (Kadokawa et al. 2011). The polysaccharide content in a natural sample is dissolved in the ionic liquid, while the impurities are filtered away. The dissolved polysaccharide is sedimented and dispersed by a series of heat and solvent treatments. It is then purified by vacuum evaporation and freeze drying (Kadokawa et al. 2011; Man et al. 2011). The products are usually in the form of a film of nanowhiskers.

The advantages of using ionic liquids as solvents to extract polysaccharides are based on their properties, including their chemical and thermal stability, nonflammability, low vapor pressures, and ability to design their characteristics for specific uses (Earle and Seddon 2000). In addition, ionic liquids are not consumed during the extraction processes and are therefore reusable. A limitation of using ionic liquids is that the thermal stability of the resultant products drops after the extraction as compared to their native forms (Man et al. 2011). Furthermore, small amounts of the ionic liquid may remain in the product and are difficult to removed

due to the formation of hydrogen bonds between the polysaccharide and the solvent (Kadokawa et al. 2011).

2.2 Model Polysaccharide Surface

Model polysaccharide surfaces are often used to study the surface properties of natural polysaccharides. The most common model surface used for surface property studies is cellulose. Compared to natural polymers, model surfaces can be prepared more rapidly and reproducibly and have defined supramolecular structures, defined surface morphologies, and defined thicknesses. In addition, model surfaces allow surface interactions, such as adsorption and desorption, which are difficult to study in natural polymers, to be measured.

2.2.1 Model Cellulose Films

In contrast to natural polysaccharide samples, model cellulose films are ideal for surface interaction studies due to their well-defined surfaces. Adsorption, wettability, electrostatic interactions, surfactant activities, bioactivities, as well as surface characterization are easier to perform on cellulose model films because of their homogeneity.

Cellulose films are usually prepared using the Langmuir–Blodgett (LB) technique by spin coating or dip coating an LB film onto an inert solid substrate (Schaub et al. 1993; Hitrik et al. 2011). Cellulose LB films can be prepared by homogenizing a cellulose ether, such as trimethylsilyl cellulose (TMSC), in an organic solvent (e.g., *n*-hexane or chloroform). After coating the TMSC film on a solid substrate (e.g., gold, silicone, or indium tin oxide), the film is hydrolyzed by HCl vapors to cleave the TMS side groups. The thickness of a film can be controlled by the number of layers that are coated on the substrate (Hitrik et al. 2011). The hydrolysis process can generate free OH groups on the film surface, which decreases the surface hydrophobicity and consequently the surface free energies. Carefully controlling the hydrolysis reaction conditions allows the tailoring of the wettability and thickness of the film (Mohan et al. 2012a, b).

The selection of solvent systems for LB film preparation is crucial because some solvents can react with cellulose to a certain extent. For example, 1-alkyl-3-methylimidazolium can react with cellulose at its reducing end (C-2) and form a carbon–carbon covalent bond (Ebner et al. 2008) which results in a lack of purity of the cellulose phase. In addition, salts (e.g., LiCl) added to the solvent to break up the hydrogen-bonded network may lead to rough model surfaces.

Using model cellulose films for studies of polysaccharide surface properties is, however, not always ideal. For instance, model films consist only of cellulose II (the crystalline state) which do not represent other supramolecular cellulose structures. Conclusions drawn from cellulose films are therefore not generally applicable to other polysaccharide surfaces, although cellulose II is the most common cellulose allotrope present in manufactured products (e.g., regenerated cellulose fibers). For this reason cellulose I (the amorphous state) films have been

synthesized and characterized in some surface property studies (Habibi et al. 2007; Ahola et al. 2008b). It should also be noted that due to the well-defined surface morphology of model cellulose films, results of analysis of surface structure are more meaningful for natural polysaccharide samples than for model films. For this reason the intended application of model cellulose films needs to be defined before choosing them for surface property studies. For example, model films are useful for studies of surface interactions, such as adsorption, wetting, hydrophobicity, and electrostatic interactions, which are strongly dependent on surface morphology and chemical composition (Notley and Wågberg 2005; Eriksson et al. 2007).

2.2.2 Bacterial Cellulose (BC) Films

Cellulose produced by bacteria (e.g., *Gluconacetobacter*) has a specific network structure, high crystallinity, high mechanical strength, and high water retention ability. For these reasons it has been suggested to be a potential model of cellulose in native plant cell walls. Plant cell walls have been extensively studied in terms of their chemical properties, structural properties, surface properties, and interactions with the external and internal plant environment. The part of plant cell walls which contribute most significantly to these properties is the primary cell wall which consists of a polysaccharide matrix. This polysaccharide matrix is in a network structure consisting of approximately 30 % cellulose acting as the backbone of the network, 50 % pectin, and 20 % hemicelluloses (e.g., xyloglucan) filling in the pores of the network (Cybulska et al. 2010). Work has been conducted on isolation and characterization of cell wall materials from natural plant tissues. Current methods for isolation, however, do not allow the acquisition of cell wall fragments of sufficient size to perform many tests (e.g., mechanical tests, bacterial interaction tests). Bacterial cellulose has a similar chemical composition, similar network structure, similar physicochemical properties, and comparable mechanical strength as native cell walls and is therefore an ideal substitute for conducting such studies (Cybulska et al. 2010; Mikkelsen et al. 2011).

BC is produced by growing *Gluconacetobacter xylinus* in Hestrin–Schramm broth containing 2 % glucose (Mikkelsen et al. 2011). The bacteria synthesize uridine diphosphoglucose from glucose and polymerize it into long and unbranched chains of cellulose. The chains then assemble into a network and form a film on the top of the broth. The resultant BC films have been repeatedly shown to have similar structural and mechanical properties as native plant cell wall samples (e.g., apple tissues, wheat) (Quero et al. 2010; Mikkelsen et al. 2011). Images (SEM) of a BC network are shown in Fig. 1a. It is clear that the cellulose chains form a web-like network and cells of *Gluconacetobacter xylinus*, which can be removed by NaOH treatment, are trapped inside. In order to further imitate native plant cell walls, other cell wall polysaccharides, such as pectin and xyloglucan, can be added to the network by mixing them with the broth prior to inoculating the bacteria. The resultant BC composites will have these polysaccharides incorporated into the network (Fig. 1b) and have been shown to have a similar chemical composition as native plant cell walls (Cybulska et al. 2010).

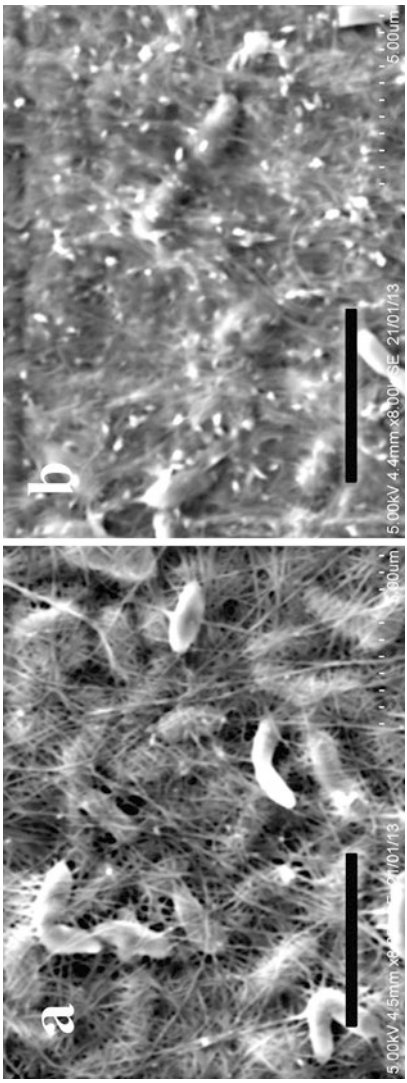

Fig 1 Scanning electron microscopy images of (**a**) bacterial cellulose and (**b**) bacterial cellulose composite with pectin and xyloglucan incorporated into it

Bacterial cellulose has been widely used in research for different purposes. The first attempt to use BC as a model of plant cell walls for studies of surface interactions with bacteria was carried out by Tan et al. (2013). The attachment of *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Listeria monocytogenes* to BC with pectin and xyloglucan incorporated in them was investigated and was compared to apple tissues in terms of the number of attached bacteria and the rate of attachment. Results analyzed by a multifactor ANOVA model showed that this plant cell wall model can well represent apple tissues with respect to bacterial surface interactions.

2.3 Surfaces of Natural Polysaccharides and Regenerated Fibers

Model surfaces cannot always represent natural polysaccharides and regenerated fibers (e.g., textile fibers, pulp fibers, and foils) since their surface properties are strongly dependent on surface structures such as surface topography, chemical composition, networking, and crystalline/amorphous state. For example, natural cellulose (e.g., cotton, flax, ramie) contains ~10-15 % of non-polysaccharide compounds such as proteins, pigments, and minerals, which gives them different surface morphology, chemical networking, and side groups on the surface and consequently different levels of charge, hydrophobicity, and water-retaining abilities. Differences also exist between different types of cellulose, for instance, foils exhibit extremely hydrophilic characteristics due to their relatively low surface roughness, despite having the same chemical composition as other types of cellulose (Stana-Kleinschek et al. 2012). In addition, the degree of crystallinity and amorphousness of cellulose results in different surface properties. For example, as compared to pulp fibers, modal fibers are more crystalline resulting in different morphologies and higher surface energy (Krässig 1996) and lower zeta potential (which is a result of a relative lower amount of acidic groups on the surface of modal fibers) (Jacobasch et al. 1985). Amorphous fibers also have a higher reactivity and a different adsorption activity. Model polysaccharide surfaces are therefore not always ideal representation of natural polysaccharides due to their homogeneity which does not occur in nature. Careful consideration is therefore required for sample selection and preparation of polysaccharides for analysis of surface properties.

3 Methods of Analysis

In the characterization of polysaccharide surfaces, interfaces, and interactions with compounds in the liquid phase, some surface properties such as morphology, chemical composition, surface tension/hydrophobicity, charge, and adsorption are of particular importance. Methods for studying these properties are briefly described in this section and summarized in Table 1.

Table 1 Frequently used methods for surface characterization of polysaccharides

Surface property	Methods	Notes	References
Surface chemical composition	XPS	Careful sample preparation required	(Johansson et al. 2005)
	MALDI-TOF	Provides 2-D maps, limited by "matrix effect"	(Belu et al. 2003)
Surface morphology	SEM	Specific sample preparation required	(Tan et al. 2013)
	AFM	Expensive and time consuming; no specific sample preparation required	(Katsikogianni and Missirlis 2004)
Surface layer thickness	Sarfus	Real-time visualization, provides 3-D maps	(Ausserre and Valignat 2006)
	Ellipsometry	Allows in situ analysis, allows measurements of multilayers	(Losurdo et al. 2009)
Hydrophobicity and surface energy	Contact angle measurements	Results can be affected by surface morphology and wetness	(Yasuda and Okuno 1994)
	AFM	Expensive and time consuming; results can be affected by surface charge	(Ducker and Senden 1992)
Surface charge	Charge titration	Quantifications of immobile charges	(Fras et al. 2004)
	ZP measurements	Measurements of the electric potential at Debye layer, ZP values are converted from electrokinetic measurements	(Bellona and Drewes 2005)
Surface adsorption	QCM	Real-time measurement; high sensitivity	(Marx 2003)

3.1 Surface Chemical Composition

The presence and number of specific functional groups on the surface of polysaccharides result in particular surface properties. For example, acid groups give the surface negative charges and hydroxyl groups contribute to hydrophilicity. It is therefore of interest to identify and quantify functional groups on the surface of polysaccharides.

3.1.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) can be used to determine the atomic composition of the outermost few nm of a solid surface (Gray et al. 2010). A surface will emit photoelectrons when exposed to X-ray photons. The binding energies of the emitted photoelectrons are then measured and compared to those of elements, and their oxidation states, with known values. The intensity of the emitted photoelectrons directly correlates to the atomic distribution of elements on a surface. It is a quantitative indicator of the relative atomic composition and

stoichiometric ratio of the elements. The resultant spectrum graph is a plot of intensity against binding energy which provides information on elemental composition on the surface, thickness of the surface layer, electronic state of the elements, and material density. The detectable range of XPS covers most elements except hydrogen. The XPS technique has been applied to a range of cellulose samples, such as pulp and paper, to study their surface chemistry and morphology (Maximova et al. 2001; Gustafsson et al. 2003; Johansson and Campbell 2004; Johansson et al. 2005).

A major challenge of the XPS technique is that the detectable depth is limited to the top few nm on the surface. Samples must be prepared very carefully since minor amounts of contamination can spoil the results.

3.1.2 Matrix-Assisted Laser Desorption/Ionization and Time-of-Flight Mass Spectrometry (MALDI-TOF)

The XPS technique provides proportional measurements of atomic composition but does not allow determination of the spatial distribution of atoms or chemical groups on the surface. The MALDI-TOF technique, which determines the type and quantity of ionizable chemical groups at different tilts on the sample surface, has been used in combination with XPS and/or imaging technology to provide an insight into the gradients and distributions of elements in the surface layer (Belu et al. 2003).

The MALDI technique involves application of a laser beam of primary ions to the sample surface, the energy of which is absorbed by matrix molecules and transferred to the macromolecules on the surface. This causes ionization of the macromolecules which then emit secondary ions. The emitted ions are separated according to their mass-to-charge ratio in a mass spectrometer which then plots a spectrum of signal intensity against mass-to-charge ratio. MALDI-TOF also provides two-dimensional chemical maps to aid imaging and allows depth profiling, which can be used to establish surface homogeneity (Belu et al. 2000; Pan et al. 2007).

A major limitation of the MALDI-TOF technique is the so-called matrix effect where the intensity of a given fragment can, in some cases, be highly dependent on its surrounding environment and therefore not directly related to its concentration on the surface. For example, oxygen and cesium enhance the yields of positive and negative ion, respectively, emitted by other elements by several orders of magnitude (Delcorte 2006). Fortunately for most polymeric biomaterials such as proteins and polysaccharides, the matrix effects are minimal.

3.2 Surface Morphology

3.2.1 Scanning Electron Microscopy (SEM)

The use of SEM allows visualization of the surfaces of biomaterials at submicron scales. An SEM emits a high-energy electron beam which is scanned over the sample surface and receives secondary electrons released from the atoms on the surface. The surface topography of the specimen is imaged from the received

secondary electrons. The sample surface must be electrically conductive or, for most biological samples, be sputtered by powdered conductive materials (e.g., Au, Pd). The SEM technique has been used for surface analysis of many polysaccharides such as pectin, cellulose, and carrageenan (MacArtain et al. 2003; Andrade et al. 2009; Tan et al. 2013).

3.2.2 Atomic Force Microscopy (AFM)

Atomic force microscopy is a method that can be used to profile surfaces and to measure their roughness at a sub-nm scale (Katsikogianni and Missirlis 2004; Warning and Datta 2013). An AFM scans a surface using a sharp tip attached to a cantilever with a curvature of several nm. The deflection of an inclined cantilever tip is measured with a laser beam reflected from the back of the cantilever and is used to construct a topographic image.

For hard surfaces, the cantilever makes direct contact with the sample surface, and the deflection of the tip is measured as a feedback signal. For soft surfaces, the tip gently taps on and scans over the surface. The tapping is gentle enough for soft biomaterials, even in a liquid medium. Using this method, the surface morphology of different samples can be visualized, and the surface roughness can be measured. The AFM technique can also be used to measure surface physicochemical forces (e.g., van der Waals, dipole–dipole, dispersion, electrostatic) using a noncontact mode. When scanning a surface, the tip is oscillating above, but not in direct contact with, the surface. The forces between the tip and the surface change the oscillation which is measured and expressed as a force–distance curve. Using this technique a force map of the surface can be obtained.

Two major advantages of the AFM technique are that it can profile a diverse type of surfaces in three dimensions at high resolutions and that it does not require specific sample pretreatments and can therefore be used to investigate biological samples under in situ conditions.

3.3 Surface Layer Thickness

3.3.1 Sarfus

Sarfus is an optical technique for determining surface layer thickness by measuring the change of the polarization of light reflected from a specific support (surf) caused by the thickness of the sample layer (Ausserre and Valignat 2006). The surfs amplify the contrast of light but do not change the polarization after reflection. The polarization is changed only by the sample surface mounted on the surf and is affected by the sample thickness. This technique has been used for thickness analysis of chitosan and cellulose (Mohan et al. 2011; Spirk et al. 2013).

An advantage of the sarfus technique is that it visualizes thin films directly in real time requiring no vacuum conditions and provides three-dimensional presentations which also allow measurements of surface roughness. The measurable range of thickness is, however, limited to 70 nm.

3.3.2 Ellipsometry

Ellipsometry is an optical technique employed to determine the thickness of thin layers by measuring their dielectric properties (Losurdo et al. 2009). It analyzes the dielectric function tensor by determining the polarization of light reflected from the sample surface. It can be used to measure the thickness of single layers or multilayers ranging from sub-nano to micron scales. Ellipsometry is a nondestructive, nonintrusive, noninvasive, and contactless optical technique used for the characterization of surface structures, interfaces, and composites of bulk materials and thin films.

An advantage of ellipsometry is that specific sample preparation or surface modification is not required which allows real-time in situ analysis of biomaterials. It is, however, limited by the need for establishing reference surfaces for measurement purposes.

3.4 Hydrophobicity and Surface Energies

3.4.1 Contact Angle Measurements

Contact angle measurement is the most commonly used method to determine hydrophobicity, surface tension, and total surface energy of a solid substrate. These properties are, in general, a direct function of the contact angle of a liquid with known surface tension energies and can be calculated from the contact angle data using Young's equation (van Oss et al. 1988). Contact angles cannot, however, be directly correlated to surface tension which is contributed to by both Lifshitz–van der Waals (LW) and Lewis acid-based (AB) interaction energies. The total surface energy, which represents the hydrophobicity of a solid substrate, is obtained by calculating the components of the two interaction energies (1. LW component of surface tension, 2. electron acceptor parameter of the AB component of surface tension, and 3. electron donor parameter of the AB component of surface tension) using contact angle data (Wang et al. 2014). As a consequence of having three unknown factors to determine the total surface energy, contact angles of three liquids with different and known surface tension energies are deposited on a substrate and measured. In addition to the information derived from static contact angle measurements, the determination of contact angle hysteresis, which is the difference between the advancing contact angle and receding contact angle, gives insights into the dynamics of the interactions (Yasuda and Okuno 1994). The contact angle method has been used for characterization of surface tensions of different polysaccharide gels and surface coatings (Yasuda and Okuno 1994; Morra and Cassineli 1999).

Contact angle measurement is an inexpensive, rapid, and simple method which allows the contributions of different surface tension parameters to be distinguished. It is, however, limited by discrepancies in the results caused by surface morphology (e.g., roughness) and the wetness of the sample surface.

3.4.2 Surface Force Measurements Using AFM

As mentioned above in addition to measuring surface roughness, AFM can also be used for measurements of surface forces between the cantilever tip and a surface, which represent hydrophobicity, at a nanoscopic scale. It can be applied to different polysaccharide films to measure and compare the surface forces. Alternatively, the forces between a polysaccharide sphere fixed to the cantilever tip and standard surfaces (e.g., silica, metal) can also be measured using this technique (Ducker and Senden 1992). The surface forces are determined as a function of the displacement of the cantilever. This method is, however, expensive and time consuming, and the results can be greatly affected by the surface charge of the material.

3.5 Surface Charge

3.5.1 Charge Titration

It is of interest to quantify the immobile charges, contributed by weak acid groups, located on the surfaces of polysaccharides. High-precision titration techniques designed to determine complex equilibriums in solutions are often used for quantification of surface charge of dispersed polysaccharides (Fras et al. 2004). Titration methods are diverse and various indicators can be used (e.g., pH, conductivity, streaming potential).

For example, the potentiometric titration method determines the concentration of hydrogen ions dissociated from weak acid groups on the sample surface after the stepwise addition of NaOH. The concentration of hydrogen ions represents the electrical potential of the sample surface. The titration process is performed in an electrochemical cell, the potential of which is premeasured in order to determine the proton concentration. This method has been used to measure the charge of different fibers and pulp products (Laine et al. 1996; Lindgren and Öhman 2000; Räsänen et al. 2001).

Instead of the concentration of hydrogen ions, conductometric titration measures the conductance which is an additive function of the concentration and equivalent conductance of each type of ion present in the solution. The conductance of a solution is dominated by the two most conducting ions, namely, hydrogen and hydroxyl ions. This method has also been widely used for polysaccharides (Fras et al. 2004).

Another titration method for charge quantification is polyelectrolyte titration. This technique uses charged polyelectrolytes as the titrant and streaming potential as the indicator. A counter-charged substance is added to the sample solution and is absorbed on the sample molecules. The unadsorbed part is titrated by a polyelectrolyte until charge neutralization is achieved and the charge of the sample can be determined. For surface charge measurements, high molecular weight polyelectrolytes which neutralize the surface charges only are used. For total accessible charge measurements, low molecular weight polyelectrolytes that can neutralize both surface and interior charges are used. This method has been used for a range of polysaccharides (Wågberg et al. 1989; Zhang et al. 1994).

3.5.2 Zeta Potential (ZP) Measurement

The electrostatic charge density on a surface is usually expressed ZP. The ZP is defined as the difference in the electric potential between the surface double layer and the mobile liquid phase. This is the primary interaction area for substances diffusing from the liquid bulk phase toward the solid surface. The level of ZP depends on the chemical and physical properties of the surface as well as the liquid medium and is therefore a parameter describing the interaction properties of a solid surface in contact with a liquid. The ZP is often used to estimate the effect of surface charge on different physicochemical processes such as aggregation, adsorption, and dispersion.

Direct measurements of ZP are not experimentally feasible, and it is therefore necessary to employ indirect means such as electrokinetic methods (e.g., electrophoresis). For example, the electrophoretic mobility method determines the mobility of reference colloid particles in an electric field generated by the surface charges of the solid sample (Bellona and Drewes 2005). The result (mobility) can be converted into ZP.

Streaming potential measurement is also widely used for determinations of surface charge. It measures the potential induced when an electrolyte solution flows across a stationary and charged surface (Childress and Elimelech 1996). A limitation of these methods is that results can be greatly affected by surface morphology (roughness and topography). Similar methods for charge measurements, such as sedimentation potential and electroosmosis assays, are also used.

3.6 Surface Absorption: Quartz Crystal Microbalance (QCM)

Adsorption of polysaccharides at solid–liquid interfaces is of interest with respect to technological and biomedical applications. Use of the QCM is a promising technique that provides direct information on solid–liquid interactions. It gives information about adsorption/desorption processes at the solid–liquid interfaces. The QCM detects a sub-monolayer of adsorbate on the surface by measuring the change of the oscillating frequency of a piezoelectric quartz crystal device upon mass loading (Marx 2003). Real-time measurements of the frequency change and energy dissipation caused by mass changes and viscoelastic properties give information about the absorbed layer. This technique has been used for adsorption studies of different polysaccharides such as cellulose and chitosan (Indest et al. 2008; Lee et al. 2014).

An advantage of this method is its sensitivity that allows the detection of small amounts of adsorbate (at the ng level) in situ. A disadvantage is that it does not measure the adsorbed mass directly. The results need to be approximated using Sauerbrey equation (Marx 2003) to calculate the adsorbed mass.

4 Potential Applications: Surface Engineering

While they are widely used across a range of industries, the applicability of polysaccharides is, in some cases, limited by their surface properties. For example, chitosan is a good candidate for food packaging materials due to its nontoxicity,

biodegradability, and antimicrobial activity. Its applicability is, however, limited by its low mechanical strength. In order to overcome these limitations, characterization and measurement of polysaccharides by the above techniques, and subsequent directed surface chemical and physical modification techniques to improve their features for specific applications, can be used.

4.1 Charge Modification

The strength of the surface charge on polysaccharides is dependent on the surface chemical composition and affects surface chemical and physical interactions. Modification of surface charges of polysaccharides can greatly alter their surface properties and interactions and consequently change or enhance their research and industrial applicability. Both cations and anions can be induced on polysaccharide surfaces.

A common method for charge modification is surface ionization via induction of chemical groups. Anions on polysaccharide surfaces are contributed by acid groups which can ionize the surface without changing the pH to extreme values. Methods for inducing acid groups are diverse and include but are not limited to: oxidation which uses hydrogen peroxide, nitrogen dioxide, or ozone to oxidize hydroxyl groups on polysaccharide chains to charged carboxyl groups (Zeronian and Inglesby 1995); addition of sulfonic acid groups to fibers to produce sulfoxine cellulose (Zih-Perényi et al. 1998); and carboxymethylation, which replaces the proton on a hydroxyl group with a carboxymethyl group (Song et al. 2012). The increases in charge and acid groups on polysaccharide surfaces are usually determined by charge titration methods in combination with XPS. These chemical processes not only modify polysaccharides with respect to surface charges but also their structural, mechanical, and biofunctional properties. A good example of such a product is carboxymethyl cellulose (CMC) which is often used as an adsorbent for cotton to improve its functionality. After being adsorbed by cotton, CMC facilitates higher water adsorption of the cotton and improves its ability to adhere to moist surfaces, its biodegradability and biocompatibility, and its antimicrobial activity.

The cationic functionalization of polysaccharides is also of great importance in industry. The most common cationized polysaccharide is nitrogen-coated cellulose. Water-soluble cellulose coupled with tertiary amino groups, which is prepared by the interaction of cellulose acetate with *N,N*-diethylepoxypropylamine, has been extensively studied for its application in industry (Liesiene 2010). Due to its increased charge, this cationic cellulose can be used as a flocculant for wastewater treatment, as an additive in paper making, as a thickener for mineral processing and oil recovery, and as a conditioner in personal care formulations (Finch 1996).

While charge modification is widely used to enhance the applicability of polysaccharides, it should be noted that the chemical treatments used to modify charges may also result in undesirable changes in physical and chemical properties such as texture, surface morphology, rigidity, and toxicity. Precautions and a thorough

investigation of the characteristics of charge-modified polysaccharides need to be taken before employing them, especially in food industry.

4.2 Polysaccharide Hybrid Materials

The specific properties of individual polysaccharides are frequently used in industry and research. The application of a single polysaccharide is, in some cases, not sufficient due to the limitations of the nature of the polysaccharide itself. For example, cellulose nanoparticles can be used as a bionano reinforcement for formulating biobased nanocomposites (Samir et al. 2005) because they are a renewable and environmentally friendly material with low density, high mechanical strength, and a nonabrasive nature. This applicability is, however, limited by a low compatibility with other materials that does not allow acceptable dispersion in nanocomposites. Chitosan composites represent an example of this. Chitosan is a very abundant material in nature and is characterized by nontoxicity, biocompatibility, biodegradability, and antimicrobial activities. These features make it suitable for various biomedical applications such as drug delivery, tissue engineering, wound healing, and food packaging (de Mesquita et al. 2010). Its water sensitivity and relatively low mechanical strength limit its use for a wider range of applications, especially in moist environments. To overcome these limitations, hybrid materials of two or more polysaccharides or of a polysaccharide and a non-saccharide material have been developed for industrial and research purposes. A hybrid polysaccharide material of cellulose and chitosan has been intensively studied in recent years. Due to the presence of glucosamine monomer units, chitosan is chemically compatible with a number of polysaccharides and in particular with cellulose. The adsorption of chitosan onto cellulose surfaces involves the protonation of the primary amine groups on the surface of chitosan and is therefore irreversible. The amine groups also increase the charge levels of cellulose by cationizing the surface. Čakara et al. (2009) established a chitosan–cotton model and found a relationship between pH and the increase in charge using potentiometric titration. de Mesquita et al. (2010) developed a new biodegradable nanocomposite using layer-by-layer assembly of nanocellulose and chitosan. Potentiometric titration and ZP measurements were performed to determine the charge of the product, and SEM was used to visualize the surface morphology. The surface was relatively smooth and negatively charged. Nanocellulose was incorporated into chitosan layers in high amounts resulting in a dense and homogeneous distribution. After the incorporation of nanocellulose, chitosan displayed a significantly increased mechanical strength and excellent thermal stability and water resistance.

Development of organic-inorganic hybrid materials provides another approach to improve surface physicochemical properties of polysaccharides. For example, introduction of silica into polysaccharides can affect the oxygen permeability, hydrophobicity, and mechanical and thermal properties (Suzuki and Mizushima 1997; Tian et al. 1997; Liu et al. 2004). Liu et al. (2004) combined chitosan and silica at different ratios using sol–gel chemistry and determined the properties of

the products. It was found that an increase in silica–chitosan ratio resulted in a decrease in water contact angle, an increase in thermal stability, and a decrease in mechanical strength. This shows that the physicochemical properties of hybrid materials can be manipulated by adjusting the ratio of precursors.

Hybrid materials are able to combine the advantages of different polysaccharides and/or non-saccharide materials, which can greatly enhance their applicability in different areas. This approach is limited by the compatibility of the components in terms of solubility and different reaction conditions such as pH and temperature. Careful consideration must be given to material selection based on the conditions of under which the materials will be produced and used.

4.3 Chemically Modified Polysaccharide Surfaces With Bioactivities

Chemical modification of polysaccharides may provide bioactivities that can be used in the industrial and medical spheres. A well-known example is the derivatization of the hydroxyl groups of cellulose that provides specific bioactive properties. A research area of growing interest is the synthesis of sulfated polysaccharides that possess antithrombotic features. Polysaccharides often used for sulfation are cellulose, chitosan, and galactoglucomannans. Negatively charged sulfate groups are introduced to these polysaccharides, usually at the C-6 position, in different solvents based on their solubility. For galactoglucomannans organic solvents are used, whereas chitosan is only soluble under acidic conditions and cellulose requires very specific solvent systems. When sulfating chitosan consideration must be given to the fact that the NH_2 group at C-2 needs to be preserved during the process for further reactions. Important characterization techniques for sulfated polysaccharides include infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and inductively coupled plasma mass spectroscopy (ICP-MS) (Fasl et al. 2010).

The antithrombotic activities of sulfated cellulose, chitosan, and galactoglucomannans were determined and compared to their original forms as well as heparin, a well-known anticoagulant, using activated partial thromboplastin time (APTT), free hemoglobin, and QCM methods (Doliška et al. 2012a, b). Results showed that the sulfated form of the three polysaccharides exhibited high antithrombotic activities. These polysaccharide sulfates can effectively prevent blood from clotting and are therefore excellent examples of how surface engineering can improve the biocompatibility of artificial polymers.

Another example of the chemical modification of polysaccharide surface characteristics is carboxymethylation of lacquer polysaccharide (LP) isolated from the sap of the lac tree, which enhances bioactivity against leukopenia induced by cyclophosphamide (Yang and Du 2003). LP was carboxymethylated using monochloroacetic acid, and the product was characterized using gel permeation chromatography (GPC), Fourier transformation infrared spectroscopy (FT-IR), and NMR. The bioactivity assay was performed in mice, and the results showed that

carboxymethylated LP exhibited a significantly higher activity against leukopenia as compared to its unmodified form.

5 Conclusions and Outlook

This chapter aims to provide an overview on methods and instrumentation used to study surface properties of polysaccharides and to provide examples of surface modification of polysaccharides measurable by these techniques that are applicable in the medical, food, and material science. An overview of this information allows a better understanding of the methods used to investigate interfacial processes in polysaccharides and provides insight into future polysaccharide material development and processing. There are a number of challenges with respect to research into, and future applications of, the methods used to characterize surfaces of polysaccharides. First, development of methods for sample preparation that can effectively extract polysaccharides from the natural source while preserving the chemistry, structural features, and inherent properties is still beyond reach. Second, analytical methods for one property are often unable to eliminate interference from other properties (e.g., results obtained from contact angle measurements are often affected by surface morphology and wetness, hydrophobicity and charge often affect the determination of each other). Third, the surface properties and molecular/supermolecular structure of polysaccharide in the dry state are already well described and understood, while these properties in the wet state, where most technological and natural processes take place, are still barely known. Knowledge of the chemical/physical interactions between polysaccharides and liquid media, such as the swelling processes and the kinetics of penetration, is very limited. Future research on polysaccharide surfaces and the development of methods associated with this are challenging and require effort from multidisciplinary expertise in areas such as surface chemistry, engineering, and instrumentation.

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Computerized Models of Carbohydrates

47

Alfred D. French

Contents

Abstract

Some history of computerized modeling is furnished to set the stage for modern work, including the development of the justification for using flexible monosaccharide residues. Modeling studies of mono- and disaccharides, primarily by the author and colleagues, have been reviewed and additional analyses were furnished that relate the conformations found in crystal structures to the adiabatic energies through a Boltzmann-like distribution. Those decreases in the numbers of observed crystal structures with increasing energy, discussed earlier by Dunitz and coworkers, suggest that the consequences of crystal packing forces are equivalent to the distribution of structures that would be found at about 500 K for non-crystalline molecules. A closing study of a large oligosaccharide, a cycloamylose with 26 glucose residues (CA26), revealed the extent to which energy minimization studies could retain molecular features found in crystal structures of a large carbohydrate. As also done for the disaccharides, both empirical force fields (molecular mechanics) and density functional theory

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(quantum mechanics) were employed. Some intrinsic features of the CA26 were reproduced, but the detailed results from the different modeling methods diverged. Results from the CA26 calculations based on normal termination criteria changed significantly when tighter criteria were employed; it was not practical to tighten the criteria for the quantum calculations.

Keywords
Monosaccharides • Disaccharides • Oligosaccharides • Polysaccharides • Fructose • Cellobiose • Cycloamylose • CA26 • Validation • Energy • Molecular mechanics • Quantum mechanics

1 Introduction

Carbohydrates are chiefly composed of carbon, hydrogen, and oxygen, with occasional substitution of the oxygen atoms with nitrogen, sulfur, and other atoms. Included are mono-, di-, oligo-, and polysaccharides. Structural knowledge of a polysaccharide logically includes the knowledge of the smaller units, so it might seem ironic that the earliest computer model of a carbohydrate was of the cellulose polysaccharide. That model was intrinsic to the fiber diffraction study of Jones (1960), who used the Manchester University Mark I and DFUCE electronic computers. His efforts sought a model that would be satisfactory from a stereochemical standpoint and satisfy the X-ray intensities on the fiber diffraction pattern. At that time, there were just a few relevant crystal structures to provide approximate positions of the carbons and all but one of the oxygens of the glucose monomer.

Shortly afterwards, Ramachandran et al. (1963) and his modeling method captured the collective imagination of the biopolymer field because his approach was focused on predicting the most likely shapes of a molecule through conformational analysis. That goal is more widely held than Jones' need to account for a diffraction pattern from a reasonably well defined molecular shape. Conformational analyses are founded on the Boltzmann relationship that states that the most probable shapes will have the lowest energies. Thus, a conformational analysis evaluates the relative energies of the various potential molecular shapes that might occur, with the shapes having the lowest energy being most probable. In early studies (Rees and Skerrett 1968; Goebel et al. 1970; Lemieux et al. 1980), the geometry of individual monomers was assumed and not varied (although different authors assumed slightly different structures); the very first "energies" were based on interatomic contact distances. They were rated as allowed (low enough), partially allowed, or disallowed (too high in energy).

In analogy with Ramachandran's work on peptides and proteins, it was understood that the energy of a disaccharide would depend on the values of the torsion angles describing the amount of rotation about the bonds to the jointly held glycosidic oxygen atom. The conformations of two-bond-linked disaccharides were studied by systematically calculating the energy for all of the combinations of two torsion angles now widely named ϕ and ψ. The standard visualization of the

results is a ϕ-ψ map. If the dipeptide or disaccharide linkage conformation was being studied as a model for the polymer, then all linkages of the polymer were assumed to have the same geometry, resulting in a regular helix. In discussions of the exact shapes, a helical cavity is not required for a helix to exist. The only requirement is for screw-axis symmetry, and the helix can have nonintegral numbers of residues per turn. The relationship between ϕ and ψ values and the helix descriptors of number of residues per turn (**n**) and the advance per monomer along the helix axis (**h**) was worked out by Shimanouchi and Mizushima (1955). Thus, a disaccharide's ϕ-ψ map can also depict the **n** and **h** values for the related polysaccharide with a given monomeric geometry and glycosidic linkage bond angle.

When studying proteins or polysaccharides, it is also necessary to account for the excluded volume principle, namely to avoid conflict with more distant parts of the molecule and also to recognize any attractive interactions. Thus, while useful information is available from the simple allowed, marginally allowed, and disallowed ratings of the earliest computer models (Ramachandran's earliest models were physical!), the many forces in play gave a strong impetus to incorporation of quantitative energy calculations.

During the ensuing decade, it was realized that carbohydrates have special needs that are less important or even inapplicable when modeling proteins or other organic molecules. With the abundance of hydroxyl groups of carbohydrates, hydrogen bonding is a major complication and the still-controversial anomeric effects (including the exo-anomeric effect) are another (Grindley 2008). Departures from established understanding continue to be called "effects" even after they become part of the conventional thinking. Several other effects that affect carbohydrates are described by Allinger (2010).

What wasn't initially appreciated was a third item, the flexibility of the monomeric unit. In the case of ring-form carbohydrate monomers, there is a substantial variability among the positions of the ring atoms, even if all rings being considered can be described as having a chair form. The detailed variability is indicated by the different values of the endocyclic torsion angles. This variability can radically affect the relative disposition of the bonds to the inter-monomeric linkage oxygens. To fully appreciate the effects of monomer flexibility, it is useful to think in terms of connecting particular individual glucose monomers with given glycosidic bond angle and ϕ and ψ linkage torsion angles. In the case of models of the amylose component of the starch polysaccharide that has α-(1,4)-linked glucose residues, many monomers with feasible structures can be taken from the extensive literature of crystal structures. The shapes of the model starch molecules will depend importantly on the particular monomer shape. Because each of the glucose monomer shapes from crystal structures had been experimentally observed, it must be considered as a potential monomer shape in an oligosaccharide or polysaccharide.

While the distance between the C1 and C4 atoms of α-glucopyranose is relatively constant, the O1–O4 distance varies over a range of more than 0.8 Å (French and Johnson 2011), with roughly 15° ranges each for the C4–C1–O1 and C1–C4–O4 virtual bond angles in either α- or β-glucopyranose. These ranges were found with a new search of the Cambridge Crystal Structure Database

(Groom and Allen 2014) that was conducted for this review. The virtual torsion angle O1–C1–C4–O4 in α-glucose $^4C_1$ rings have values ranging from −14° to +22° (French and Johnson 2007). The resulting variations in polymer shape are important in that some observed polymer shapes cannot be modeled unless the correct monomer shape is incorporated. Of course, the problem of variable monomer geometry is magnified when an overall shape, such as a chair, cannot be assumed and other ring shapes must also be considered.

The variability of the O1–O4 distance has strong implications for the shape of some model oligomers of the amylose component of starch. Because of the relatively constant C1′–O–C4 glycosidic bond angle, the oligomers with short O1–O4 distances lead to helices with small values of the helical parameters **n** and **h**. Longer residues lead to helices with larger values of **n** and **h** (French and Murphy 1973). Cyclic model oligosaccharides such as cyclomaltohexaose (α-Schardinger dextrin) have **h** = 0.0. Stereochemically correct models of the cyclomaltodextrins can be built with five to nine residues that have increasing O1–O4 distance of the constituent residues. Goebel et al. (1970) reported a maximum of six residues per turn for amylose when modeled with one glucose geometry and nine residues per turn when another geometry was used, without attributing the difference to any particular geometric feature of the glucose residues. The O1–O4 distance variability is discussed further in Sect. 4.

A second manifestation of the need to consider flexible residues was less obvious. A clear example of this need was given by a Ramachandran analysis to understand the range of observed variation in torsion angles of the glycosidic linkages in the crystal structures of molecules that contained sucrose moieties (Ferretti et al. 1984). Their computer models were based on rigid monomer geometries from crystalline sucrose. Their calculated energy for the sucrose moiety in the raffinose trisaccharide, relative to the sucrose conformation in its own crystal, exceeded 100 kcal mol^{-1}. A relative energy of 10 kcal mol^{-1} indicates severe problems for an observed structure, let alone 100 kcal mol^{-1}. Ferretti et al. observed that the extremely high energy was probably an artifact due to the use of rigid residue models. Subsequent calculations that allowed the monomer geometries to relax during energy minimization reduced that energy to 8–10 kcal mol^{-1}, and a hybrid quantum mechanics/molecular mechanics (QM::MM) calculation in the same paper reduced the relative energy to about 3 kcal mol^{-1} (French et al. 2000a).

Conformational energy calculations for reducing disaccharides that had been based on energy minimization with MM had been reasonably predictive of the observed conformations. Therefore, it was hypothesized that the parameterization for the linkage torsion angle energies was insufficient for nonreducing sugars. When both carbon atoms of the glycosidic linkage serve as anomeric centers, there could be an overlapping exo-anomeric effect that was mentioned earlier by Schleifer et al. (1990). Such effects may be modeled more reliably with QM.

At first glance, the emphasis on Ramachandran mapping of disaccharides might seem excessive, considering that there are other carbohydrate conformation questions of interest. For example, the shapes of five-membered furanosyl rings are important, as there is substantial variability potentially present and modeling could

assist understanding of the properties of molecules having these small rings. The six-membered pyranosyl rings are usually found in a chair form with the maximum number of equatorial substituents, but a few sugars, especially idose, are found in both chair forms (Angyal and Pickles 1972) and a skew-boat form (Snyder and Serianni 1986). Idose chair forms have mixtures of axial and equatorial substituents. The study of the shapes of the various rings, however, required development of potential energy functions that could be considered reliable. In the early work, therefore, it was more attractive to try to explain the polysaccharide shapes based on monosaccharide geometries that were known with some confidence.

By the early 1990s, there was a wider range of carbohydrate modeling studies. Rigid-residue modeling had been mostly discarded as a viable approach and was replaced with relaxed-residue energy mapping. In addition to empirical potential energy functions, electronic structure theory (quantum mechanics) methods were being applied to small molecules such as five-membered rings (Garrett and Serianni 1990). Models of crystals were also being studied, including attempts to solve the crystal structures for simple polysaccharides such as cellulose Iα, which has only one chain with two glucose residues per unit cell (Aabloo and French 1994). Computer software was expanded to handle such large problems, and increases in processor performance and memory size enabled these advances.

One of the most important divisions was the development of molecular dynamics (MD) methods. MD has numerous capabilities that are not available in energy minimization methods. Although MD is usually carried out with empirical (often called molecular mechanics or MM) force fields, it is also possible to use electronic structure theory (quantum mechanics, QM) methods to compute the energy of the system at each increment of time. The first advantage of MD is that entropy is explicitly considered. A molecule may possibly spend more time in a conformation with slightly higher enthalpy because the range of allowed variation is larger than for a structure with a lower enthalpy but a small allowed range. The time spent in each conformation is equivalent to its relative probability, and the probability is a function of the free energy. Thus, information on free energy is available. Another advantage is that the orientations of exocyclic groups of carbohydrates are varied "naturally" because of thermal motion. However, it is still important to make sure that the necessary variations are sampled during the dynamics.

Perhaps the biggest reason for MD studies is the ability to explicitly incorporate water molecules. By placing the solute molecule inside a "box" that is filled with hundreds or thousands of water molecules, the influence of water on the properties of the carbohydrate can be studied. Because the addition of thousands of water molecules adds considerably to the computational expense (the amount of time and computer memory capacity used), the added water molecules are treated with their own specific parameters. These parameters are combined to produce SPC (Berendsen et al. 1981), TIP3P (Jorgensen et al. 1983), or a number of other water models. Some observed properties of carbohydrate molecules are apparently the result of changes in the structure of the water around the carbohydrate; MD studies can test those ideas (Liu and Brady 1996; Mason et al. 2005). Another important function of MD modeling is the study of the effects of temperature

change, for example calculation of the glass transition temperatures for polysaccharides (Caffarena and Grigera 1997, Mazeau and Heux 2003), as well as other temperature-based phase changes.

With the current ability to do multi-nanosecond or even microsecond simulations for models of reasonable sizes, the Boltzmann equation makes it possible to back-convert the populations (or probabilities) of the various increments of geometry to give an energy map. When the MD studies also include water explicitly, the calculated population distribution depends on both the intrinsic forces as well as the forces from the water and the effects of the water on the intrinsic forces. The conversion of the populations gives a "potential of mean force" that is plotted on free-energy versions of Ramachandran maps. Simulations by Hatcher et al. (2011) have been able to determine energy values as high as 6 kcal mol^{-1}. Thus, reliable populations were obtained for structures that were only 0.000045 as probable as the global minimum structure.

With this list of MD's important attributes comes an impressive list of extra challenges. One important challenge is the degree of sampling needed to assure that all of the important conformations are given due consideration. The structures can remain trapped inside of local wells and never access the global minimum structure because of high barriers. There are several schemes for overcoming this "multiple minimum" problem, with examples in Perić-Hassler et al. (2010), Spiwok et al. (2010), and Campen et al. (2007). This problem also exists with energy minimization methods. Methods for overcoming the problem with MD could be said to add to the complexity of MD methods.

Another MD problem is that the methods of calculating the energy are usually compromised because of a need for rapid calculations. The empirical force fields used are based on simple potential energy functions to speed up the calculation, and the most widely used force fields are specific to narrow classes of molecules. The force field is usually refined to work with a particular water model, and the force field may then not be optimal for other environments.

At present numerous topics in carbohydrate chemistry are under study with various modeling methods. With the worldwide interest in sustainable bioenergy, much effort is expended on the study of cellulose. Of particular interest is the so-called biomass recalcitrance, i.e., the resistance of the biomass to degradation to individual fermentable glucose residues. That recalcitrance is usually attributed to the crystallinity of the cellulose. Therefore, crystal modeling is the subject of many studies. The resulting models are tested by the calculation of numerous observables, including FT-IR (Fourier transform infrared), Raman, and NMR (nuclear magnetic resonance) spectra (Kubicki et al. 2013), crystal dimensions and retention of proposed hydrogen bonding schemes (Matthews et al. 2006; Yui et al. 2006; Nishiyama et al. 2008), and X-ray diffraction patterns (Nishiyama et al. 2012).

Another fairly new area is the study of the weak interactions within or between molecules with either Natural Bond Order (NBO) methods or Bader's Atoms-in-Molecules theory. NBO methods have been used to study the anomeric effect, based on various interactions (Takahashi et al. 2007; Cocinero et al. 2011). AIM has been exploited to a limited extent for detecting in an objective manner the various

atomic interactions in both experimental X-ray diffraction studies and purely theoretical QM calculations, as well as studies that use both theory and experiment (Jaradat et al. 2007; Stevens et al. 2010; French et al. 2014).

This chapter will touch on modeling studies in order of molecular size, avoiding, with apologies, as much as possible the numerous areas in which the author has little or no first-hand experience. A general thought is that the arc of modeling progress bends towards fewer assumptions, but the effort involved increases exponentially with the loss of each assumption.

2 Monosaccharides

2.1 Acyclic Sugars

Over the years little effort has been spent on acyclic monosaccharides. When a hexose is acyclic there are many variables that have complete freedom. For example, all the torsion angles have surmountable barriers to full rotation so a complete determination for the hexose chain would have three backbone torsions plus five hydroxyl group torsions to consider. This would result in some 3^8 (6,561) conformers for which the energy is calculated, assuming that the low-energy forms would correspond to, or be accessible from, one of the three staggered conformations. Such a project, involving the added torsion angle in D-glucaramide (3^9 or 19,683 starting structures), was completed using the empirical force field program MM3 (Allinger et al. 1990) in batch mode on a Windows PC that was considered obsolete in 2002 (Styron et al. 2002). One complication, often encountered in MM projects (either MD or energy minimization), was the need to develop torsion angle parameters. In the glucaramide case, parameters were needed for the N–C–C–O sequences. That was done with the aid of HF/6-31G* QM calculations. Another complication is simply the bookkeeping that is needed for so many thousands of calculations. Follow-on work involved a Monte Carlo method running under Linux to more efficiently search for the low-energy forms (Dowd et al. 2011). The method is also applicable to larger structures, but since an explicit search of all possibilities had been conducted for glucaramide, it was a good target for validation.

2.2 Ring-Form Sugars

Monosaccharides of greatest interest often have ring shapes. As in the case of the acyclic structures, bond-length variations are not considered to be meaningful for describing ring shapes, nor are conventional bond angle differences. Torsion angles are again the major factor in conformational change, although changes in torsion angles will result in some variation in the bond lengths and bond angles. Because there are too many endocyclic torsion angles to mentally monitor, however, conformational analysis is based on the fact that ring formation results in a structure for which there is a meaningful mean plane. Deviations of the atoms from that plane

result in different descriptors for the ring shape, and the descriptors indicate the position(s) of maximum deviation from the plane as well as the extent of the deviation. If just the exocyclic torsions are considered for an aldohexose, there are $3^6 = 729$ conformations to consider for each ring shape. A brief study (French and Johnson 2011) showed that only 150 combinations of exocyclic orientations were stationary for α-glucopyranose when it was in the $^4C_1$ shape. Of these, 125 were true minima at the B3LYP/6-31+G(d,p) level and the remaining 25 were saddle-point structures. The range of energies for these structures was 13.8 kcal mol^{-1} in the vacuum calculations.

2.3 Ring Puckering

Although many studies ignore variations of ring shape, it is critical to understand it. The simplest important form is the furanose ring. Many aspects of conformational analysis can be illustrated with furanose rings and they are small enough that fairly complete analyses can be carried out with minimal assumptions. The conformations of furanose rings were recently reviewed in great depth by Taha et al. (2013).

Furanose rings. Figure 1 shows both an aldofuranose, ribose, and a ketofuranose, fructose, with the carbohydrate-specific numbering of the atoms. Because three points define a plane, a conformational analysis of furanose rings can be carried out by driving the starting structure into different shapes by moving the remaining two atoms perpendicular to the plane. Then, a determination of the resulting ring shape is performed. Five-membered rings are described as having either Envelope (E) or Twist (T) shapes. E shapes have one atom that is either above or below the plane formed by the other four atoms, with a total of ten different E shapes. T shapes have two adjacent atoms deviating in opposite directions from a plane formed by the other three atoms, and in characteristic T shapes, the two deviating atoms will be the same absolute distance from the plane. The 10 T shapes are intermediates between the adjacent E forms. For example, the $^3T_2$ form would be a transition shape between the ^3E and the E_2 forms.

Consider a furanose ring with conventional ketose numbering (Fig. 1a). If the C2, C3, and C5 atoms are fixed in a plane with z-coordinates of zero, the ring oxygen and C4 can be fixed at different distances from that plane, for example in increments of 0.1 Å up to ± 1 Å. Of course, this particular procedure depends on the modeling software to have the facility to fix the coordinates of the atoms in one dimension, but not in the others. Although the ring oxygen can be moved by itself to create starting models for energy minimization, movement of C4 requires coordinated movement of its attached exocyclic groups (H4 and O4–H). Thus, substituents on C4 will require the varying C4 z-coordinate value to be added to their original z-coordinate value. Movements of the atoms from the plane would more naturally follow arcs, so when puckered ring shapes are initially constructed by simply adjusting the z-coordinates of the deviating atoms, very strained rings occur. However, energy minimization will quickly relieve that strain. The process of giving 21 values of the z-coordinates to the O5 and C4 atoms (for a total of

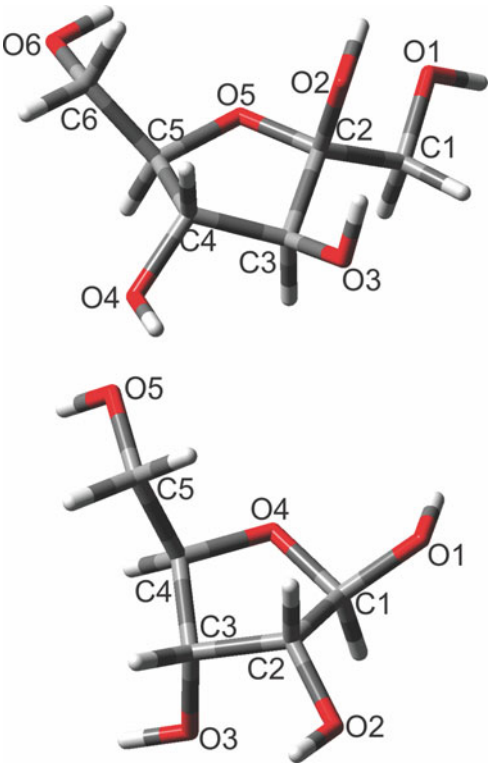

Fig. 1 β-D-Fructofuranose (*above*) and β-D-ribose (*below*). The fructose ring has the $^{4}T_{3}$ shape, and the ribose ring is $^{2}T_{3}$ in this drawing

441 structures) generates a reasonably complete range of furanosyl ring conformations (French and Dowd 1994).

Numeric values can be associated with the deviation from planarity for furanoid molecules because there are 10 E forms, interspersed with 10 T forms for a total of 20 characteristic (or canonical) conformations. In the case of cyclopentane, the E and T forms are nearly isoenergetic, and the equilibrium state is dynamic in solution or the gas phase as the structures interconvert, with different atoms being out of plane. The process is called pseudorotation, as one E form converts to a T form and then on to another E form. For example, the ring form can easily change from ^{1}E to $^{1}T_{2}$ to E_{2} and then to $^{3}T_{2}$. Thus, the location of "puckering" moves around the ring. For a five-membered ring, numeric values can be given to describe the puckering. Namely the characteristic conformations are spaced 18° (360°/20) apart on a "conformational wheel," allowing intermediates to the characteristic conformers to be designated with an intermediate numeric value. Figure 2 illustrates conformational wheels for both aldoses and ketoses, and in both the Altona and Sundaralingam (1972) and the Cremer and Pople (1975) puckering nomenclature. Additionally, the broad North–South-East–West classifications are indicated, as well as the obsolete "endo" and "exo" notation. Further, the amplitude of deviation from the mean plane can be expressed in distance units such as Å. A useful, if not rigorous, conversion between the Altona-Sundaralingam and Cremer-Pople

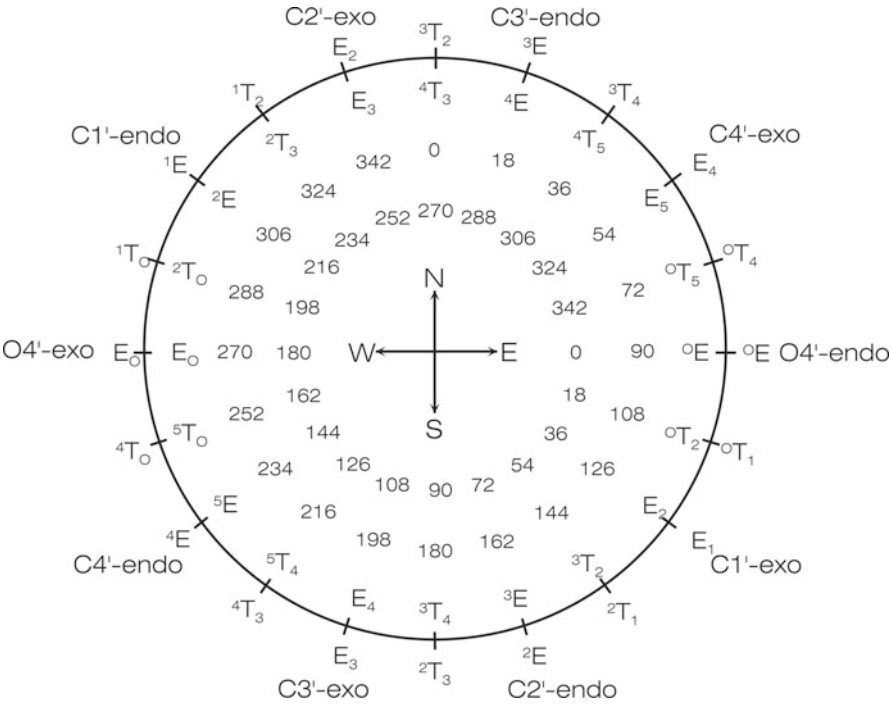

Fig. 2 Conformational Wheel for aldo- and keto-furanose rings. The outer designations apply to the aldofuranoses and the inner ones to ketofuranoses. The inner ring of puckering phase angles applies to the Altoona-Sundaralingam puckering parameters; the outer ring to Cremer-Pople phase angles. The obsolete endo and exo notation is also given for the aldofuranoses

nomenclatures is to just add 90° to the Cremer-Pople phase angle (Φ) and multiply the Cremer-Pople amplitude (q) by 100.

Unlike cyclopentane, the various conformers of furanosyl sugars have ranges of energy values so it is of interest to conduct a conformational analysis based on the puckering parameters. One way to carry out such an analysis is to calculate the energy at each of the 441 points mentioned previously (30 points corresponded to very distorted structures and were not included). One "small" problem is that the orientations of the exocyclic groups affect the calculated energy. Hexafuranosyl sugars such as fructofuranose have seven different exocyclic bonds about which groups can be rotated. So an "adiabatic" conformational energy surface can be calculated by finding the lowest of the 2,187 energies for each of the 441 points, i.e., while holding the ring in the prescribed shape with the O5 and C4 atoms held at different distances from the z = 0 plane held by the other three carbon atoms. The puckering parameters for the 441 ring shapes are determined by software provided by Cremer (Ring 88), or other suitable routines, e.g., PLATON (Spek 2009). Because most plotting software is not able to provide plots for the plane polar coordinates that describe the puckering, they are converted to Cartesian coordinates, after attending to the need to actually plot the coordinates based on 360°-Φ so

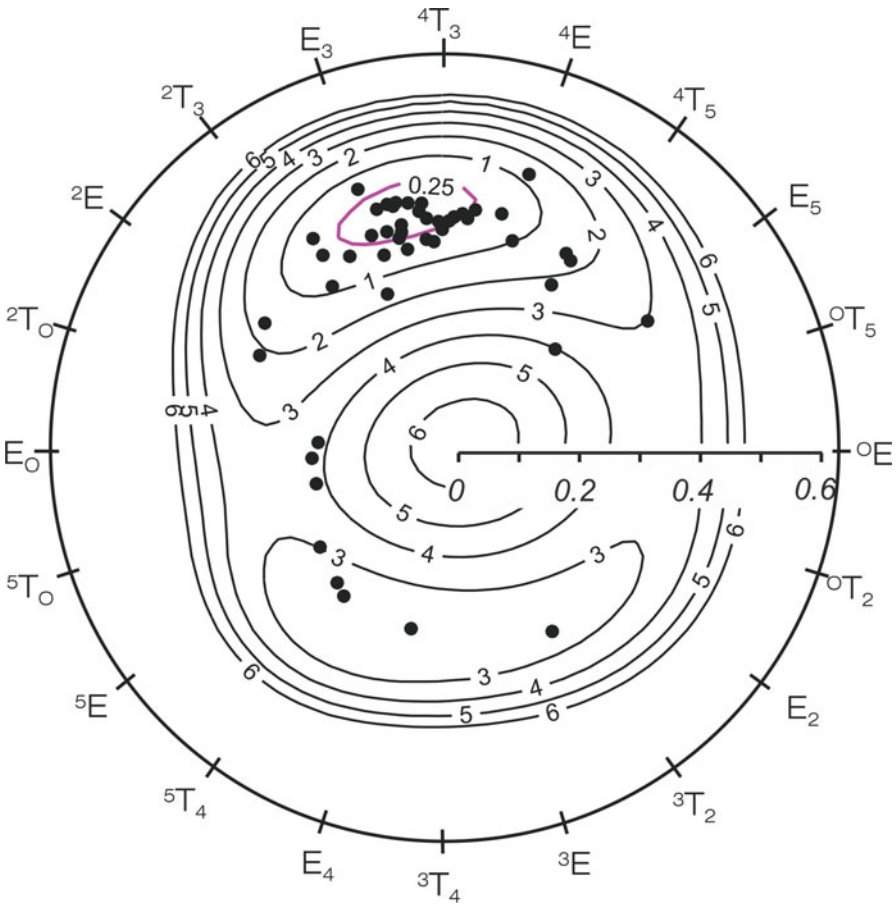

Fig. 3 MM3 energy contours for β-D-fructofuranose plotted on conformational wheel. The Cremer-Pople puckering amplitude is shown by the *horizontal line* from the *center* of the pattern to the ^{O}E conformation. Observed ring conformations from crystal structures for many fructose-related compounds are shown as *large dots*

that a clockwise system results. That allows the plot to correspond to the ring shape when the ring is oriented according to the convention, as in Fig. 1.

The Cartesian coordinates for 441 different conformations and their associated energies can then be fed into a surface contouring routine. The Surfer program (Golden Software, Golden, Colorado) has most of the necessary facilities, and its output is shown in Fig. 3 for β-fructofuranose. In this plot, the energies were calculated with MM3 at a dielectric constant of 4.0 (French et al. 1997) and the points representing the experimental puckering parameters from a new survey of the Cambridge Crystal Structure Database (CSD) are displayed. The plot shows the puckering amplitude scale as well. All but six of the 47 different conformations fall into the northern, global minimum, with the southern structures generally having

smaller puckering amplitudes than the northern ones. This decrease in amplitude for the lowest-energy southern structures is also indicated by the energy contours, which have their local minimum closer to the center of the wheel.

It is not universally agreed that such an analysis can be expected to predict the conformations found in crystal structures. Crystal neighbors deform the ring shape in different ways and this is shown in Fig. 3. Chemical substitution seems to have less influence on the variation than does crystal packing. The 'rules' for inclusion of a particular structure from the CSD were that there be no rings involving the exocyclic groups that would hold the ring in a particular shape. But there are substantial chemical differences, with sucrose octaacetate and sucrose octasulfate both included as well as numerous hydroxyl-bearing oligosaccharides. Conformations of the furanose rings in the trisaccharide, 1-kestose are $\Phi = 254.4°$, $Q = 0.417$ Å and $\Phi = 100.6°$, $Q = 0.298$ Å. Thus it has both northern and southern rings in the same molecule. They are about as different as the rings in the two substituted sucrose molecules (for the octaacetate, $\Phi = 302.7°$, $Q = 0.328$ Å; for the octasulfate $\Phi = 129.2°$; $Q = 0.280$ Å).

The hypothesis that the puckering of furanose rings can be predicted from isolated-molecule calculations for native, substituted, and complexed crystal structures is subject to quantitative tests. Previously, a similar proposal for another set of conformational indicators, the linkage torsion angles for disaccharides, was tested by examining the distribution of conformational energies that corresponds to the observed conformations. To be a successful prediction, the frequencies of the corresponding energy values should decrease in an exponential manner as the energies increase (Bürgi and Dunitz 1988).

Figure 4 shows a "cumulative frequency" plot of the corresponding energies for the puckering parameters from the crystallographically determined fructose structures in Fig. 3. Those energies are obtained with the "residual" function of the Surfer software. Cumulative frequency plots perform the function of a histogram, but are much more suited to small data sets. Although the *y*-axis is labeled

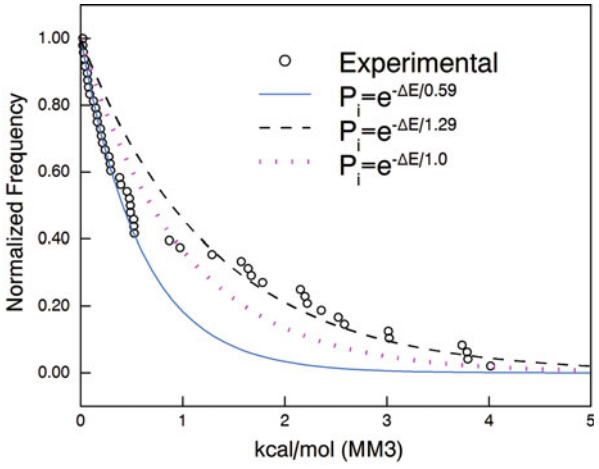

Fig. 4 Cumulative frequency plot for observed crystal structures of fructofuranose, with corresponding energies taken from Fig. 2. *Ideal lines* of probable frequency (P_i) are plotted for different temperatures, with the exponent's denominators of 0.59, 1.0, and 1.29 being the products of multiplication of the universal gas constant (R), × 298°, 505°, and 651°K

normalized frequency, the actual value plotted is cumulative frequency, or "1.0 – (position in a sorted list/number of points)." Three lines are shown, all based on $P_i = e^{-\Delta E/RT}$ with RT (the universal gas constant R, 1.98 kcal (mol^{-1}), times a temperature in Kelvin). When RT = 0.59, the line represents the exponential decay expected at room temperature. When RT = 1.0, the line corresponds to 505 K, and RT of 1.29 corresponds to a temperature of 651 K. The latter value was derived by fitting an exponential function to the data, forcing the function to have a value of 1 at 0 kcal using a spreadsheet program.

Although there is not perfect agreement between any of the lines and the experimental data, it is clear that the number of points decreases as the energy increases. The fitted line with RT = 1.29 appears to give too much weight to the high-energy structures, while the arbitrarily selected room temperature line (RT = 0.59) does not explain the structures above 0.5 kcal mol^{-1} well. The equally arbitrary RT = 1.0 line appears to be a compromise. It is important to not overanalyze this result. For one thing, the limited number of structures is spread over a wide range of conformations. More crystallographic results could change the perceived distribution. For another, Fig. 3 is a very complex hypersurface and errors of 1.0 kcal mol^{-1} could dramatically alter the graph in Fig. 4. Another factor is that the arrangement of the hydroxyl groups found for minimum-energy isolated molecules is almost never the same as found in crystal structures (French and Csonka 2011).

Digressing to further discuss the cumulative frequency plots, French et al. (2000a) showed an example (Fig. 3b in that work) of a cumulative frequency plot for sucrose structures, with corresponding energies from a simplified model compound that did not have the primary alcohol group at C2 of the furanose moiety. That omitted group resulted in a cumulative frequency plot that was not predictive. A different cumulative frequency plot in that work (its Fig. 3a) was based on hybrid energies with a dielectric constant of 3.5 for the full sucrose molecule. Compared with Fig. 4 of this work, that plot has a somewhat better fit, with RT of 1.16, equivalent to 585 K. In cumulative frequency work with disaccharides (French et al. 2000b), the 153 corresponding energies from eight disaccharides were pooled. Combining the corresponding energies when they were all calculated with the same method makes it possible to judge the validity of a force field when the data for any single molecule are limited. Three different dielectric constants were employed as well. At $\varepsilon = 1.5$, the fitted line corresponded to 942 K; at $\varepsilon = 3.5$, to 480 K, and at 7.5 to 419 K.

When the energy is lowered by formation of hydrogen bonds in some locations on Ramachandran's surfaces but not in others, the surfaces generally become flatter (and more predictive) as the dielectric constant is elevated (French et al. 2000b; French 2012, Fig. 10). The same phenomenon was observed with quantum mechanics calculations for the cellobiose disaccharide when a continuum solvation model was added (French et al. 2012). In turn, this flattening of the surface should reduce the effective temperature of a cumulative frequency analysis. When combined with a visual examination of the energy surfaces to see whether all of the low-energy regions are suitably populated (French et al. 2000b), the cumulative frequency

analysis can be a useful tool to judge the quality of the conformational analysis. It is significant that the distortions in crystals of the primary conformational variables correspond to conformational distributions that would be observed in the vapor phase at about 500 K, at least according to the present calculations and two previous studies.

Pyranose rings. There are three puckering parameters for pyranose rings: an amplitude, Q, the phase angle, Φ, and another angular variable, θ . When a chair-form pyranose ring is viewed from the side, the choice of the word "puckering" (a gathering into small wrinkles) to represent the ring shape becomes obvious. Instead of a 2-dimensional conformational wheel, a 3-d sphere is appropriate. The amplitude is represented by the distance from the center of the sphere to the surface, whereas the Φ angle corresponds to the longitude on a globe. The θ parameter is equivalent to the latitude. This conformational space can be represented by a 3-dimensional solid that encloses all structures with an energy less than a certain value, or perhaps more conveniently, the amplitude can be ignored and the space is therefore only 2-dimensional. The amplitude, or deviations from a mean plane, systematically varies depending on the ring shape. For example, if a chair form ring is constructed with alternating torsion angles for the ring atoms of ±59°, its amplitude will be about 0.60 Å, but a boat with two torsion angles of 0° and the others with ±59° will have an amplitude of about 0.82 Å. These differences occur despite having kept the bond lengths constant between the two rings. Thus, the amplitude of a chair is inherently less than a boat so amplitude is primarily of interest in comparing different examples of the same ring shape. For example, a molecule subjected to energy minimization in isolation might have somewhat greater puckering amplitude than one found in the crystal structure. Also, the two chairs for a given pyranose may have interesting differences in the puckering because of the interactions of the exocyclic groups in axial or equatorial dispositions.

There is some disagreement on the most correct and convenient way to calculate the ring puckering. Although the work of Cremer and Pople is the de facto standard, Haasnoot (1992), Bérces et al. (2001), Ionescu et al. (2005), and Hill and Reilly (2007) are among the numerous authors who have proposed alternate algorithms. Barnett and Naidoo (2010) adopted the Hill-Reilly approach, which reports the puckering in terms of the angles of three rotatable planes to a central reference plane, in their comparison of several semiempirical methods for modeling both furanose and pyranose ring shapes. Incidentally, Barnett and Naidoo found that none of those methods (AM1, PM3, PM3CARB-1) were suitable for modeling carbohydrates.

Because the puckering space has been repeatedly described for pyranose rings (e.g., Jeffrey and Yates 1979; French and Brady 1990; Ernst et al. 1998; Babin and Sagui 2010) and the concepts for the furanose rings were already discussed herein, treatment in this chapter will be brief. The 38 characteristic or canonical conformations comprise two chairs, six boats, six skew boats or twists, 12 half-chairs and 12 envelopes. They have letter designations of C, B, S, H, and E, respectively. The relationship between the various letter designations and the Φ and θ parameters is shown in Fig. 5. The aldopyranose puckering can be obtained from Fig. 5 by subtracting one from the super- and subscripted numbers associated with the C,

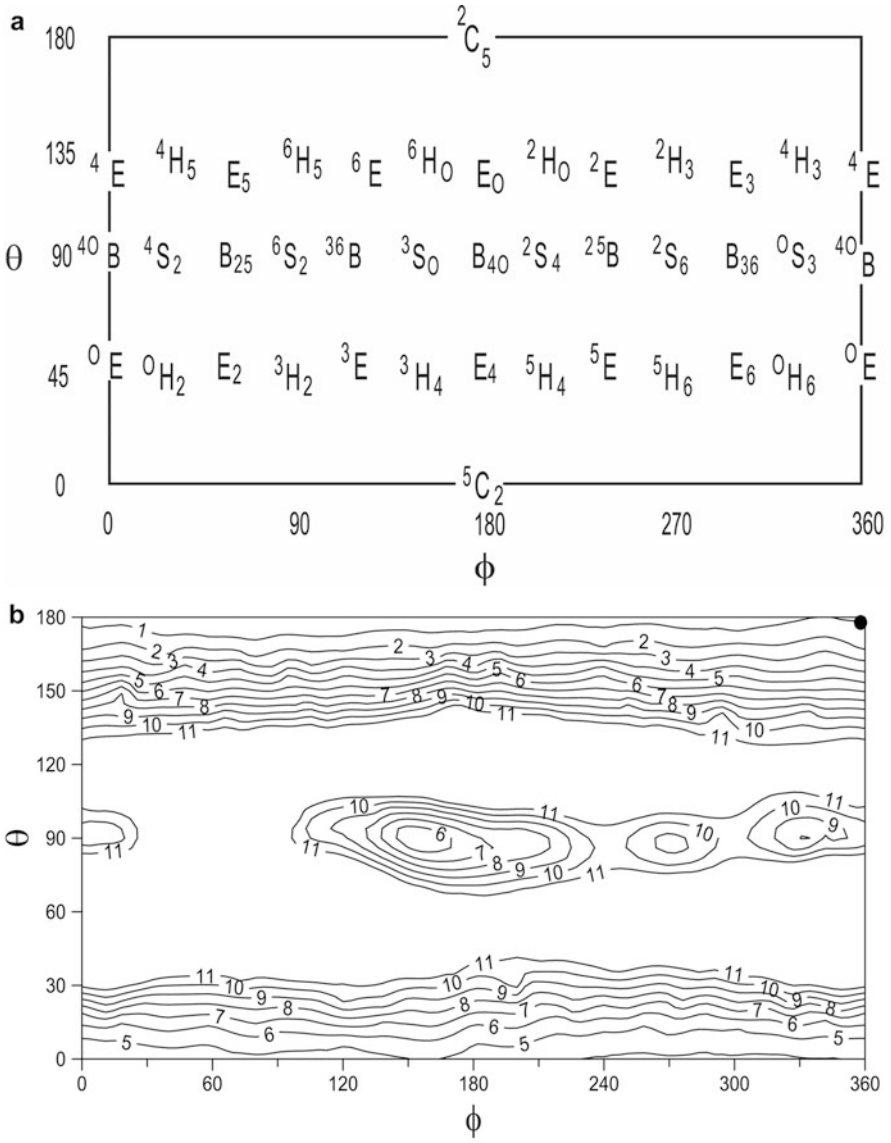

Fig. 5 (**a**) (*Upper*) Ketopyranose puckering chart, (*lower*) β-psicose energies. The designations of conformations are the same for the α- and β-anomers. The puckering for the crystal structure of psicose is plotted in the *upper right corner* of (**b**) as a *large dot*

B, S, H, and E letters. Note that the puckering maps will often reverse the θ axis, so that the $^{5}C_{2}$ (or $^{4}C_{1}$) chair is at the top of a depiction. Just as at the north or south poles of a globe where longitude is meaningless to describe the position, values of Φ are irrelevant for all structures having θ values of 0° or 180°.

In a manner analogous to the study of fructofuranose described previously, an energy surface can be created for pyranosyl sugars on the surface described in Fig. 5a. However, that 2-dimensional surface ignores the puckering amplitude values. This is a reasonable compromise because the amplitude depends on the θ values. Such a surface is again typically a hypersurface that depicts the lowest energy value at each increment of ring shape variation but does not reveal other information such as the variation of the exocyclic group orientations and puckering amplitude. Of course that information is available during such a study if desired. In our studies there were 4,913 ring shapes based on 0.1 Å vertical shifts from -0.8 to +0.8 Å for three alternating ring atoms (e.g., O5, C2, and C4 for an aldopyranose). For the 16 aldohexapyranoses (Dowd et al. 1994), psicose (French and Dowd 1994) and fructopyranose (French et al. 1997) the 729 combinations of staggered exocyclic group orientations were only evaluated for the 38 characteristic conformations. An updated example from that body of work is shown in Fig. 5b. The subject molecule, β-psicose, is of special interest because it was thought to be impossible to crystallize due to a complex equilibrium in solution. More recently (Kwiecień et al. 2008), psicose was crystallized; its $^{2}C_{5}$ puckering is consistent with the prediction. Note that the energies in Fig. 5b are somewhat different than shown in the 1994 paper. In that work the energy map lacks periodicity in the Φ puckering direction because of a lack of data points at $\Phi = 360°$. However, the calculated values at $\Phi = 0°$ were copied and used at $\Phi = 360°$ to make the new map.

The pentopyranoses xylose, lyxose, and arabinose only have four rotatable exocyclic side groups and so all 81 combinations of staggered orientations were tested at each of the 4,913 ring shapes (Dowd et al. 2002). Deoxy- (L-fucose, D-quinovose, and L-rhamnose) and dideoxyhexapyranoses (D-digitoxose, abequose, paratose, and tyvelose) were also studied exhaustively with 81 or 27 combinations of exocyclic group orientations (Rockey et al. 2001). Ribose and deoxyribose have also been studied exhaustively (Dowd et al. 2000). In all of these studies, there were hundreds of thousands of starting structures for each molecule. Because they were done with the MM3 empirical force field, the computer time, while considerable, was not the only obstacle to completing the work. The bookkeeping for that many structures required planning and organization.

Given the advances in computational resources since that time, it has been possible to begin to repeat this work with QM (Mayes et al. 2014). In that work, all staggered combinations of exocyclic group orientations for each of the 38 characteristic conformers were evaluated at the M06-2X/6-31G(d) level for the two anomers of glucose and β-mannose, as well as 972 for β-*N*-acetylglucosamine, 81 for β-xylose, and one of oxane. The major difference is that the puckering amplitude was not a variable in the screening work, whereas in the work of Dowd et al., the 4,913 ring shapes included the 38 conformations of Mayes et al. and many other variations such as intermediates and various puckering amplitudes. Mayes et al. assumed that vacuum calculations would mimic the electrostatic environment similar to the interior of a glucosyl hydrolase enzyme, on the grounds that the dielectric constant of the protein-carbohydrate complex would be around 4.0. However, a change from 1.0 for the vacuum to 4.0 for the complex amounts to a

fourfold reduction in the strength of the electrostatic contribution to a hydrogen bond. As calculated from the data in Řezáč et al. (2011, see more below), the electrostatic contribution to the dimerization energy of methanol in vacuum is nearly 4.1 kcal mol^{-1}. Reducing it to one fourth to account for the reciprocal relationship of the dielectric constant to electrostatic stabilization would yield a value of 1.0 kcal mol^{-1} as the appropriate amount of stabilization for the complex. A dielectric constant of 78, as for water, would reduce the electrostatic contribution to just 0.05 kcal mol^{-1}. Still, 1.0 kcal is closer to 0.05 kcal than it is to 4.1 kcal at dielectric 1.0. Therefore, use of an aqueous solvent model in the calculations would have been more appropriate if some solvent model with an intermediate dielectric constant were not deemed more appropriate.

Because of the large number of calculations involved in the semi- or fully-exhaustive energy minimization approach and the inability to explicitly account for solvation and free energy, molecular dynamics approaches have also been utilized. One example is the study of methyl glucuronic and iduronic acids by Babin and Sagui (2010) mentioned in the context of puckering calculations. Their work used a combination of Adaptively Biased Molecular Dynamics and Replica Exchange protocol along with Amber and the Glycam06 force field for carbohydrates and was presented on Hammer-Aitoff equal-area projections of puckering space. Preliminary implicit solvation calculations were used along with TIP3P explicit solvation in the production simulations.

At nearly the same time as Babin and Sagui's paper, Spiwok et al. (2010) published a force field comparison with the metadynamics extension of the GROMACS software (Van Der Spoel et al. 2005) (Fig. 6). The molecule studied was β-D-glucopyranose. The modification to GROMACS is called GROMETA 2.0 (Camilloni et al. 2008), and the Glycam06, GROMOS 45a4, and OPLS force fields were evaluated by both vacuum and solvated calculations. Again, the dynamics simulation is forced by a bias to sample the puckering space. The results were

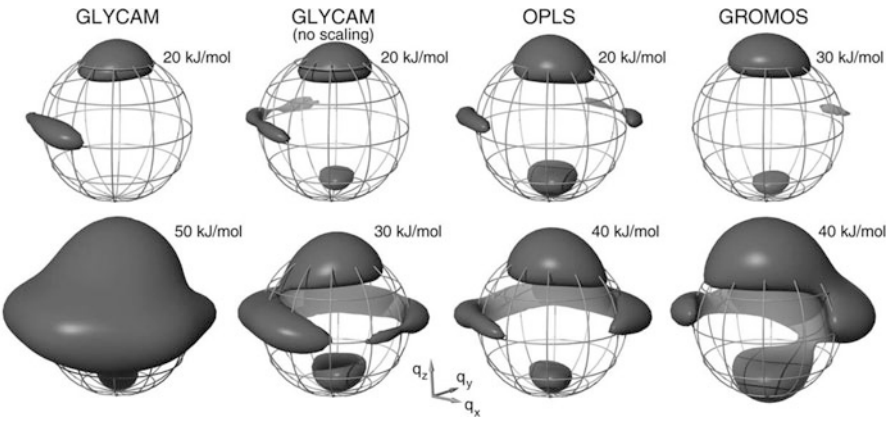

Fig. 6 Puckering surfaces for β-D-glucopyranose from GROMETA/Glycam metadynamics (From Spiwok et al. (2010), used with permission)

compared to glucose conformations observed in structures found in the Protein Data Bank. In crystals of small molecules, β-glucose residues are invariably in the $^4C_1$ conformation, but there are a number of examples in enzyme complexes where the glucose residues are distorted as part of the hydrolytic cleavage of the polysaccharide. Interestingly, a few examples of α-glucose with ring shapes other than $^4C_1$ have been observed residues in substituted cyclodextrins having seven glucose monomers per cycle (Steiner and Saenger 1998; Anibarro et al. 2001; French and Johnson 2007). Just as the complexation of the carbohydrate substrate at the catalytic site of an enzyme can result in distortion of the pyranosyl ring (Phillips 1966), a crumpling of an empty derivatized cyclodextrin because of van der Waals forces can make it advantageous to the system for one of the rings in the cyclodextrin to take an alternative form.

Somewhat earlier, Biarnés et al. (2007) carried out quantum mechanics studies of β-glucopyranose with the Car-Parrinello molecular dynamics (CPMD) method that incorporated metadynamics. Again, the molecular dynamics simulation was forced to explore various ring shapes, although in this case only conformations having puckering θ values of less than 90° were considered. In that work, the authors utilized a diagram published by Stoddart (1971) that represented the same conformational space that they explored with CPMD. This diagram is also widely used in studies of glucoside hydrolases, but it has some misleading implications. For example, Envelope conformations are not included, and the indicated interconversion pathways go directly through a canonical Half-chair form to a particular ring form on the Boat/Skew itinerary where $\theta = 90°$. The canonical or characteristic conformations are only descriptive landmarks in conformational space and too much significance should not be attached to them.

Limitations in that work included a very short simulation (44.4 ps, whereas a microsecond is sought in current MD simulations with empirical force fields) as well as the lack of consideration of the $^1C_4$ form and conformation space leading to it. Their energies based on plane wave basis functions and the PBE density functional theory (DFT) method were compared with results from calculations by a more frequently used DFT method, B3LYP, and the /6-31+G* Pople basis set. The differences of ±0.6 kcal mol^{-1} did not change the order of the preferred conformers. Their values for a ^2S_O structure were 4.12 and 3.0 kcal for ΔE and ΔG, which were also compared with literature values of 4.75 and 3.4 kcal mol^{-1} from Appell et al. (2004) who used B3LYP/6-311++G**. Dowd et al. (1994) reported a relative MM3 energy for a ^2S_O structure of 6.5 kcal mol^{-1}, but that calculation involved an elevated dielectric constant to account for a condensed phase while the other calculations were for the gas phase.

2.4 Electron Density Analyses

Descriptive analysis of the electronic structure in terms of the electron density (instead of orbitals) is being applied to carbohydrates. The electron density can be probed by both experiment and QM calculations. Although not applicable to

carbohydrates because of their nonplanar structure, specialized Atomic Force Microscopes with a carbon monoxide molecule on the tip have produced images of the bonding structure at both the covalent and hydrogen bonding levels (Gross et al. 2012; Zhang et al. 2013). High-resolution X-ray crystallography also can yield information on the electron density because the X-rays are scattered by the electrons (Koritsánszky and Coppens 2001). Although the diffraction patterns are normally solved by assuming the atoms to be spherical, for electron density studies they are assumed to take a shape described by a combination of mono-, di-, quadra-, octa-, and hexadecapoles. Experimental X-ray diffraction studies of electron, or charge, density can use "difference density" methods in which the electron density as modeled by the multipole method is compared with the usual spherical density for the atoms. For example, this data can provide resolution of the lone pairs of electrons on oxygen, for example (Longchambon et al. 1985; French et al. 2014).

The work of Bader (1990) has added topological analysis to quantitative studies of electron density with X-rays and QM. In Bader's Atoms-in-Molecules (AIM) analysis, the molecular structure is described in terms of critical points, bond paths, and basins. For example, a point at which the electron density is a maximum in all three directions is a "nuclear attractor" or nucleus, and a point from which the electron density increases in all directions is a "cage critical point." "Ring critical points" are located where electron density is at a minimum in two directions but a maximum in the third. The last to be mentioned is the "bond critical point" (BCP) at which the electron density is at a minimum between two atoms but a maximum in a plane perpendicular to a line connecting the two atoms. A bond path connects the BCP to the nuclei in question. The bond path need not be straight. For example it is curved for C–C bonds in cyclopropane (Patai 1992). The sign of the Laplacian (the second derivative of the electron density at the BCP) indicates whether the bonding is covalent (negative values) or a weaker, "non-bonded" interaction (positive values). Another descriptor of the electron density at the BCP is the ellipticity, which can indicate the extent of π-bonding, for example. A depiction of the nuclei and bond paths constitutes a molecular graph, which usually looks like a "ball and stick" model.

Because the electron density extends to infinity around each atom, it is usual to use a cutoff of the electron density, perhaps at the 0.001 electron per atomic unit (a cubic bohr, which equals 0.14735 $Å^3$). Quantum chemists favor the atomic units and crystallographers favor $Å^3$. This cutoff distance is equivalent to the van der Waals surface of a molecule, defined by objective criteria for each atom. Each nucleus is said to exist in a basin defined by the minima in the surrounding electron density and the outer van der Waals surface. Thus the volume of each atom in a molecule can be calculated. Each basin can be said to contain the electrons associated with the particular nucleus, so the atomic charge on each atom is also defined. Unfortunately, the shapes of these basins make them generally unsuited for providing parameters for empirical force fields.

Figure 7 shows two different representations of QM-derived electron density for α-D-glucopyranose. The upper plot shows the 0.021 e/a.u. (darker green) and 0.018 e/a.u. (pea green) isodensity surfaces, rendered by the MOLISO software (Hűbschle

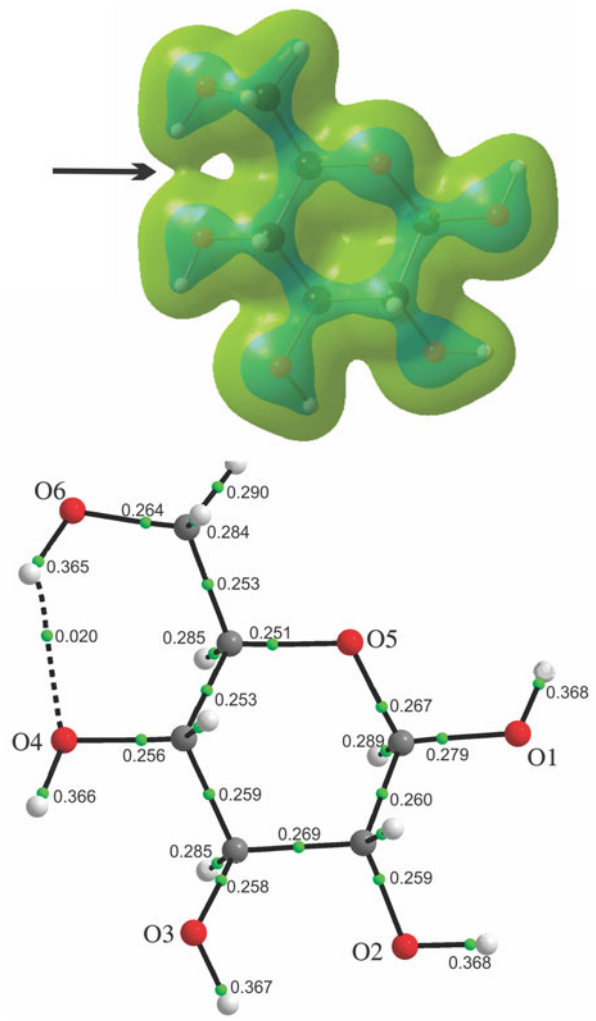

Fig. 7 Electron density analysis with 0.23 e/au isodensity surface (*upper*) and AIM critical point and bond-path analyses (*lower*). Electron densities are shown in the *lower* image for the sugar with the hydroxyl groups in the counter-clockwise orientations

and Luger 2006). The lower plot shows the rendition of the same structure by the AIM Studio software that is part of the AIMAll package (Keith 2013). This molecular graph shows the labels for the oxygen atoms and the values of the electron densities for the BCP. The electron density was derived from energy minimization at the B3LYP/6-31+G* level and the final electronic structure provided by B3LYP/6-311++G** theory.

Although depictions of bonds have been described as "cartoons," the darker green regions in the MOLISO plot clearly depict the concentrations of electron density that correspond to the molecule's covalent structure. Although it is not useful to compare the electron densities for bonds that involve different elements, for a given type of bond, the bond order (e.g., single or double) is related to the

electron density. The arrow points to the concentration of electron density that represents a hydrogen bond between the hydrogen on O6 and O4.

As previously indicated by Klein (2002), there is a hydrogen bond formed for this particular structure of glucose, with O6 in the experimentally rare *tg* orientation and the O4 hydroxyl group pointing counter-clockwise. This is despite the absence of hydrogen bonding for the other adjacent hydroxyl groups, reported initially by Csonka et al. (1997) and by Klein (2002). Although the secondary hydroxyl groups in Fig. 7 are all oriented in a counter-clockwise direction around the glucose ring, there are no bond critical points or bond paths to indicate hydrogen bonding. This is despite the fact that such an arrangement and its clockwise counterpart are routinely found to correspond to the minimum energy arrangements of the hydroxyl groups (French and Csonka 2011), leading to a claim of hydrogen bonding in many studies.

Investigation of this problem indicated that the proposed intramolecular hydrogen bonds between vicinal hydroxyl groups have O–H. . .O angles of less than 115° as well as H. . .O distances greater than 2.4 Å (Table 1). Quantum mechanics calculations for 1,2-dihydroxycyclohexane showed that there was a drop of about 2 kcal mol^{-1} when the "donor" hydroxyl group was in an optimal orientation vs. an increase of about 2 kcal mol^{-1} when oriented in an opposite direction (French and Csonka 2011). (A zero of relative energy was established subsequent to the 2011 work by placing the second hydroxyl group in a nonvicinal position.) Additionally, the 2 kcal mol^{-1} attraction was eliminated by calculations that added the SMD implicit solvation model. This evidence points to the idea that such vicinal hydroxyl hydrogen bonds, if they are to be called that, are based on only the electrostatic component of the hydrogen bond, whereas the AIM analysis depends on the dispersive or van der Waals component.

A breakdown of water-water, water-methanol, and methanol-methanol hydrogen bonds, all with optimal geometries, showed ratios of dispersive to electrostatic interactions of 0.29, 0.35, and 0.40 (Řezáč et al. 2011). The corresponding total ΔE values from that work were 4.92, 5.59, and 5.76 kcal mol^{-1} at the CCSD(T)/CBS level. The breakdown was achieved with Symmetry-Adapted Perturbation Theory (Jeziorski et al. 1994; http://www.physics.udel.edu/~szalewic/SAPT/index.html). Thus, the AIM calculations, which are not oriented towards finding electrostatic bonds, are failing to find a van der Waal component of the hydrogen bonding because of the geometry, but the QM calculation is finding the electrostatic component, albeit reduced because of the longer H. . .O distance that is a consequence of the vicinal geometry. Small changes in that geometry, such as for vicinal

Table 1 Interactions among hydroxyl groups in β-D-glucose of Fig. 7

Interaction	AIM HB?	H. . .O (Å)	O–H. . .O (°)	O. . .O–H (°)
O6–H. . .O4	Yes	2.06	134.8	31.1
O4–H. . .O3	No	2.40	106.5	54.3
O3–H. . .O2	No	2.50	102.7	58.1
O2–H. . .O1	No	2.55	99.7	60.9

hydroxyl groups on a septanoid ring, do allow the calculations to show a bond path and BCP (French and Csonka 2011).

While the AIM approach has been used to question one class of proposed hydrogen bonds, it has strengths in the detection of C–H. . .O and general van der Waals interactions. Particularly interesting was the detection of C–H. . .O interactions with complexed water in crystalline trehalose dehydrate by both crystallography and QM calculation (Stevens et al. 2010). Several C–H. . .O and van der Waals interactions in crystalline native cellulose were detected by AIM on QM calculations based on the coordinates proposed from fiber diffraction. Fiber diffraction does not provide sufficient data for electron density studies, but basing the AIM analysis on QM calculations on the fiber diffraction coordinates appears to be an effective way to detect weak interactions in such samples.

In overview, the AFM work dramatically illustrates and validates the work on charge density that has been underway in the crystallographic and QM communities at admittedly low levels of intensity for some 40 years. For carbohydrates, three approaches can be used: QM calculations alone, QM calculations based on crystal coordinates from a conventional determination, or analysis of the high-resolution X-ray data. Combination of the latter two approaches allows insights into the accuracy of the approximations at given levels of QM theory. Although Bader's book (1990) offers interesting examples such as detection of covalent bonds in propellane, perhaps AIM analyses are likely to be most fruitful in the carbohydrates arena for detection of weak interactions that are generally considered to be "non-bonded."

3 Disaccharides

The most critical subject in understanding polysaccharide structure has been the disaccharide linkage. This writer has written extensively on Ramachandran mapping studies based on energy minimization studies, including a methods chapter (French 2014) and a justification of methods that we have used (French 2011). Disaccharide maps for various force fields and QM maps are described at some length in French (2012). The present chapter will concentrate on results obtained with energy minimization, with some observations on some molecular dynamics efforts. This focus on energy minimization is justified from a similarity of results for a given method of calculating the energies, as indicated in Fig. 8. That work shows the explicit solvation Amber/Glycam Replica Exchange MD data from Shen et al., with results from energy minimization using dielectric constant 8 Amber/Glycam calculations on 150 different starting geometries.

For those wishing to create relaxed-residue Ramachandran maps of disaccharide energies, there are a few important guidelines. The first is that maps of the entire $\phi-\psi$ conformation space must be periodic, i.e., the values at the edges must be the same on opposite sides of the map. This will not generally be the case if "dihedral driver" or "scan" capabilities that are built into modeling software are used. Most of those utilities carry out an energy minimization at a certain conformation and then

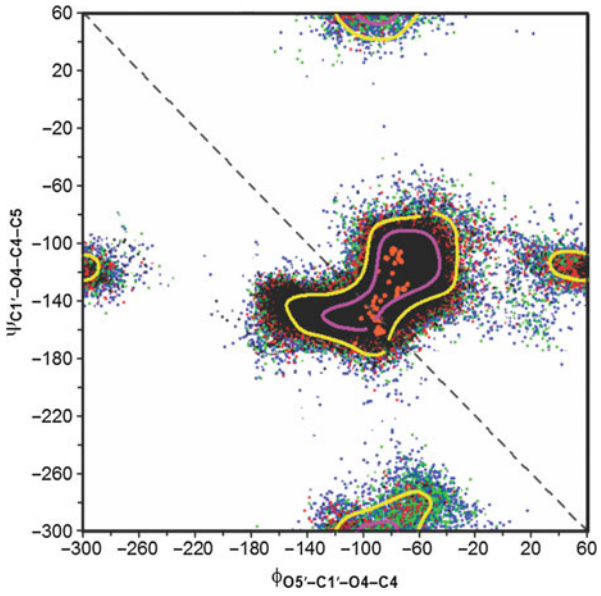

Fig. 8 Merged Replica Exchange MD and energy minimization plot (Modified from Shen et al. (2009) and French (2012)). Crystal structure conformations of molecules related to cellobiose are plotted as *large orange dots*. *Black dots* are for room-temperature structures, and *colored dots* indicate conformations accessed at progressively higher temperatures. The *contour lines* are from the dielectric 8.0 Amber/Glycam map (French 2012), with *magenta* and *yellow contour lines* for 1 and 2 kcal mol^{-1}, respectively. The *diagonal line* indicates conformations that would result in twofold screw-axis symmetry when applied to an infinitely long cellulose molecule

make a small change in the conformation and carry out another minimization for the next point on the Ramachandran grid. This does not work for carbohydrates because the structures are transformed by the energy minimization process and do not recover. Therefore, on an energy map from $-180°$ to $+180°$ with intermediate steps, the minimized structure at $-180°$ will not generally be the same as at $+180°$. So it is necessary to start each minimization with the intended starting structure (French 2011).

Another point is that a variety of starting geometries must be used if it is desired to find the global minimum. In a practical sense, this means that individual maps for the various combinations of the clockwise and counter-clockwise orientations of the exo-cyclic groups must each be calculated. The various combinations of primary alcohol group orientations are also needed. The desired adiabatic map can be generated by finding the lowest energy from all of the starting geometries at each increment of ϕ and ψ. Our recent QM adiabatic maps for cellobiose (French et al. 2012) consisted of energies from about 30 different starting geometries. A caution for those constructing adiabatic maps is that to be predictive in general, such maps must include some concession to a condensed phase, such as continuum solvation or an elevated dielectric constant.

When a method is in doubt, it would be useful to compare the results for the backbone of the disaccharide molecule having no hydroxyl groups with those from a HF/6-31G* QM study for analogs with various linkages (French et al. 2001). These analog energy surfaces are remarkably independent of the level of theory, although higher levels have been subsequently used for less specific models in the parameterization of the most recent CHARMM force field (Guvench et al. 2009).

Even when adhering to these rules it is possible to get results that do not seem predictive when using MM methods. Although our MM models of many disaccharides gave sensible results, one did not. For reasons that are not fully understood, modeling the sucrose disaccharide with MM was not satisfactory, based on the distribution of crystal structures on the Ramachandran map. However, a hybrid QM::MM hybrid method was reasonably predictive (French et al. 2000a). Another situation that was resolved with QM calculations involved the conformational preference of α,α-trehalose. Based on MD simulations with CHARMM in both vacuum and solvent, it was decided that the vacuum and solution conformations were very different (Liu et al. 1997; Engelsen and Pérez 2000). Taking advantage of the chemical symmetry of the molecule and a reduction in conformation space because of very high energies in some regions for an analog molecule stripped of hydroxyl groups (Tvaroška and Václavik 1987; French et al. 2001), mapping studies showed that the $\phi = 180°$, $\psi = 180°$ conformation had 7 or 8 kcal mol^{-1} higher energy despite a modeled hydrogen bond. This was the result from three different levels of QM theory. The $\phi = 60°$, $\psi = 60°$ conformation, which is in accord with the exo-anomeric effect had the lowest energy and was in full agreement with conformations of some 25 crystal structures. A dozen new crystal structures are also found in that same gauche-gauche energy well, once three determinations are corrected for the inadvertent reporting in as the mirror images of the correct structure. There was a substantial variation in energy surfaces because of the different levels of theory that were employed and a particular combination that was thought to underestimate hydrogen bonding energies (HF/6-311++G**// B3LYP/6-31G*) gave the best rationale for the observed crystal structures. In any case, the idea of a dramatic change in conformation from the gas phase to aqueous solution was refuted for trehalose by the QM studies.

More recently, such a solvent effect was supported by a much more extensive QM study of cellobiose (French et al. 2012). That work pointed out the critical role of the solvation model, as well as inclusion of a sufficient number of starting geometries. Although the preference for a conformation of cellobiose was indicated by several modeling studies, including the work of Appell et al. (2004), it was not until the QM-aided experimental work of Cocinero et al. (2009) that the idea of a very different vacuum conformation was widely accepted. A QM study by Schnupf and Momany (2011a) was based on 18 starting structures and attempted to build an adiabatic map based on energy studies around each of the local minima. Also, the COSMO continuum solvation model was used. Our following effort used the same QM computation strategy with the B3LYP method and mixed basis set for minimization, namely /6-31+G* for the oxygen atoms and 4-31G for the carbon atoms, with final energies by B3LYP/6-31+G*. However, we used the SMD solvation

model and some twenty more starting geometries, previously extracted (French and Johnson 2006, 2008) from HF/6-31G* studies. In the vacuum calculations, the $\phi = 60°$, $\psi = -120°$ conformation was favored by 3.5 kcal mol^{-1} but in solvent the $\phi = -100°$, $\psi = -140°$ form was favored by 2.0 kcal mol^{-1}.

Figure 9 shows the entire SMD energy surface as well as a close-up view of the central area where most of the crystal structures are located. The close-up view includes a diagonal line that corresponds to a twofold screw axis when applied to cellulose itself. The twofold screw conformation for cellulose has been controversial, with various MM software programs giving higher energy right along the line, owing to a short contact between H4 and H1′ on the linkage carbon atoms. That is not so much a problem with the levels of QM theory that have been applied (French and Johnson 2009).

A different set of crystal structures was plotted, based on a new survey of the CSD for this work. It discriminates between the crystal structures that are for molecules that have hydroxyl groups in the 2- and 3-positions so that inter-residue hydrogen bonds can form across the glycosidic linkage and those β-(1,4)-linked structures that are derivatized in those locations or missing the oxygen atom. On Fig. 9, there is considerable overlap between the two types of crystal structure, with the non-H-bonding structures slightly exceeding the range of the structures that can form hydrogen bonds, whether they do or not. Structures with ψ values greater than −130° do not form the classic 03-H. . .O5′ hydrogen bonds.

Using the same cumulative frequency analysis as previously used herein for fructofuranose, these all-inclusive and hydrogen-bonding ability only populations can be evaluated on the energy surface. (The heavily derivatized structure (Ernst and Vasella 1996) at the bottom of the map could not be included for technical reasons.) The cumulative frequency plots (Fig. 10) differ in a fairly subtle manner. However, the upper plot has an equivalent temperature of 533 K, and, according to the fitted exponential decay line, some 61 % of the structures should have energies less than 1 kcal mol^{-1}. The comparable figures for the structures that can form hydrogen bonds across the linkage are 413 K and 70 %. In a room temperature gas, 82 % of the structures would have energies of less than 1 kcal mol^{-1}.

This type of analysis must be kept in perspective. Selection of the crystal structures to plot is obviously important. In the case of the data set used herein, for example, the structures of cellotetraose and methyl cellotrioside are included. They alone contribute 14 different linkage geometries to the statistics, and once the molecule is in the flat-ribbon, twofold shape, crystal packing can be very efficient. The shapes of carbohydrates in complexes with enzymes are another matter. Not only are ring shapes distorted, but the glycosidic torsion angle values indicate that the linkage may be in a higher-energy shape (Johnson et al. 2009).

Another argument is that the exocyclic groups in the actual structures are involved in mostly intermolecular interactions, and the lowest-energy forms bear little similarity in that regard to the experimental structures. Still, the basic idea that a molecule in a crystal is perturbed in one direction or another from its ideal shape by crystal packing forces seems reasonable enough. When a sufficient number of different but closely related molecules are examined in their crystalline

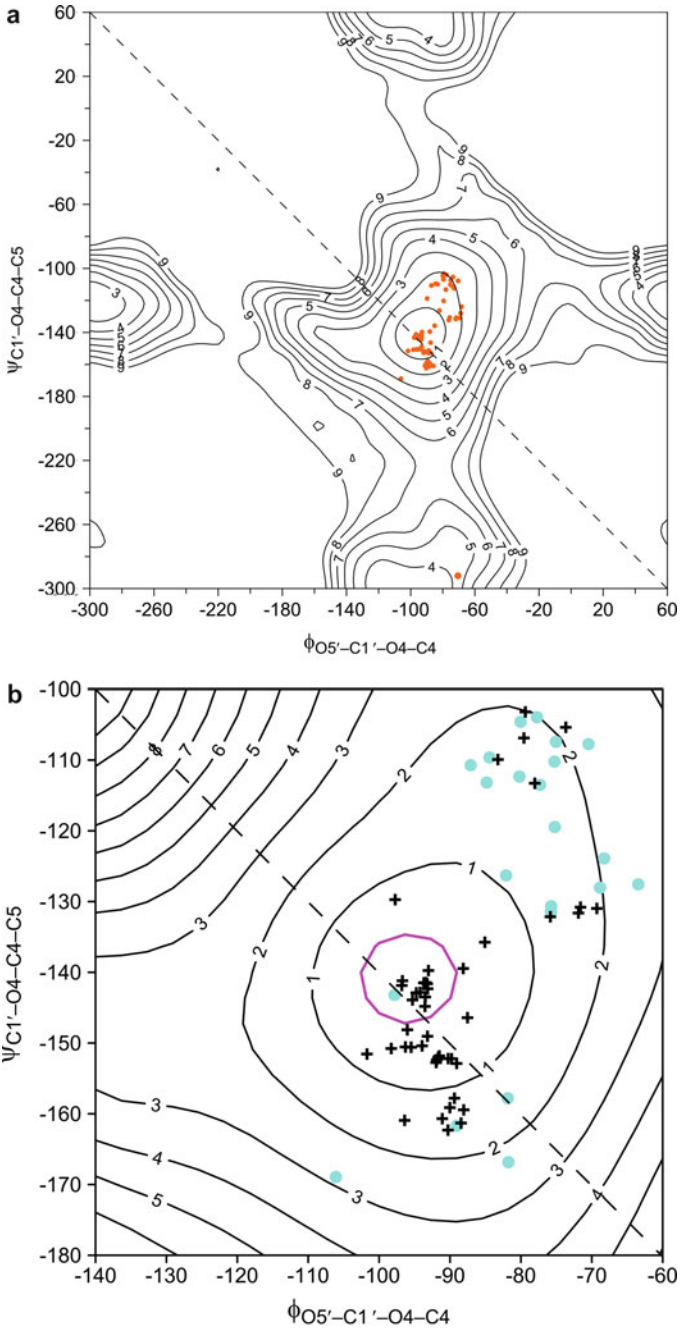

Fig. 9 (**a**) QM solvated cellobiose energy surface using the full 6-31+G* basis set. The *orange dots* represent conformations found in crystal structures of β-(1,4)-linked disaccharides such as

environments, the perturbations will appear random but have a distribution that approaches exponential decline with increasing corresponding energy. Even if the details are fuzzy, it seems that the information provided by crystal structures can provide useful information on the validity of a particular approach to modeling. It can also give valuable insights on the factors that determine conformation. Another point is that even with the structural diversity of the different crystal structures, they are confined to a relatively small portion of conformation space.

4 Oligosaccharides and Polysaccharides

The structures of simple oligosaccharides such as cellotetraose can be readily understood in terms of the material on monosaccharides and disaccharides, with just more linkages (Shen et al. 2009). The relative importance of MD simulations increases substantially, however, because exploration of the increased number of dimensions of conformation space is expedited by the fundamental basis for MD. Namely, thermal motion can overcome minor energy barriers.

The complex branched oligosaccharides that are on the surfaces of proteins or recognized by proteins, on the other hand, are a more complicated topic. Rather than try to address it here, the reader is referred to a substantial body of work in that area by the Woods groups, located at the University of Georgia and at the National University of Ireland in Galway. Among their papers that should be known among all carbohydrate modelers are generally applicable works such as a review of available carbohydrate force fields by Fadda and Woods (2010) and Foley et al. (2011). The description of the Glycam force field that they developed (Kirschner et al. 2008) should be of interest. One of the major drivers for the latest version of the Glycam force field is the ability to mix protein and carbohydrate structures. The Glycam web site is a potential resource for many users. http://glycam.ccrc.uga.edu/. Among the publications from that group and colleagues specifically on oligosaccharides and their binding to enzymes, readers can get an idea of the state of the art from reading Woods and Tessier (2010), DeMarco et al. (2010), Kadirvelraj et al. (2011), Nivedha et al. (2014), or Martin et al. (2014). Other efforts on the conformation of oligosaccharides include Widmalm (2013), Fernandes et al. (2010), and Wood et al. (2013).

This review will instead cover the conformation of a large cyclodextrin, a field that is less complicated but still quite challenging. The biosynthetic cyclomaltooligosaccharides and cycloamylose molecules comprise as few as five glucose residues (Nakagawa et al. 1994) up to hundreds and a number of them have

Fig. 9 (continued) cellobiose. The *dashed diagonal line* represents structures with twofold screw axes. Near the *line*, structures above the *line* are left-handed and below it they are right-handed. Energies taken from French et al. 2012. (**b**) QM solvated cellobiose energy surface, zoomed in view of (**a**). + *signs* represent crystal structure conformations for molecules that could (or did) form inter-residue hydrogen bonds, *cyan dots* show β-1,4-linked structures without that capacity

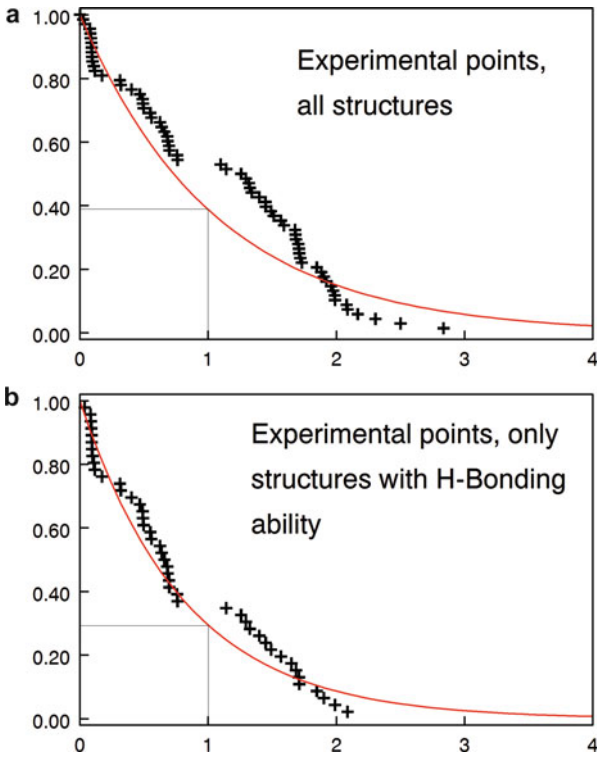

Fig. 10 Exponential decay of corresponding QM energies for crystal structures containing cellobiose moieties. (**a**) All structures; (**b**) only those that can make H-bonds

crystallized (Saenger et al. 1998). These molecules are made by the action of enzymes on the mostly linear amylose component of starch. The prediction of cyclodextrin structures with various molecular modeling approaches has been reasonably popular (Ivanov and Jaime 2004; Maestre et al. 2007). One of the most interesting is CA26 or cycloamylose with 26 glucose residues per cycle (Fig. 11). The interest in CA26 comes from its two helical segments that closely resemble some of the "V" polymorphic structures proposed from fiber and powder diffraction patterns for amylose (Rappenecker and Zugenmaier 1981), one of the two components of starch. Their helical cavities can host guest molecules and structures of crystalline complexes of CA26 with triiodide (Nimz et al. 2003), undecanoic acid, and dodecanol (Nimz et al. 2004). Those crystal structures are in addition to the triclinic and orthorhombic packings of the hydrated but otherwise empty carbohydrate (Gessler et al. 1999; Nimz et al. 2001). In all, eight crystallographically unique molecules are in the literature; two of the molecules have C2 symmetry but both halves of the molecule are all included in the analyses below.

The two helices are connected by two folding (or flipping) regions, where the ψ_H torsion angles take on values near 180° instead of about 0° taken by the other 24 linkages. Such folds were indicated by Manley (1964) from electron microscopy of amylose crystals but no details were provided. In solutions of CA26, the ^{13}C NMR spectrum shows only six unique chemical shifts, i.e., all glucose residues are

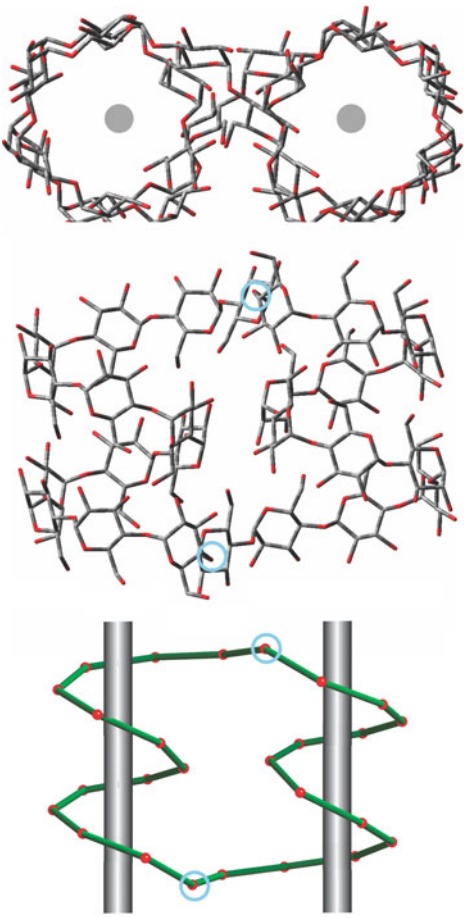

Fig. 11 CA26 molecule showing a schematic of two turns each of two six-residue/turn helices with helix axes (*lower*); atomistic drawing in same view (hydrogen atoms deleted, *middle* image) and looking down the helix axes (*upper* image). Disordered oxygen atoms were removed. *Light blue circle* indicate the two linkages where the amylose chain "flips" with ψ values very different from the other linkages

equivalent on the NMR timescale in water at 50 °C (Gessler et al. 1999). Therefore, there is an interconversion and the flipping linkages rapidly migrate through the molecule on the NMR timescale similar to pseudorotation in furanosyl rings.

Modeling studies of CA26 before the crystal structure was solved did not reveal the correct structure so further studies should be useful in improving modeling methods for large carbohydrates (Gessler et al. 1999). Figure 12 shows that the ϕ and ψ linkage conformations fall into the two major minima for the maltose disaccharide (the shortest amylose molecule), with the two flipping linkages from each of the eight molecules located within the secondary minimum surrounded by a 3 kcal·mol^{-1} contour. The energy contours on that surface were calculated with a composite, or hybrid, method. Energies for the backbone, a suitably linked pair of tetrahydropyran rings, were calculated with HF/6-31G* theory and remaining atoms were calculated with MM3 (Allinger et al. 1990) and a dielectric constant of 3.5. In this case, the dielectric constant was not critical to a successful modeling calculation as the other two maps in Johnson et al. (2007) would have also provided

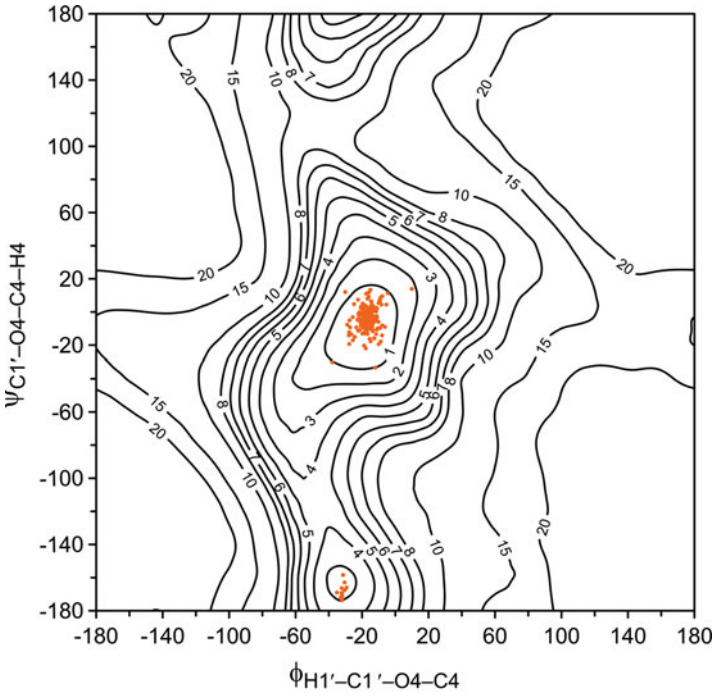

Fig. 12 Energy contours for maltose from MM3, dielectric constant 3.5 with HF/6-31G* correction for the tetrahydropyran rings and glycosidic linkage (Johnson et al. 2007), with crystal structure conformations from CA26 (Note that the torsion angles are defined with the hydrogen atoms, rather than our recent practice of using the ring oxygen and C5 atoms)

suitable energy wells for the CA26 conformations. In Johnson et al., however, an elevated dielectric constant was quite important to providing a low-energy region that matched the distribution of linkage conformations of the noncyclic di- and oligosaccharide crystal structures. While the HF/6-31G* correction of the hybrid map was crucial to successfully modeling the sucrose disaccharide (French et al. 2000a), it played a minor role in mapping for the maltose disaccharide (compare with maltose maps in Dowd et al. 1992). This map is very different from the vacuum CHARMM map of Hatcher et al. (2011) who made extensive use of QM in the parameterization. They found another significant ($<$2.0 kcal mol^{-1}) secondary minimum around $\phi_H = 180°$. Their solution map is similar to Fig. 12 and also to the QM::MM3 hybrid map at dielectric 7.5 (Johnson et al. 2007).

Although CA26 is a large oligosaccharide, some modeling studies can be carried out with DFT methods. Schnupf and Momany (2011b) evaluated several different methods with their mixed basis set that uses 4-31G for the carbon atoms and 6-31+G* for the oxygen atoms. Besides the widely used B3LYP method, B97D (the D is for dispersion) and the same method with PCM continuum (implicit) solvation were applied, based on the B3LYP geometry as a starting structure. A potentially interesting alternative quantum mechanical method (SCC-DFTB) that

has similarities to semi-empirical methods (Elstner 2006) was also evaluated. They also made a comprehensive comparison of the different CA26 crystal structures. One motivation of the work was to study the V-amylose helix based on the computed information starting with a half-segment of the CA26 structure. In that they found the polysaccharide to be reasonably stable under the calculations; a misleading point compared the average 7.9 Å pitch of single V helices to a value of 10.57 Å for the native starch B form (Takahashi et al. 2004). That 10.57 Å value is the crystallographic repeating distance for the double helix; the individual strands of the double helix repeat in 21.14 Å, so there is a much larger conformational change in transitioning from V amylose to B amylose, besides the mysteries of the double helix formation by amylose.

Despite the necessity of a continuum solvation model to model cellobiose crystal structure conformations (French et al. 2012), Schnupf and Momany found that the B3LYP method with their mixed basis set and no solvent gave the lowest Root Mean Square Deviation with the CA26 crystal structure that was used to derive the starting geometry. The preparation of the starting structure included deleting one of the disordered positions for several of the oxygen atom positions and adding hydrogen atoms that were not located crystallographically. The crystal structures were heavily hydrated but the water molecules were deleted before starting the calculations.

Given the approximations to the physical situation resulting from the preparation of the starting geometry, it is not surprising that the coordinates from the modeling calculation and from the crystal structure were not identical. The RMSD values for the B3LYP model were between 0.77 and 1.20 Å. However, that range was similar to the range of RMSD values among the various CA26 crystal structures, so comparison with experiment did not invalidate the modeling method.

For the present paper we essentially repeated their vacuum B3LYP calculation with the mixed basis set. However, we started with the other molecule in the original triclinic structure determination (Gessler et al. 1999). Our initial crystal structure was equivalent to Schnupf and Momany's molecule 1A (their starting structure was based on 1B), and we used different software to remove the extra atoms (Chem-X, no longer available) resulting from the disorder and to add hydrogen atoms (OpenBabel, O'Boyle et al. 2011). While Schnupf and Momany (2011b) used PM3 to adjust the hydrogen atom positions, we used MM4 (Allinger et al. 2003; Lii et al. 2003a, b, c, 2005) for energy minimization of all atomic positions. We then used that MM4 structure for the B3LYP calculations. We also carried out an MM3 minimization (Allinger et al. 1990) with the same input file as for MM4. The MM3 and MM4 calculations were done with the standard termination criterion based on energy change, and the B3LYP calculations used Gaussian 09 (Frisch et al. 2009) with the "Loose" keyword. Even with "Loose," the QM calculations required some 26 days using four processors. The initial MM3 and MM4 calculations were considerably more economical at 2 and 4 min, respectively. Both MM3 and MM4 calculations were done with a dielectric constant of 1.5. Note that a dielectric constant of 1.5 is designated for use in MM3 and MM4 for calculations on isolated molecules (in a vacuum, except for the molecule itself).

Because experimental values are known for the parameters that have been modeled, it is fair to question why such calculations are useful. There are several excellent reasons for this exercise. The first is to learn whether the geometric features that have systematic variation are predictable with theoretical calculations. Another way of asking that question is to find out whether there are features that are outside our understanding of the general principles of molecular structure. Similar to the case of branched oligosaccharides, the CA26 structures have a considerably wider range of inter-residue interactions than a disaccharide, and therefore constitute a more stringent test of the reliability of the computational methods. In particular orthorhombic CA26 has some short inter-helical contacts including a network of C–H. . .O hydrogen bonds (Nimz et al. 2001). Because of the similarity of the interactions within a CA26 molecule to those of oligosaccharides for which crystal structures are unavailable, the quality of the models of a known structure is of substantial interest. Another interesting question regards the symmetry of the CA26 molecule. Two of the crystal structures have C2 symmetry, i.e., the two halves are related by rotational symmetry. Is this the minimum energy condition, or is the asymmetric structure the preferred structure? Is the symmetrical condition a consequence of crystallization, or is asymmetry?

To rigorously address these questions for such a large, complex molecule with computations is difficult. The final energy hypersurfaces are likely to be fairly flat, with numerous variations on the global minimum structures that have small differences in energy. In this work, we have conceded that the starting structure is not likely to minimize to the global minimum. Another issue is whether the structure has actually completed the energy minimization? Has the termination criterion stopped the process before the geometric details are settled to a reasonable approximation? In the case of the QM calculation, the "Loose" keyword was a practical necessity. In the case of MM3 and MM4, however, a tenfold increase in computer time would not be an issue. Their default termination criterion is an energy change of 0.00008 kcal mol^{-1} times the number of atoms. That is appropriate for small molecules, but when there are 546 atoms, as in CA26, the energy is still changing by 0.0437 kcal mol^{-1} for each cycle. When evaluating different computational methods, it is important that the results for each method represent the capability of the method, so we did decrease the termination criterion by a factor of 10 for MM3 and 8 for MM4 (the difference was due to a local technical consideration). In general, the resulting longer minimizations would allow the atoms to move further from their starting positions. That makes comparison with the B3LYP calculation more difficult.

Table 2 shows the various RMSD values computed for structures minimized by various methods. The additional minimization time in the longer runs did increase the deviation from the starting structure, except for the MM4 determination of the glycosidic angle. Not revealed by the glycosidic angle data in Table 2 is that the MM3 values of the glycosidic angle decreased on average by about 1.9° and increased by MM4 by about 1.1°.

Another way to compare the effect of minimizing with the various methods is shown in Table 3. A fully minimized model should have parameters near to the

Table 2 RMS differences from starting structure

	RMSD values for various methods		
Parameter	MM3	MM4	B3LYP-r
$\phi_{\text{standard termination}}$	4.4°	3.9°	7.2°
$\phi_{\text{small termination (tight)}}$[a]	5.9°	6.8°	
$\psi_{\text{standard termination}}$	6.4°	4.2°	9.7°
$\psi_{\text{small termination (tight)}}$[a]	9.2	9.2°	
$O1–O4_{\text{standard termination}}$	0.054 Å	0.044 Å	0.103 Å
$O1–O4_{\text{small termination (tight)}}$[a]	0.071 Å	0.063 Å	
$C1–O–C4_{\text{standard termination}}$	1.7°	2.2°	1.2°
$C1–O–C4_{\text{small termination (tight)}}$[a]	2.4°	1.8°	

[a]"Tight" indicates termination criteria of 0.0546 and 0.0437 kcal mol^{-1} for MM3 and for MM4, respectively

average of the experimental structures. Table 3 compares the average, minimum, and maximum values of the four descriptive parameters. On a positive view, none of the modeled values are grossly outside of the range of observed experimental values. Average values of the modeled parameters were reasonably close to the experimental values, with the MM3 values for the glycosidic (C1–O–C4) angle being slightly low, as mentioned above. Several discrepancies merit comment. The two B3LYP studies are impressively dissimilar, considering that the same software was used for the final results. However, the initial crystal structures were not the same, and several different procedures were used to create the models that were minimized with B3LYP. The O1–O4 distance is an interesting case; the MM3 average was closest to the experimental average, with the MM4 and B3LYP methods being 0.05 Å longer. The ranges of minimized values, 0.51 Å and 0.55 Å for MM3 and MM4, respectively, were somewhat larger than the B3LYP ranges of about 0.47 Å, but all were smaller than the experimental range of 0.75 Å.

An overall assessment of the crystal structures and the models suggests that there is a substantial flexibility of the molecule. Still, as shown by Schnupf and Momany, there is a substantial similarity of the structures of the molecules in the crystals and the models are also all easily recognizable as having the same structure for chemical or biological purposes.

The questions of whether the minimized models tend towards a symmetric structure, and whether there is a coupling of parameters can be addressed graphically. Figure 13a shows the ϕ and ψ angles for two complete trips (residue numbers 1–27 and 27–53) around the final MM3 and MM4 models of CA26 to illustrate the degree of symmetry and coupling. The large spikes in the lower curve for the ψ angle are matched by smaller, downward spikes in the upper, ϕ angle line. These spikes occur for the "flipping" linkages. Smaller fluctuations between the peaks for the 1st and 14th (and 27th, 40th, and 53rd) residues are not so well matched, with four and five small "up" peaks in the two different intervals for the upper plot's ϕ values. On the lower curve's ψ values there are three, somewhat larger "up" peaks in each of the same intervals. One might conclude from the models that there is an atom-conflict based need for the ψ values to alternate even within the helical

Table 3 Comparison of experimental averages and ranges with those from minimized models

Parameter	Experimental (all crystal structures)[a]	MM3	MM4	B3LYP/r (our calculations)	B3LYP/r (Schnupf and Momany)[b]
$\phi_{average}$	102.3°	101.7°	100.2°	103.0°	101.6°
$\phi_{minimum}$	82.1°	92.0°	86.7°	86.3°	76.2°
$\phi_{maximum}$	129.9°	108.4°	110.7°	119.2	111.9°
$\psi_{average}$	−109°	−111.7°	−107.9°	−107.7°	−107.4°
$\psi_{minimum}$	−153.2°	−146.7°	−135.4°	−138.1°	−147.7°
$\psi_{maximum}$	81.6°	80.4°	84.2°	76.1°	84.7°
$O1–O4_{average}$	4.374 Å	4.375 Å	4.421 Å	4.421 Å	4.437 Å
$O1–O4_{minimum}$	4.043 Å	4.150 Å	4.194 Å	4.183 Å	4.210 Å
$O1–O4_{maximum}$	4.790 Å	4.660 Å	4.743 Å	4.659 Å	4.670 Å
$C1–O–C4_{average}$	118.0°	115.5°	118.5°	117.9°	117.7°
$C1–O–C4_{minimum}$	114.0°	112.0°	114.8°	116.1°	113.6°
$C1–O–C4_{maximum}$	121.6°	118.6°	121.5°	119.9°	120.5°

[a]Average values include all 208 experimental values. Minimum and maximum values are the largest and smallest values in the sets of 208 values

[b]Taken from values in Schnupf and Momany (2011b). These averages are based on their 25 reported values. Their ψ values were converted from C1′–O–C4′–C3′ to C1′–O–C4′–C5′ by addition of 120°

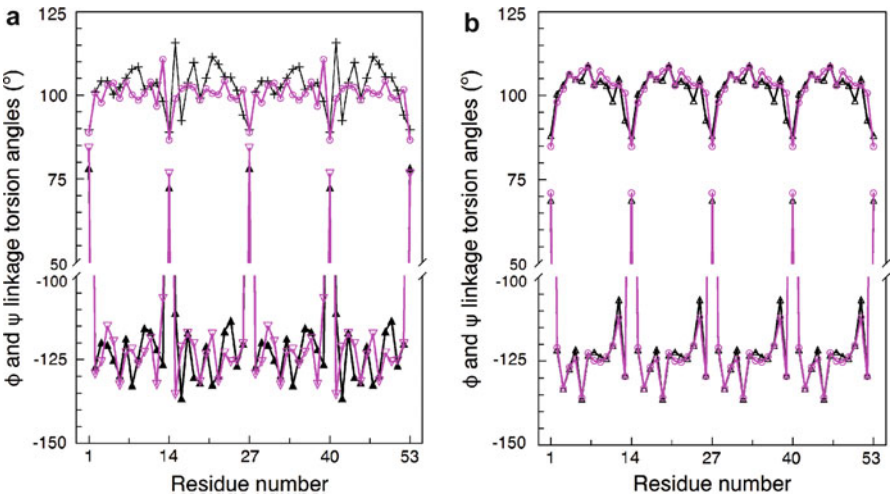

Fig. 13 ϕ and ψ torsion angles for CA26. (**a**) MM3 (*black*) and MM4 (*magenta*) values for ϕ (*upper graph*)/ψ (both parts of *split graph*) data on sequence plot. (**b**) Experimental structures from the symmetrical barium iodide and dodecanol crystals are shown in *black* and *magenta*, respectively. *Torsion angles* are ϕ (O5'–C1'–O4–C4) and ψ (C1'–O4–C4–C5). Two trips around the model (26 residues each trip) are shown to help visualize the periodicity and reproduction of intrinsic variation

portions of CA26. Figure 13b is a similar plot for the two symmetrical CA26 molecules [barium iodide complex II (Nimz et al. 2003) and dodecanol complex (Nimz et al. 2004)]. The ϕ oscillations are smaller and both molecules are quite similar. The ψ torsion angle values are similar for both experimental molecules, and their ranges of values, even when excluding the flipping linkages, are larger than for the ϕ torsions. There is reduced variation in ψ values for residues 7–9 (and 20–22, etc.); those groups of residues are on the inner sides of each helix. That observation of reduced variation possibly due to inter-helix forces is not confirmed by the models in Fig. 13a.

Figure 14a shows the O1–O4 distances for the MM3, MM4, and 14b shows values for the two symmetric experimental structures (see previous paragraph). In particular the MM4 plot indicates strong pseudo symmetry; the MM3 values are somewhat different between the two halves of the molecule (split at the flipping linkages). In the two experimental structures, which are nearly identical to each other, the variation is somewhat different in both the flipping regions (residues 1–3, 13–16, 26–29, etc.) and the helical regions (residues 4–12 and 17–25).

The values of the O1–O4 distance for the 16 residues that were considered to be part of the six-residue per turn helices in the MM3, MM4 our B3LYP structures are 4.33 Å, 4.38 Å, and 4.37 Å, whereas the average value from the eight crystal structures is 4.29 Å. That value is closest to the prediction of 4.25 Å for V_6 amylose in French and Murphy (1973) based on a variety of rigid-residue models. The average O1–O4 distance for the residues in the tetrasaccharide sections associated with the

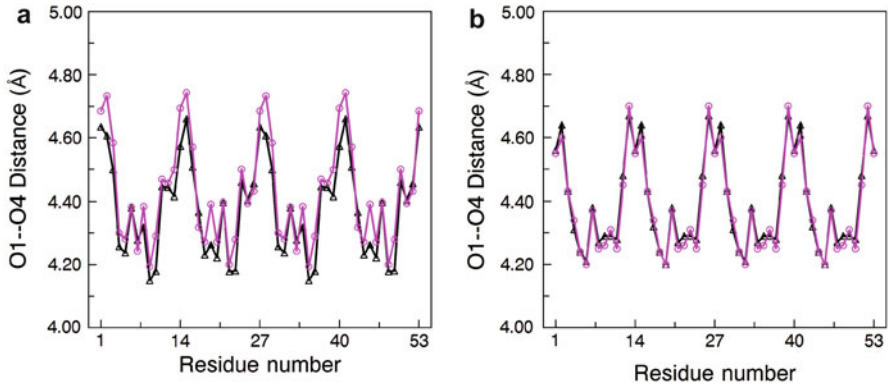

Fig. 14 O1–O4 distance for each residue number showing the stretching of the residues at the flip zones (residues 1, 14, 27, 40, and 53). (**a**) The *black line* is for the MM3 geometries, and the *magenta line* for the MM4 calculation. (**b**) The O1–O4 distances are shown for the symmetrical experimental structures. The *black line* is for the barium iodide complex with CA26, and the *magenta line* for the dodecanol structure. Two trips around the model are shown to help visualize the periodicity and reproduction of intrinsic variation

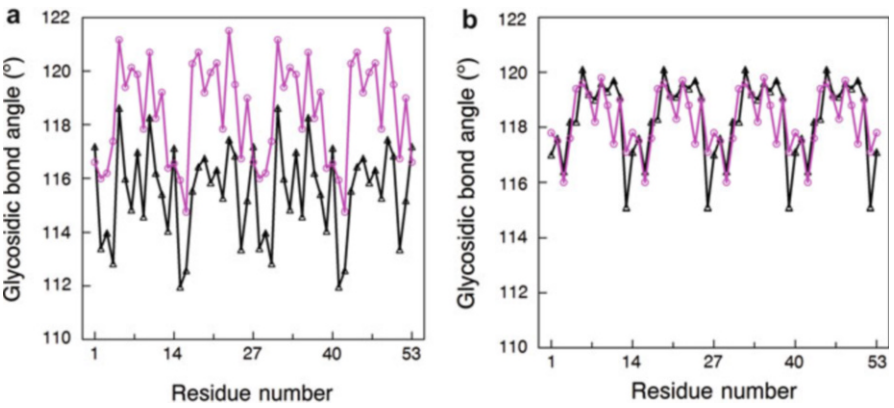

Fig. 15 C1–O4–C4 glycosidic bond angle for CA26. (**a**) MM3 (*black*) and MM4 (*magenta*) bond angles. (**b**) Values from the symmetric experimental structures, with the *black line* for the barium iodide complex and the *magenta line* for the dodecanol complex structure. Two trips around the model are shown to help visualize the periodicity and reproduction of intrinsic variation

flipping linkages was 4.56 Å. These values required an offset in the matching of the O1–O4 distance with tabulated linkage parameters in Table 1 of Nimz et al. (2001) for the orthorhombic structure to match with the groupings of Schnupf and Momany.

Figure 15a, b show the glycosidic bond angles (C1′–O4–C4) by MM3 and MM4 and the two symmetric CA26 molecules from crystal structure determinations. For the bond angle, the B3LYP method was superior (not shown), with the MM values being systematically low (MM3) or high (MM4), each by about a degree.

5 Conclusions

The material on CA26 provides a perspective on computerized modeling of carbohydrates, indicating both some successes and some remaining issues. It shows that there can be substantial intrinsic variation in molecular and linkage geometries for chemically identical moieties, and that modeling can reproduce numerous aspects of that variation. The relatively small set of methods had been shown in earlier parts of this chapter to perform credibly in reproducing features of precisely known crystal structures, such as the puckering of sugar rings and linkage geometries of disaccharides. In the original work it was already established that some acknowledgement of a condensed phase environment was needed to predict all of the known experimental geometries. The predictions were made objective by calculating a "temperature" that would give a comparable distribution of corresponding energies, with values in the neighborhood of 500 K. New calculations of corresponding energies from updated lists of crystal structures were made based on previously published energy surfaces.

In the CA26 study, it was not so convenient to construct a starting model from atoms or even glucose residues; it was far easier to take advantage of an existing crystal structure. Even so, the existing crystal structures required modification before calculations because of missing hydrogen atoms or disordered atoms that are in the coordinate lists twice. Because the limits of our capability to calculate many atom structures with quantum mechanics were being tested, the numerous water and other guest moieties such as iodide or dodecanol were deleted. In our work, the missing hydrogen atoms were added in an arbitrary manner. Most ironically, the acknowledgement of the condensed phase, established for either somewhat elevated dielectric constants or continuum solvation, was not employed in the CA26 calculations.

There are many simplifications or approximations in these calculations, but some conclusions can be tentatively drawn regarding the ability to model complex carbohydrate structures. The substantial variation in some of the critical properties of the experimental structures is met with a substantial variation in the same properties of the models calculated by different methods. However, the major intrinsic variations in linkage torsion angles and O1–O4 distances were retained by the models. There were no obvious differences that would affect the chemistry or biochemistry of this complicated molecule. The average values of the four major variable parameters were reasonably well predicted, but some of the modeled values were slightly outside of the range of the experimental structures. These were models, after all. A certain amount of tension in the structures, both experimental and modeled, was indicated by the persistent variation in parameters even in the helical regions, although the tension is resolved in different ways in the various models and actual crystal structures.

One important lesson is to pay attention to the termination criteria. When operating with a large molecule such as CA26 with 546 atoms, it was useful to continue the energy minimization beyond its default point. The structural details changed significantly afterwards when some ten times as much computer time was

used. With the empirical force fields, this increase was not a significant issue but it was not feasible to increase the precision of the "loose" B3LYP calculation. The B3LYP calculation that we did afford furnished results closer to experiment for only one of the four variables. As will be obvious to many readers, much work remains to be done for a comprehensive study of CA26, and the extensive computer time needed for the B3LYP calculations indicates that a more reasonable approach would involve empirical force fields when only basic structural information is needed. Studies starting with both the symmetric and the other asymmetric crystal structures and some acknowledgement of the condensed phase environment would be critical issues in a more comprehensive study. A molecular dynamics study would be especially interesting to learn whether the equivalency of all of the linkages on the NMR time scale can be reproduced. But this is a review, with some supplemental calculations, rather than a new computational effort.

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Carbohydrate Microarrays

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Denong Wang, Jin Tang, Russell D. Wolfinger, and Gregory T. Carroll

Contents

Abstract

Carbohydrates, like nucleic acids and proteins, are essential biological molecules. Owing to their intrinsic physicochemical properties, carbohydrates are capable of generating structural diversity in a multitude of ways and are prominently displayed on the surfaces of cell membranes or on the exposed regions of macromolecules. Recent studies highlight that carbohydrate moieties are critical for molecular recognition, cell-cell interactions, and cell signaling in many physiological and pathological processes, and for biocommunication between microbes and host species. Modern carbohydrate microarrays emerged in 2002 and brought in new high-throughput tools for "glyco code" exploration. In this section, some basic concepts of sugar chain diversity, glyco-epitope recognition, and the evolving area of glyco-epitomics and biomarker discovery are discussed.

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Two complementary technologies, carbohydrate antigen arrays and photogenerated glyco-chips, serve as models to illustrate how to apply carbohydrate microarrays to address biomedical questions.

Keywords

Antibodies • Carbohydrates • Carbohydrate microarrays • Glycans • Glycoepitopes • Glycoconjugates • Glycome • Glycomics • Lectins • Oligosaccharides • Polysaccharides

Abbreviations

Ab	Antibody
Ag	Antigen
AGOR	Agalacto-orosomucoid
ANOVA	Analysis of variance
ASOR	Asialo-orosomucoid
BSA	Bovine serum albumin
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (also known as CD209)
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GBP	Glycan-binding protein
Gn	GlcNAc
GNA	Galanthus nivalis lectin
Hi-D FACS	High-dimensional fluorescence-activated cell sorting
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KLH	Keyhole-limpet hemocyanin
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
Man9	Man9Gn2Asn moiety
m-II	Multivalent type II chains
Neu5Ac	*N*-acetylneuraminic acid
OR	Orosomucoid
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
P-Man	Yeast phosphomannan Y-2448
PHA-L	Phaseolus vulgaris-L lectin
RAR	Relative antibody reactivity
SARS CoV	Severe acute respiratory syndrome-associated coronavirus
SNA	Sambucus nigra I agglutinin
Stpv	Streptavidin
TI	T-independent

Tn	GalNAcα1→Ser/Thr
Tri/m-Gn	Tri-antennary and multivalent GlcNAc cores
Tri/m-II	Tri-antennary and multivalent type II chains
Tri-II	Tri-antennary type II (Galβ1→4GlcNAc) chains

1 Introduction

Cellular carbohydrates are present in multiple structural configurations, including mono-, oligo-, and polysaccharides, as well as various glycan-hybrid molecules. The latter include, but are not limited to, glycolipids, glycoproteins, proteoglycans, and glycosaminoglycans. Unlike proteins, which are composed of amino acids that are connected solely by one possible peptide bond, carbohydrates utilize many possible glycosidic linkages so as to extensively diversify their structures. For example, two amino acid residues, such as two alanines, can produce only one possible dipeptide; however, two molecules of glucose have the potential to generate 11 different disaccharides. A trimer of any of the nine common sugar residues of the human body theoretically can give rise to 119,736 different structural isomers; this is in striking contrast to the maximal construction of 8,000 tripeptides using 20 different amino acid residues. Theoretically, sugar chain structures can have unlimited variation.

In human and virtually all animal species, cell display of specific complex carbohydrates is characteristically associated with the stages or steps of embryonic development, cell differentiation, and transformation of normal cells to abnormally differentiated tumor or cancer cells (Feizi 1982; Hakomori 1985; Crocker and Feizi 1996; Focarelli et al. 2001). In plants, a highly complex set of polysaccharides are associated with structural proteins and lignin to form cell walls (Avci et al. 2012). Even in a given tissue or cell, cell wall layers and domains may have very different carbohydrate structures and express different glyco-epitopes (Albersheim et al. 2010; Avci et al. 2012). Many microbial organisms also carry unique glycosylation systems and produce specific sugar signatures for almost every microorganism in the living world (Dochez and Avery 1917; Heidelberger and Avery 1923; Ezzell et al. 1990; Robbins and Schneerson 1990; Mond et al. 1995; Wang and Kabat 1996). The term "glycome" has been recently introduced to cover the universe of carbohydrate moieties in all living organisms.

Importantly, multiple carbohydrate recognition systems are present in living species. For example, there are numerous anti-glycan antibodies produced by human and other animal species that play key roles in protecting a host from microbial infections (Behring and Kitasato 1890; Wang and Kabat 1998; Lucas et al. 2008) and families of lectin-like glycan-binding proteins (GBPs) that are evolved for carbohydrate-mediated cell-cell communication (Drickamer 1988; Sharon 2007; Varki 2009). The well-known GBPs that are associated with cell signaling and immunomodulation are the receptors of the innate immune system, such as Dectin-1 that recognizes fungal β-glucans (Brown et al. 2002), mannose

receptor that recognizes carbohydrate moieties on multiple pathogens and is involved in the clearance of inflammatory molecules in vivo (Gruden-Movsesijan and Milosavljevic Lj 2006), and DC-SIGN (dendritic cell-specific ICAM-3-grabbing non-integrin) that selectively detects viral glycoproteins, such as HIV-1 gp120 glycoprotein (Curtis et al. 1992). The interaction between HIV-1 gp120 and DC-SIGN plays an important role in the CD4-independent association of HIV with human cells (Curtis et al. 1992). Thus, carbohydrates are suitable for storing biological signals in forms that are identifiable by other biological systems.

In the immunological and glycobiological literature, "glyco-epitope" is often used to specify the carbohydrate moiety that is recognized by an antibody or by a GBP. The antibody-binding glyco-epitopes are also classified as B cell epitopes or antigenic determinants. The term "glyco-epitome" was recently introduced to describe the entire repertoire of glyco-epitopes, including the B cell epitopes and those that are recognized by GBPs. Differing from the term "glycome," which covers all the existing carbohydrate molecules in living organisms, glyco-epitome refers to a unique subset of carbohydrates that serves as the sugar signatures for molecular recognition and biosignal transmission. "Glyco-epitomics" is, thus, an area of glycomics research focusing on identifying, characterizing, and understanding the carbohydrate moieties that serve for multiple levels of biocommunication.

It is noteworthy that glyco-epitope characterization requires not only carbohydrate structural analysis but also immunological studies. The structural aspects of glyco-epitomics focus on the elucidation of the glycan structures that display glyco-epitopes. This research area has been substantially enhanced by the development of advanced profiling and structural characterization strategies. Notably, these include high-resolution chromatography methods coupled with exoglycosidase digestions (Campbell et al. 2008; Royle et al. 2008), modern mass spectrometry (Babu et al. 2009; Goldberg et al. 2009; North et al. 2009) and nuclear magnetic resonance spectroscopy analyses (Petrescu et al. 1997; Wormald et al. 2002; Petrescu et al. 2006) of carbohydrates and state-of-the-art methods of glycan structural modeling (Woods and Tessier 2010; Jo et al. 2013).

However, availability of carbohydrate structural information alone is not sufficient in defining a glyco-epitope unless its specific binding by an antibody or a GBP is also demonstrated immunochemically and/or cryptographically. For example, chemical determination of a tetrasaccharide that decorates the spore of *Bacillus anthracis* appears to be an important discovery in microbial glycomics (Daubenspeck et al. 2004). Based on past knowledge of immunogenic carbohydrate moieties, this structural glycomics progress may suggest that this unique sugar moiety may have potential in an immunological application (Saksena et al. 2005, 2007). However, whether such a carbohydrate moiety preserves a B cell epitope or a potent antigenic determinant must be determined immunologically, including at least demonstration of its antibody-binding specificity and capacity in eliciting immune responses in vivo (Wang et al. 2007). It was the integrated structural and immunological investigation of glyco-epitopes (Wang et al. 2007; Lucas et al. 2008) that has revealed anthrose tetrasaccharides as key immunological targets of *B. anthracis*.

2 Classification of Carbohydrate Microarrays

Four research articles about carbohydrate microarrays first appeared in the scientific literature in 2002 (Borman 2002, 2012). These include polysaccharide and glycoconjugate microarrays, reported by Denong Wang's group at Columbia University's Genome Center (now at SRI International, CA, USA) (Kiessling and Cairo 2002; Wang et al. 2002); monosaccharide chips, by Milan Mrksich and coworkers at the University of Chicago (Houseman and Mrksich 2002); arrays of natural and synthetic neoglycolipids, by Ten Feizi's group at Imperial College Faculty of Medicine, Harrow, UK (Fukui et al. 2002); and arrays of synthetic oligosaccharides in microtiter plates, by a Scripps Research Institute group led by Chi-Huey Wong (Bryan et al. 2002). A specialized book, "Carbohydrate microarrays, Methods and Protocols (Humana Press)," recently edited by Dr. Yann Chevolot of Université de Lyon in France (Chevolot 2012), provides a comprehensive summary of the emerging technologies for construction of carbohydrate microarrays.

2.1 Spotting Carbohydrates with Different Structural Characteristics

Given the various structural characteristics of carbohydrates displayed on chips, carbohydrate microarrays are classified as monosaccharide chips (Houseman and Mrksich 2002; Park and Shin 2002), oligosaccharide arrays (Bryan et al. 2002; Fukui et al. 2002; Blixt et al. 2004; Wang et al. 2007), and microarrays of carbohydrate-containing macromolecules (Wang et al. 2002; Willats et al. 2002). The latter includes polysaccharides and various glycoconjugates. These different sugar chips or arrays were developed to accommodate multipurpose applications in carbohydrate research. For example, mono- and disaccharide microarrays are suitable for screening and characterizing novel carbohydrate-binding proteins or carbohydrate-catalyzing enzymes and for identifying novel inhibitors of carbohydrate-protein interactions.

A large class of carbohydrate-binding proteins, called lectins, was initially classified by their binding specificities to monosaccharides and recently by disaccharides. However, there are lectins and many antibodies with anti-carbohydrate reactivities that bind to larger and more complex carbohydrate ligands or antigenic determinants. Mono- and disaccharide sugar chips are not sufficient for investigations involving such molecular targets. Oligosaccharide, polysaccharide, and glycoconjugate microarrays fill this gap by displaying carbohydrates of complex structures or longer sugar chains on the chips.

2.2 Strategies for Carbohydrate Immobilization on a Chip

Based on the technologies that are applied to immobilize carbohydrates on bioarray substrates, the various methods to construct carbohydrate microarrays can be classified as distinct technological platforms. These include technologies that directly utilize underivatized carbohydrates in microarray construction,

technologies that require chemical modification of carbohydrates before microarray fabrication, methods of non-covalent immobilization of carbohydrates, and methods of covalent coupling of saccharides on array substrates.

The use of underivatized saccharides for microarray construction has the advantage of preserving the native structures of the carbohydrate molecules. It requires, however, a ready-to-use microarray surface with appropriate surface chemistry that can be directly used to fabricate comprehensive carbohydrate microarrays with underivatized carbohydrates from a wide range of sources. Methods currently in use include non-covalent binding of underivatized carbohydrate antigens by passive adsorption on a chip, such as nitrocellulose-coated glass slides (Wang et al. 2002) or black polystyrene surfaces (Willats et al. 2002), and methods for covalently immobilizing underivatized carbohydrates on a slide surface by appropriate chemical linking techniques (Angeloni et al. 2005; Lee and Shin 2005; Carroll et al. 2006; Zhou and Zhou 2006; Wang et al. 2007; Zhou et al. 2009, 2012).

Carbohydrate microarrays can also be fabricated by using derivatized carbohydrates. Due to their small molecular size and hydrophilic nature, most oligosaccharides cannot be directly immobilized onto nitrocellulose or black polystyrene surfaces for microarray applications. However, an oligosaccharide probe can be modified with a tag or coupled to a larger carrier molecule for non-covalent immobilization. Methods include non-covalent immobilization of derivatized carbohydrates on microarray chips (Fukui et al. 2002; Palma et al. 2006) or on enzyme-linked immunosorbent assay (ELISA) microtiter plates (Bryan et al. 2002) and covalent immobilization of derivatized carbohydrates on microarray chips. The latter includes, but are not limited to, the popular Consortium for Functional Glycomics (CFG) printed glycan arrays (Blixt et al. 2004; Bochner et al. 2005) and various technologies of notable technical features that were developed independently (Houseman and Mrksich 2002; Park and Shin 2002; Galanina et al. 2003; Kohn et al. 2003; Park et al. 2004; Parthasarathy et al. 2006; Gerland et al. 2012a, b; Morvan et al. 2012; Goudot et al. 2013).

Affinity immobilization is another class of approaches for coupling derivatized carbohydrates to solid surfaces. For example, biotin-derivatized carbohydrates can be immobilized on a streptavidin-coated substrate through the affinity interaction of the streptavidin–biotin pair to create carbohydrate microarrays. Biotin-derivatized carbohydrates include carbohydrate ligands that are biotinylated via a short aliphatic spacer or at the peptide part of glycopeptides (Guo et al. 2004; Bochner et al. 2005; Dyukova et al. 2006). DNA-directed immobilization (DDI) is another practical strategy for immobilization of oligonucleotide glycomimetic conjugates on a chip surface for the preparation of carbohydrate microarrays (Gerland et al. 2012a, b; Morvan et al. 2012; Goudot et al. 2013).

2.3 Common Technical Features of Carbohydrate Microarrays

Despite technical differences among different platforms of carbohydrate microarrays, they are all solid-phase binding assays and share a number of common

characteristics and technical advantages. For instance, they contain the capacity to display a large panel of carbohydrates in a limited chip space, they are high-throughput quantitative assays, they make an effective use of carbohydrate substances that are often difficult or cost-inefficient to synthesize, and, as discussed below, they are highly sensitive in monitoring carbohydrate-anti-carbohydrate interactions in multiplex manners.

In a carbohydrate microarray, each carbohydrate is spotted in an amount that is drastically smaller than that required for a conventional molecular or immunological assay. This technical feature ensures a condition in which the binding of a molecule in solution phase to an immobilized microspot of ligand on the microarray substrate has minimal reduction of the molar concentration of the molecule in solution (Ekins et al. 1990). Thus, microarray-based assays are intrinsically optimized for binding equilibrium to take place, which is the basis for this class of hypersensitive binding assays (Stoll et al. 2002). Carbohydrate microarrays have higher detection sensitivity than most conventional carbohydrate analytical tools, such as carbohydrate-specific ELISA and the glycolipid-based "Eastern blot" assays that were developed in the 1980s by a number of early researchers in this field (Wood and Kabat 1981; Tang et al. 1985). Historically, this situation is very similar to the relationship between conventional blotting methods for nucleic acids or proteins, such as Southern, Northern, and Western blots, and nucleic acid–based or protein/peptide–based microarrays.

2.4 Complementary Platforms of Carbohydrate Microarrays

Carbohydrate microarrays constructed by various methods may differ in their technical characteristics and suitability for a given practical application. Some platforms may be applied complementarily to solve a practical question. For example, the method of nitrocellulose-based immobilization of carbohydrate-containing macromolecules, including polysaccharides, glycoproteins, and glycolipids, is suitable for the high-throughput construction of carbohydrate antigen microarrays (Wang et al. 2002, 2005; Wang and Lu 2004) to support the large-scale immunological characterization of carbohydrate antigens and anti-carbohydrate antibodies. However, the detection specificity of this carbohydrate microarray would be at the level of a carbohydrate antigen, not a glyco-epitope, if the native carbohydrate antigens were spotted. This is owing to the fact that many carbohydrate antigens display multiple antigenic determinants or glyco-epitopes (Cisar et al. 1975; Wang and Kabat 1998; Wang 2004). Examining the finer details of the binding properties would require the use of microarrays of defined oligosaccharide sequences. Oligosaccharide array-based binding assays can be applied, in combination with saccharide competition assays, to decipher precise saccharide components of a specific antigenic determinant or glyco-epitope (Fukui et al. 2002; Blixt et al. 2004; Wang et al. 2007; Zhou et al. 2009). These technical features of carbohydrate microarrays are further discussed in two models, i.e., a carbohydrate antigen microarray platform and a technology of photogenerated oligosaccharide microarrays in subsequent sections.

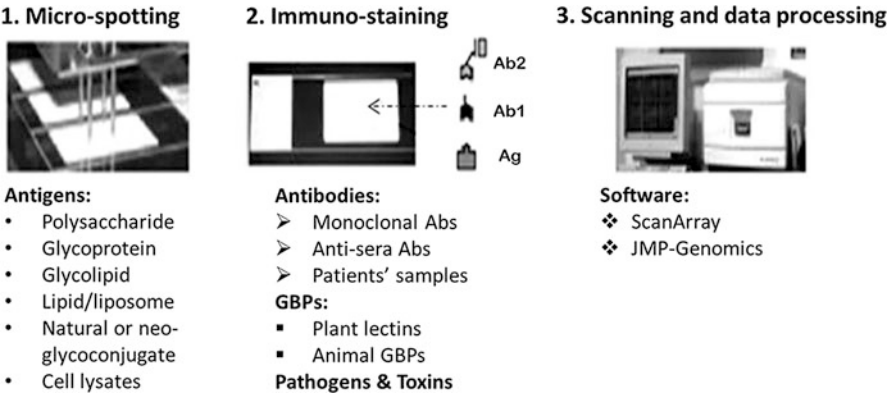


Fig. 1 A high-throughput platform for carbohydrate-based microarrays. A high-precision robot designed to produce cDNA microarrays is utilized to spot carbohydrate antigens of various structural configurations onto a nitrocellulose-coated glass slide. The microspotting capacity of this system is approximately 20,000 spots per chip. The antibody- or GBP-stained slides were then scanned for fluorescent signals with a microarray scanner that was developed for cDNA microarrays. Toxins or viral pathogens can also be applied on this platform of carbohydrate microarrays to probe potential glycan-receptors of viruses or toxins

3 A Versatile Carbohydrate Antigen Microarray Technology

A practical approach for construction of carbohydrate microarrays is to print carbohydrate antigens onto nitrocellulose-coated glass slides. This was the first reported method for high-throughput production of carbohydrate microarrays (Borman 2002; Kiessling and Cairo 2002; Wang et al. 2002). Using this technology, carbohydrate-containing macromolecules of diverse structures, including polysaccharides, natural glycoconjugates, and mono- and oligosaccharides coupled to carrier molecules, can be stably immobilized on a glass chip without chemical modification. This approach was subsequently extended to production of lipids/glycolipid and liposome microarrays (Fukui et al. 2002; Wang et al. 2005, 2014). Recently, this approach has been applied to produce integrated protein, lipid, and carbohydrate microarrays (Wang et al. 2014). Owing to the technical simplicity of this approach, anyone who has access to a standard cDNA microarray facility would be able to explore this technology for his or her own research interests (Fig. 1).

3.1 Preparing Carbohydrate Antigens for Microarray Printing

Soluble antigen preparations are generally applicable for construction of microarrays in this platform. Except certain antigens that require special solutions, proteins and carbohydrates for spotting are dissolved in phosphate-buffered saline (PBS; pH 7.4) and saline (0.9 % NaCl), respectively. Liposomes of various compositions, including *homo*- and *hetero*-liposomes, are suitable for printing on this

substrate (Wang et al. 2014). The former were produced via a single lipid preparation, e.g., phosphatidylcholine (PTC), cerebroside, and sulfatide. The latter contained two different lipid molecules with PTC as the support to display other lipid/glycolipids in desired ratios or epitope densities. For example, the *hetero*-liposome of sulfatide is composed of sulfatide and PTC at a ratio of 1:10 (wt/wt), i.e., 0.2 mg sulfatide and 2.0 mg PTC per ml of liposome suspension in saline. Methods employing sonication and extrusion (mechanical energy) to produce liposomes for microarray production were similarly described by a number of investigators (Wang et al. 2005; Palma et al. 2012).

3.2 Printing Carbohydrate Microarrays Using Conventional Microspotting Devices

Microarray printers that were designed for DNA or protein microarrays, such as PixSys 5500C (Cartesian Technologies, Irvine, CA), are suitable for spotting carbohydrates onto glass slides pre-coated with nitrocellulose polymer (FAST Slides; Schleicher and Schuell, Keene, NH) (Wang 2012). We often spot antigens in triplicate with spot sizes of 150 μm and at 375 μm intervals, center to center. The printed microarrays are air-dried and stored at either room temperature or 4 °C before application.

3.3 Staining and Scanning Microarrays

Immediately before use, the printed microarrays were rinsed with PBS, pH 7.4, with 0.05 % (vol/vol) Tween 20 and then blocked by incubating the slides in 1 % (wt/vol) bovine serum albumin (BSA) in PBS containing 0.05 % (wt/vol) NaN_3 at room temperature (RT) for 30 min. They were then incubated at RT with antibodies at an indicated titration in 1 % (wt/vol) BSA in PBS containing 0.05 % (wt/vol) NaN_3 and 0.05 % (vol/vol) Tween 20. The secondary antibodies or streptavidin conjugates applied for microarray staining are specified in the figure legends. The stained slides were rinsed five times with PBS with 0.05 % (vol/vol) Tween 20, air-dried at room temperature, and then scanned for fluorescent signals using a ScanArray 5000A microarray scanner (PerkinElmer Life Science) following the manufacturer's manual.

3.4 Producing Customized Arrays for Defined Purposes

Large-scale "repertory" microarrays containing thousands of microspots or larger are powerful means for discovering unexpected molecular targets. For example, microarray scanning of autoantibody responses allows one to "fish out" potential autoantigens in the glycome in autoimmune diseases. Customized, smaller scale carbohydrate microarrays containing a few dozen antigens are, however, suitable

for more defined purposes, such as antibody fine-specificity mapping, differential diagnosis among a number of known infectious diseases, measurement of autoantibodies for known targets in autoimmune diseases, etc. Fig. 2 shows a sub-array design where each chemically modified microglass slide contains eight separated sub-arrays. The microarray capacity is ~400 microspots per sub-array. A single slide is, thus, designed to enable eight microarray assays. Similar sub-array designs with various array capacities are commercially available (Schleicher and Schuell, Keene, NH; ArrayIt, Sunnyvale, CA).

- Each microglass slide contains twelve sub-arrays of identical content. There is a chip space for about 400 microspots per sub-array, with spot sizes of approximately 200 μm and at 300-μm intervals, center to center. A single slide is, therefore, designed to enable 12 detections. This design is typically for printing four 96-well plates of antigen preparations ($96 \times 4 = 384$).
- Repeats and dilutions: Our team usually prints carbohydrate antigens at the initial concentration of 0.1–0.5 μg/μl. The absolute amount of antigens printed on the chip substrate is in the range of 0.1–0.25 ng per microspot for the highest concentration. They are further diluted at 1:3, 1:9, and 1:27, or as specified in each experiment. A given concentration of each preparation is repeated at least three times to allow statistical analysis of detection of identical preparations at given antigen concentrations.
- Positive controls and standard curves: Fluorescent conjugates, such as BSA conjugates of FITC, Cy5, Cy3, or other dyes, are routinely applied for microarray printing to provide positive markers for each fluorescent channel. These markers are helpful for scanning calibration, alignment of microarray spots during data-capturing, the subsequent microarray data normalization, and cross-chip scaling of microarray detection. For serological studies, antibodies

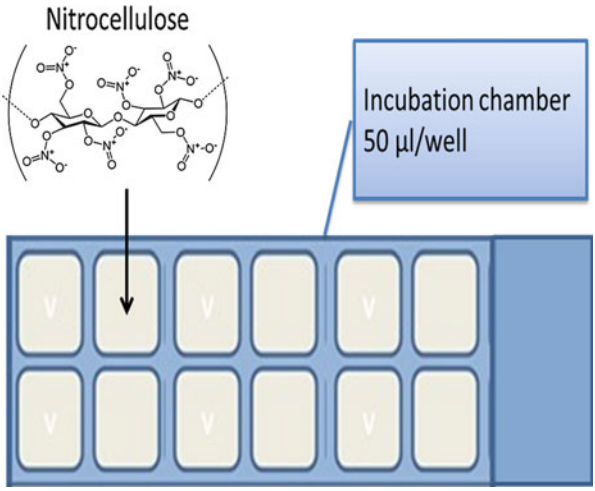


Fig. 2 Illustration of the 12-chamber sub-arrays

of IgG, IgA, and IgM isotypes of corresponding species are spotted to produce standard curves in microarray format. These curves serve as reference standards for quantifying antibody signals of a specific IgH chain isotype that are captured by spotted carbohydrate antigens.

3.5 Technical Notes of Nitrocellulose-Based Bioarray Substrate

A number of nitrocellulose-coated glass slides with different technical characteristics are commercially available. Given the structural diversity of carbohydrate antigens, examining each antigen preparation to determine the efficacy of its immobilization in a given type of substrate and the surface display of the desired glyco-epitopes in a microarray assay is essential. A practical approach is to incubate the printed microarrays with antibodies, receptors, or lectins known to react with the printed substance.

Figure 3 is an example of such analysis where lectin *Galanthus nivalis agglutinin* (GNA) and antibody 2G12 were applied to examine specific glyco-epitopes on the spotted microarrays. Inspection of both microarray images (Fig. 3a) and the quantitative datasets show that the GNA-epitopes were presented by three glycoconjugates (Fig. 3c), i.e., Man9-cluster ($4^{\#}$), M9_2G12-cluster ($3^{\#}$), and Man5-9 RB ($1^{\#}$). In contrast, 2G12-glyco-epitopes were preserved only by one of the three, i.e., M9_2G12-cluster ($3^{\#}$), on this microarray substrate (Fig. 3d). This carbohydrate microarray analysis demonstrates, therefore, an example that the same sugar chain may generate different glyco-epitopes when the sugar moiety is presented in different cluster configurations. In this case, the Man9GlcNAc2Asn moiety was coupled to the protein carriers in either (Man9GlcNAc2Asn) n- or [(Man9GlcNAc2Asn) $_4$] n-configurations (Fig. 3b). The latter but not the former preserves well the 2G12-defined broadly HIV-1 neutralizing epitope.

4 A Photogenerated Glyco-Chip Technology

Carroll and colleagues (2006; Wang et al. 2007; Carroll and Wang 2012) developed a photochemical method to covalently immobilize carbohydrates on chips. As illustrated in Fig. 4, the method employs a self-assembled monolayer to present photoactive phthalimide chromophores at the air-monolayer interface. Upon exposure to UV radiation, the phthalimide end-groups graft to surface-adsorbed carbohydrates to form a covalent bond. The amount of surface-grafted carbohydrate is enhanced when carbohydrate surface interactions are increased by the incorporation of amine-terminated molecules into the monolayer. One of the important applications of this technology is to identify immunogenic sugar moieties of microbial pathogens by screening the corresponding antisera obtained from vaccinated or infected subjects.

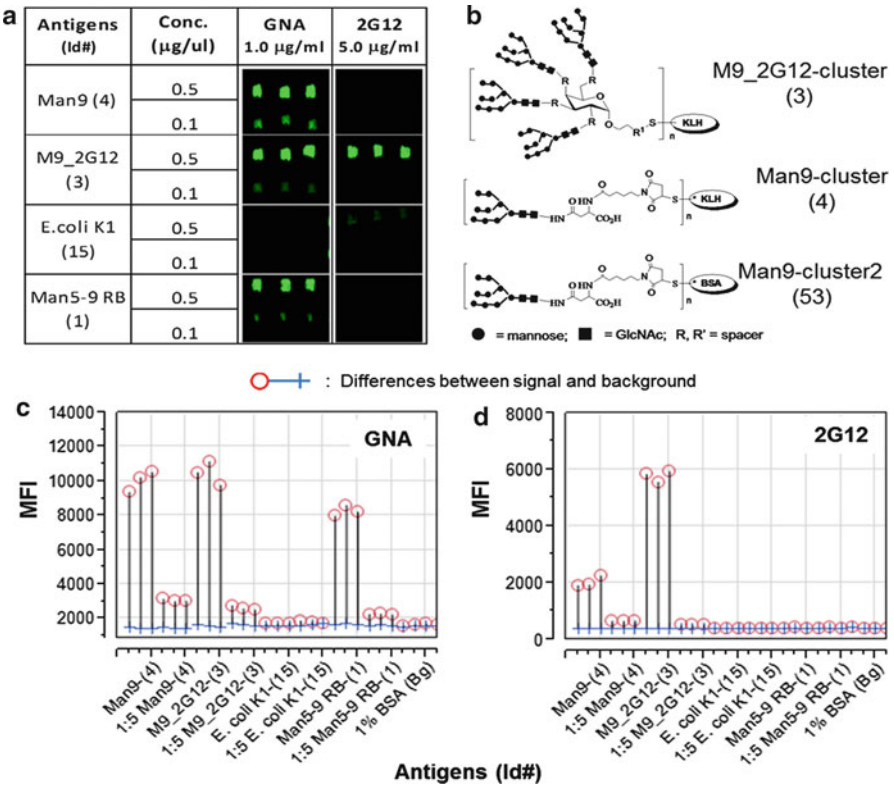


Fig. 3 A carbohydrate microarray analysis of oligomannosyl antigens for expression of the 2G12-like and GNA-like glyco-epitopes. The mannose-cluster-containing microarrays were stained with 2G12 (5 μg/ml) and a biotinylated GNA (1.0 μg/ml), respectively. The former was revealed by Alexa^{647}-tagged Goat anti-human IgG Fc-specific antibodies at 2 μg/ml and then developed with Streptavidin-Cy5 conjugate at 2 μg/ml. (**a**) Shows microarray images stained with either GNA or 2G12. (**b**) Illustrates the cluster configuration of the three Man9-conjugates. Microarray detection signals are shown as the mean fluorescent intensities (MFIs) of each microspot as captured by the ScanArray 5000A for the arrays stained with GNA (**c**) and 2G12 (**d**), respectively. Results were compared using overlay plots of the MFIs of staining signal (*red circles*) versus those of local backgrounds surrounding the antigen microarrays (*blue* +) (**c**, **d**) (Adapted from Wang et al. (2014))

4.1 Synthesis of Photoactive Compounds

The phthalimide chromophore used in the photogenerated glycan-chip was modified with a silane derivative in order to form a stable bond to glass. A 3.3 mmol portion of 11-bromoundecanetrimethoxysilane (Gelest) was added to a solution of an equimolar amount of potassium phthalimide (Aldrich) in 60 mL of anhydrous DMF (Aldrich). The solution was stirred overnight at room temperature (RT) under argon. Chloroform (50 mL) was added. The solution was transferred to a separatory flask containing 50 mL of H_2O. The aqueous layer was separated and then extracted

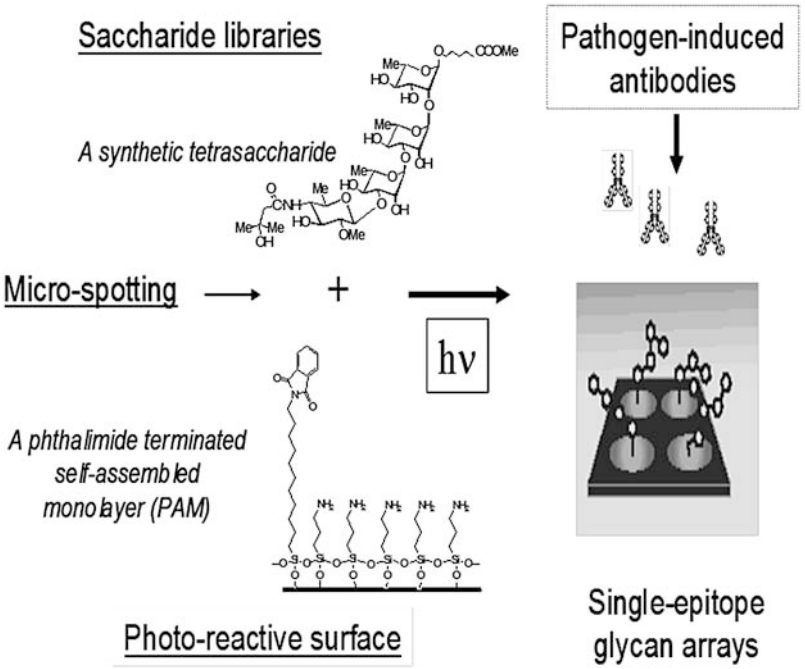


Fig. 4 Photogenerated glycan arrays for rapid identification of pathogen-specific immunogenic sugar moieties. Saccharide preparations were dissolved in saline (0.9 % NaCl) at a given concentration and spotted using a high-precision robot (PixSys 5500C, Cartesian Technologies, Irvine, CA) onto the phthalimide amine (PAM)-coated slides. The printed PAM slides were subjected to UV irradiation (300 nm) for 1 h to activate the photocoupling of carbohydrates to the surface. Pathogen-specific antisera were then applied on the glycan arrays to identify potential immunogenic sugar moieties of given pathogens (Adapted from Wang et al. (2007))

with two 20 mL portions of chloroform. The combined chloroform extract was washed with several 20 mL portions of H_2O. The chloroform was removed by rotoevaporation, and residual DMF was removed on a high vacuum line to give a pale yellow liquid (0.99 g, 72 % yield). The compound was used without further purification. Note that, for self-assembly experiments, residual DMF was not removed. 1H NMR: (CDCl3) *ä* 7.82 (m, 2H), 7.69 (m, 2H), 3.66 (t, *J* 7 Hz, 2H), 3.55 (s, 9H), 1.44–1.15 (m, 18H), 0.71–0.51 (m, 2H). LRMS-FAB+ (*m/z*): (M-H) 420.2 (experimental), 420.2 (calculated); (M-OCH3) 390.1 (experimental), 390.2 (calculated).

After synthesis of the phthalimide-silane, PAM was prepared by immersing a clean glass slide into a toluene solution containing 1 mM of the phthalimide-silane and 5 mM of aminopropyltrimethoxysilane (Gelest). The H_2O contact angle of the resulting surface was 72 ± 1°. Note that the glass slide was cleaned with a 7:3 mixture of H_2SO_4: 30 % H_2O_2. Extreme caution should be used when preparing and using such a solution, which can react violently and explosively if mixed with other chemicals.

4.2 Photocoupling of Carbohydrates onto Chips

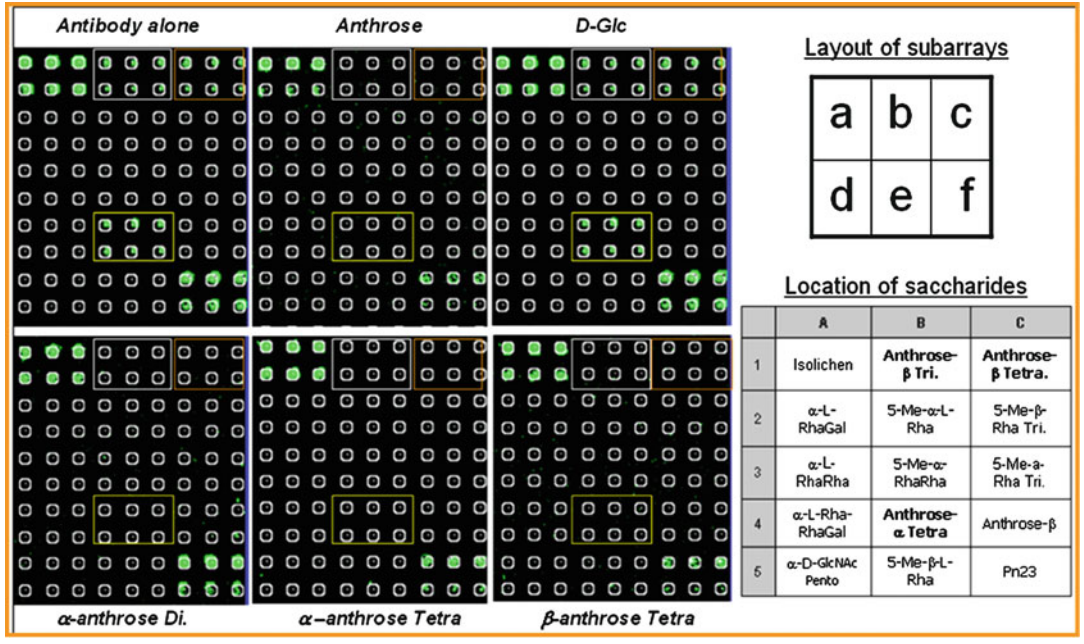
Microarray spotting is performed as outlined in the above section. After spotting of carbohydrates, the PAM slides were air-dried and placed in a quartz tube. The sealed tube was subsequently purged with argon or nitrogen before irradiation. UV irradiation was conducted by placing the quartz tube under a desktop lamp containing a 300 nm Rayonet bulb for 1 h. Precaution was made to avoid skin and eye contact with the radiation during the irradiation process.

4.3 Probing Immunogenic Sugar Moieties Using Photogenerated Glyco-Chips

The photogenerated glycan arrays were applied to probe the potential immunogenic sugar moieties of *Bacillus anthracis* spores (Wang et al. 2007). The rationale was that if *B. anthracis* spores expressed immunogenic carbohydrate structures, the spore antigen-immunized or *B. anthracis*-infected animals would be possible to mount antibody responses to these carbohydrates. This assumption was made on the basis of the fact that the host immune system is able to recognize subtle changes in sugar structures, especially those that are exposed on the surfaces of microbial pathogens that are foreign components of the mammalian hosts.

Figure 5 below is an example of photo-chip characterization of the rabbit antisera elicited by *B. anthracis* spores. The photo-chips used were spotted with a large panel of saccharide structures, including synthetic fragments and derivatives of the anthrose-containing tetrasaccharide side chain of the *B. anthracis* exosporium and a number of control carbohydrate antigens. Antibody staining was performed in the presence or absence of saccharide inhibitors. Images (a–f) display a portion of the stained glycan arrays: (a) no saccharide inhibitor; (b) anthrose; (c) D-glucose; (d) α-anthrose trisaccharide; (e) α-anthrose tetrasaccharide; (f) β-anthrose tetrasaccharide. The locations of surface-bound anthrose-containing saccharides that are recognized by the antibody in the absence of inhibitor are highlighted by colored boxes: *White*, β-anthrose-trisaccharide; *Brown*, β-anthrose-tetrasaccharide; *Yellow*, α-anthrose-tetrasaccharide. Microarray data sets are available upon request.

This analysis confirmed that a tetrasaccharide of BclA glycoprotein bears a dominant antigenic determinant, which is composed of a terminal anthrose residue and three adjacent L-rhamnoses. The terminal trisaccharide unit is essential for the constitution of a highly specific antigenic determinant. Given the fact that this carbohydrate moiety is displayed on the outermost surfaces of *B. anthracis* spores and its expression is highly specific for the spore of *B. anthracis*, the anthrose-containing tetrasaccharide can be considered an important immunological target. Its applications may include identification of the presence of *B. anthracis* spores, surveillance and diagnosis of anthrax infection, and development of novel vaccines targeting the *B. anthracis* spore.



	A	B	C
1	Isolichen	**Anthrose-β Tri.**	**Anthrose-β Tetra.**
2	α-L-RhaGal	5-Me-α-L-Rha	5-Me-β-Rha Tri.
3	α-L-RhaRha	5-Me-α-RhaRha	5-Me-a-Rha Tri.
4	α-L-Rha-RhaGal	**Anthrose-α Tetra**	Anthrose-β
5	α-D-GlcNAc Pento	5-Me-β-L-Rha	Pn23

Fig. 5 Photogenerated glycan arrays recognize immunogenic sugar moieties of the *B. anthracis* spore. A panel of 35 mono-, oligo-, and polysaccharides were photocoupled to the surface of derivatized glass slides. After incubating the glycan arrays with rabbit anti-*B. anthracis* spore polyclonal IgG antibodies (Abcam, Cambridge, UK) in the absence or presence of saccharide inhibitors, the bound rabbit IgG was revealed by a tagged anti-rabbit IgG antibody (Adapted from Wang et al. (2007))

4.4 Technical Notes for Photogenerated Glyco-Chips

A unique technical advantage of this method is the ability to produce epitope-specific glycan arrays using unmodified mono- and oligosaccharides. The quality of the surfaces obtained depends in part on cleanliness. Ideally, water with a resistivity of 18.2 MΩ-cm and total organic contaminant of less than five parts per billion should be used; however it is still possible to prepare the surfaces after rinsing with water of lower quality. Self-assembly of trimethoxysilanes can be enhanced by adding small amounts of water and acid. Such treatment was not used for the mixed surface described above but may possibly enhance the reaction times or surface coverage obtained. If the surface is allowed to dry upon removal from self-assembly solution, the aminosilane may polymerize, leaving white deposits that are difficult to remove.

5 Carbohydrate Microarray Data Processing and Statistical Analysis

Although specialized bioinformatics tools for carbohydrate microarrays are yet to be developed, some software packages developed for cDNA microarrays are applicable for carbohydrate microarrays. This is owing to the fact that these different microarray assays are commonly based on the laser fluorescence detection systems regardless of the contents of the spotted microarrays. A number of advanced microarray software packages are currently available. These include, but are not limited to, Significance Analysis of Microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/), Prediction Analysis for Microarrays (PAM http://www-stat.stanford.edu/~tibs/PAM/index.html), and JMP Genomics (www.jmp.com/genomics).

5.1 Presenting Microarray Raw Datasets and Microarray Images

A straight forward way to present the raw results of a carbohydrate microarray assay is to illustrate the microarray raw datasets and/or images. For example, carbohydrate researchers often present microarray raw datasets with or without background substraction for the carbohydrate-binding profiles of monoclonal antibodies (Palma et al. 2011), lectins (Blixt et al. 2004), or viruses (Childs et al. 2009). Figure 3 above illustrates two examples of binding profile presentations based on microarray raw data.

In this experiment, the mannose-cluster-containing microarrays were stained with mAb 2G12 (5 μg/ml) and a biotinylated GNA (1.0 μg/ml), respectively. Figure 3a shows microarray images and Fig. 3b illustrates the cluster configuration of the three Man9-conjugates. Microarray detections are shown as the mean fluorescent intensities (MFIs) of each microspot as captured by the ScanArray 5000A for the arrays stained with GNA (Fig. 3c) and 2G12 (Fig. 3d), respectively.

Results were compared using overlay plots of the MFIs of the antigen-binding signals (*red circles*) versus those of local background signals surrounding the antigen microarrays (*blue* +) (Fig. 3c, d). Analysis of such microarray raw data in association with visual inspection of the microarray image provides an initial evaluation of reproducibility and variation of this antigen microarray technology.

5.2 Statistical Tests of Carbohydrate Microarray Detection

In order to identify disease-associated biomarkers, such as antigen-specific antibodies with diagnostic and/or prognostic values, we compare microarray detection between a disease group and a normal control group or to more generally between multiple groups. Proper statistical analysis is essential for making generalizable conclusions. Typical steps include quality control, normalization, and statistical model fitting. In our general practice, carbohydrate array datasets are preprocessed and statistically analyzed using JMP Genomics software from SAS Institute.

Antigen-specific antibody reactivities are represented as microarray scores, which are the log2 transformed values normalized by some method. We have explored several different normalization methods, including mean and/or variance centering, loess, and quantile normalization, and found the interquartile range (IQR) method to provide an appropriate degree of standardization (based on distribution and correlation plots) without overly correcting the data. The IQR method sets the 25th and 75th percentiles of the microarray distributions to be equal.

After normalization, we utilize an antigen-by-antigen ANOVA model to obtain statistically significant differences. Data from triplicate spots for each antigen are included in the ANOVA model for that antigen. A cutoff to detect significant differences is determined by applying a multiple testing correction to statistical results from the ANOVA model. These procedures are further discussed below in an example of clinical sample analysis.

5.3 Clinical Sample Analysis Using Customized Carbohydrate Microarrays

Here we present an example to illustrate general statistical approaches to carbohydrate microarray analysis of clinical samples. In this case, customized autoantigen microarrays were applied to characterize the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients. MS is a complex neurological disorder in which an adaptive autoimmune response is thought to target myelin sheath in the central nervous system. We created a microarray displaying a panel of 32 carbohydrate and lipid antigens to examine MS-associated autoantibody responses.

A technical challenge to this study is the fact that the total Ig concentrations in the CSF of MS patients are higher than those in the CSF of other neurological diseases (OND) subjects, which reflects one of the hallmarks of MS

(Kabat et al. 1948, 1951; Steinman 1996; Genain et al. 1999; Raine et al. 1999; Hueber et al. 2002). However, it causes difficulty in identifying the disease-specific autoantigens and autoantibodies. In our microarray analyses, we reconfirmed this observation. Figure 6a, b show the overlay plots of antibody profiles of the two groups. The colored needles that link the pairs of group mean values provide a global comparison of the antibody profiles between the MS group (*red circles*) and the OND group (*blue crosses*). This comparison reveals global differences in antibody profiles between the two groups. Specifically, the microarray scores of CSF-antibody activities in the MS group are generally higher than those seen in the CSF of OND subjects. These include not only anti-lipid antibodies (*Right*, *26–44*$^{\#}$), as previously reported (Ho et al. 2012), but also anti-carbohydrate antibodies (*Left*, *1–22*$^{\#}$).

We further examined whether there is any selective enrichment of antigen-specific antibodies in the CSF of MS patients. We reasoned that identifying such antibodies might provide clues to pinpoint key autoimmunogenic targets of MS. For this purpose, we introduced an approach to establish RAR scores for microarray signals and then sought targets that capture the antibody signal with higher RAR scores in MS patients. Specifically, we normalized the microarray datasets by setting their IQR to be identical using the JMP Genomics software package. This statistical operation effectively "quenches" the variation seen between subjects that are due to variable antibody concentrations in the CSF. The two groups illustrate similar Ig-RAR profiles for both IgG and IgM antibody activities (Fig. 6c, d). However, a number of probes show higher IgG-RAR scores in the MS group than in the OND controls. These include two Man9-clusters (3$^{\#}$, and 4$^{\#}$), three glucose polysaccharides, dextran N279 (8$^{\#}$), B1299S (9$^{\#}$), B1355S (10$^{\#}$), and a Bacto-Agar (20 °C, extracted) antigen (13$^{\#}$) (Fig. 6c).

In Fig. 7, an antigen-by-antigen analysis of variance (ANOVA) model was applied to obtain statistically significant differences between groups in comparison. Results are graphically presented as a volcano plot (Zink et al. 2013) for a global comparison of all RAR scores between the groups in comparison (Fig. 7a) and as one-way analysis scatterplots for selected targets (Fig. 7b, c). In the volcano plot, each dot represents a statistically weighted and quantified difference between MS and OND groups. The x-axis is the normalized difference (log2 scale) and the y-axis uses –log10 (p-value) for the difference. Spots above the red-dashed line represent signatures that differ significantly between the groups after a multiple testing correction.

For the one-way scatterplots in Fig. 7b, c, each data point represents the mean of triplicate determinations. The means of the points are shown as horizontal green bars and standard deviations as green diamonds around the mean value. The comparison circles for the Student's "t"-test appear to the right of the mean diamonds to illustrate the significance of the differences among the means. These circles allow visual inspection of the statistical significance of the differences. The more the circles intersect, the less significant their difference, and vice versa.

Of the 126 antibody signatures captured in this assay (Fig. 6c, d), two were above the cutoff line [–log10 (p-value) = 2.5] as highly significant

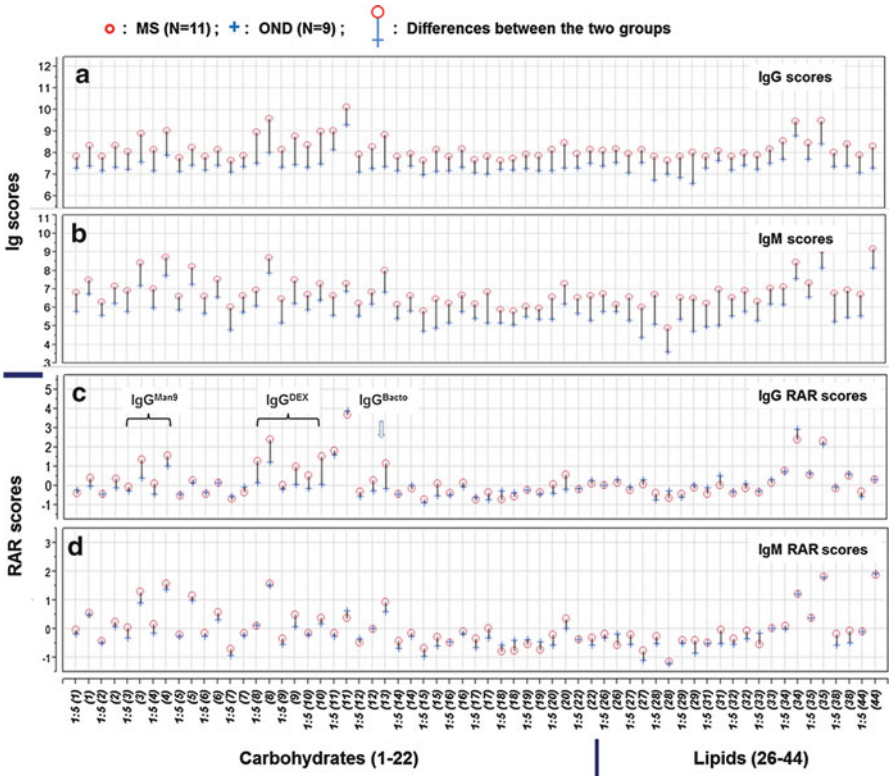


Fig. 6 Integrated lipid/carbohydrate arrays recognize globally elevated antigen-specific antibodies in the CSF of MS patients as compared to those detected in the OND CSF. CSF samples from 11 MS (10 RRMS and 1 SPMS) and 9 OND subjects were characterized using microarrays spotted with 12 lipid and 20 carbohydrate antigens. Each preparation was spotted in triplicate at 2–4 dilutions. As illustrated in the overlay plots (**a**–**d**), this microarray supports detection of 126 unique antibody signatures. These included 63 IgG (**a**, **c**) and 63 IgM (**b**, **d**) signatures. Anti-human IgG or IgM secondary antibodies were used to reveal the antigen-specific antibodies detected by these microarrays. The captured IgG were stained with an anti-human IgG antibody conjugated with Cy3 at 2 μg/ml and the captured IgM in the same array revealed by a biotinylated anti-human IgM secondary antibody at 2 μg/ml and developed with Streptavidin-Cy5 conjugate at 2 μg/ml. Microarray datasets in (**a**) and (**b**) were illustrated as microarray scores. In (**c**) and (**d**) microarray datasets were further processed using JMP Genomics to produce RAR* scores. The results are presented as overlay plots of the mean microarray scores for each group. The colored needles that link the group mean values of each pair of scores provide a global comparison of the antibody profiles of MS (*red circles*) and OND (*blue crosses*) groups (Adapted from Wang et al. (2014)). **RAR Score*: *RAR* **R**elative **A**ntibody **R**eactivity, the value of log2 transformed and IQR-standardized microarray value (mean-background). For each antigen in a given concentration, the mean value of triplicate array detections was calculated

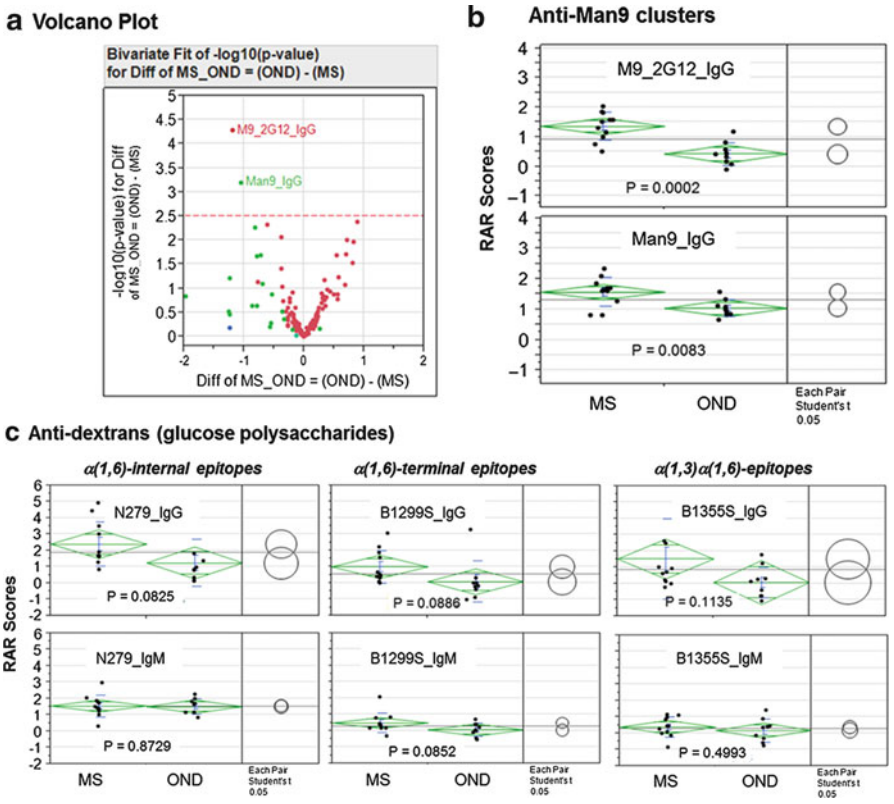


Fig. 7 MS-associated autoantibodies in CSF samples target high-mannose clusters, the cores of N-glycans. (**a**) is a volcano plot analysis of the RAR scores of all antibody signatures. In the volcano plot, each point represents a biologically unique feature captured by the microarray, i.e., a normalized difference for a specific antibody signature (RAR^{MS}-RAR^{OND}). The x-axis is the normalized difference (log2 scale); the y-axis is the –log10 (*p*-value), which weights the levels of significance of a difference. Points above the *red-dashed line* (cutoff level 2) represent signatures that differ significantly between the groups based on the Bonferroni test. Two signatures, M9_2G12_IgG and Man9_IgG, were identified by this critical statistical test as highly significant markers. In (**b**) and (**c**) one-way analysis was performed to compare group means among the selected carbohydrate antigens listed in each panel. Each point in the panels represents the mean value (RAR score) of triplicate array detections of a subject (Adapted from Wang et al. (2014))

differentiators between MS and OND. These were M9_2G12_IgG and Man9_IgG. Results of the one-way ANOVA of the two Man9 clusters are shown in Fig. 7b. All other antibody signatures had –log10 *p*-values below the cutoff line (Fig. 7a), including four signatures that were variably higher in the MS group than in the OND group. These were N279_IgG ($P = 0.0825$), B1299S_IgG ($P = 0.0886$), B1355S_IgG ($P = 0.1135$), and Bacto_Agar_IgG ($P = 0.021$). This microarray analysis identified, therefore, oligomannoses as potential immunological targets

for further investigation. Overall, JMP Genomics is a valuable tool for statistical analysis of carbohydrate microarray data.

6 Conclusion/Prospects

Modern carbohydrate microarrays emerged in 2002 (Borman 2002, 2012; Kiessling and Cairo 2002; Wang and Collins 2013) and introduced new glycomics tools to decipher the biological information content in the glycome. These technologies are especially useful in exploring the repertoire of glyco-epitomes. A number of carbohydrate microarray platforms have now reached or are very close to the technical stage of the current nucleic acid–based or protein-based microarrays that are readily available for practical uses. Technical issues that require further improvement may include, but are not limited to, optimization of existing technologies for array construction, quality control and technical standardization in both microarray production and application, and establishment of specialized bioinformatics tools to handle the massive amount of carbohydrate microarray data and to effectively extract diagnostic or research information from each microarray assay.

Nevertheless, exploring the repertoires of glyco-epitopes represents a long-term goal of glycomics research. It was estimated that the human glycome contains 10,000–20,000 minimal epitopes for glycan-binding proteins (Cummings 2009). In considering the repertoires of the "hybrid" structures that are generated by protein posttranslational modification, including both *N*- and *O*-glycosylation, the repertoires of carbohydrate-related antigenic structures can be much larger. Furthermore, the conformational diversity of carbohydrates and microheterogeneity of carbohydrate chains substantially increases the repertoire of carbohydrate-based antigenic determinants or glyco-epitopes (Wang and Kabat 1996; Wang 2014). Including carbohydrate structures of the microbial world, which are directly relevant to medicine, the sizes and diversity of the repertoires of glyco-epitopes are unpredictable.

Further development of carbohydrate microarrays requires libraries of carbohydrate antigens, including purified natural antigens and synthetic glycoconjugates, as well as anti-glycan mAbs, lectins, and other glycan-binding proteins. Naturally purified carbohydrate antigens have the advantage of preserving the native antigenic structures and often offer highly sensitive detection of antigen-specific antibodies. Availability of synthetic oligosaccharides and glycoconjugates is, however, critical for epitope determination and fine-specificity studies of carbohydrate-anti-carbohydrate interactions.

Anti-glycan mAbs and GBPs of known carbohydrate-binding specificities are required to characterize glyco-epitopes that are presented by carbohydrate microarrays. Thus, collaborative efforts by both academic and industrial sectors are required to facilitate the establishment of large collections of glycan-targeting probes. This situation is similar to established flow cytometry technology and services. Availability of specific antibody probes for CD antigens, in combination with the state-of-the-art technologies of flow cytometry (high-dimensional

fluorescence-activated cell sorting, or Hi-D FACS) (Tung et al. 2004), has revolutionized research in cellular biology and immunology and medical applications of CD antigens, especially in the clinical diagnosis of leukemia and other human diseases. Exploring the repertoires of carbohydrate-based biomarkers and targeting agents, with the aid of carbohydrate microarray technologies and other high-throughput omics tools, may represent one of the highly active areas of postgenomics research and technology development in future years.

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Advanced Nano-biocomposites Based on Starch

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Abstract

Starch as a biopolymer directly extracted from nature has received much attention in recent years due to its strong advantages such as low cost, wide availability, renewability, and total compostability without toxic residues. Starch-based materials always display properties that are less satisfactory than those of traditional polymer materials, which can be ascribed to the inherent characteristics of starch. To make such materials to be truly competitive and to widen its applications, the development of starch-based nano-biocomposites could be a promising solution. This chapter provides the fundamental knowledge related to starch-based nano-biocomposites as well as the most recent developments in this area. Various types of nanofillers that have been used with plasticized starch are discussed such as montmorillonite, cellulose nanowhiskers, and starch nanoparticles. The preparation strategies for starch-based nano-biocomposites with these types of nanofillers and the corresponding dispersion state and related properties are also largely discussed.

Keywords

Starch • Nanocomposites • Biopolymers • Biodegradable polymers • Bioplastics • Nanoclays • Cellulose nanowhiskers • Starch nanoparticles • Carbon nanotubes

Abbreviations

AFM	Atomic force microscopy
BCNW	Bacterial cellulose nanowhisker
CB	Carbon black
CEC	Cationic exchange capacity
CMC	Carboxymethyl cellulose sodium
CNT	Carbon nanotube
CNW	Cellulose nanowhisker
D	Diameter
d_{001}	Interlayer spacing or *d*-spacing
DMA	Dynamic mechanical analysis
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
FTIR	Fourier transform infrared spectroscopy
G'	Elastic modulus (rheology)
GO	Graphite oxide
HA	Hydroxyapatite
IL	Ionic liquid
L	Length
L/D	Length-to-diameter ratio (i.e., aspect ratio)
LCA	Life-cycle assessment
LDH	Layered double hydroxide
MMT	Montmorillonite
MMT–Na^+	Sodium montmorillonite

MWCNT	Multiwall carbon nanotube
O	Octahedral sheets
OMMT	Organomodified montmorillonite
OMMT–CS	Cationic starch–organomodified montmorillonite
PBAT	Poly(butylene adipate-*co*-terephthalate)
PBSA	Poly(butylene succinate-*co*-adipate)
PCL	Polycaprolactone
PEG	Polyethylene glycol
PLA	Poly(lactic acid)/polylactide
PVA	Poly(vinyl alcohol)
REX	Reactive extrusion
SEM	Scanning electron microscopy
SME	Specific mechanical energy
SNP	Starch nanoparticles
SSE	Single-screw extruder
SWCNT	Single-wall carbon nanotube
T	Tetrahedral sheets
TEM	Transmission electron microscopy
T_g	Glass transition temperature
TSE	Twin-screw extruder
UV	Ultraviolet
XRD	X-ray diffraction

1 Introduction

The increasing environmental concerns, the strong decrease of the availability on the market of some conventional refinery fractions which are more or less refined (e.g., isoprene, butadiene, bitumen, etc.), the expected shortage of fossil resources (mainly petroleum), and the necessity to develop innovative materials architectures are some of the main drivers to push the use of biobased compounds extracted from the biomass. Following this mainstream, starch, as a natural polymer from renewable resources, has experienced considerable development in the past decades in nonfood and materials applications (Avérous and Halley 2014). Starch has strong advantages such as low cost, wide availability, renewability, and total compostability without toxic residues. By using conventional polymer processing techniques such as extrusion, native starch (also known as raw starch) in the form of granules can be transformed into a molten state with the presence of low content of "plasticizers" such as water and glycerol. This converted form of starch is known as "plasticized starch," "thermoplastic starch" (although its behavior is not totally thermoplastic but more thermo-mechano-plastic (Martin et al. 2003)), or abbreviated as "TPS," which can be produced into different end-use forms such as extruded, molded, thermoformed, or blown articles (Liu et al. 2009a).

Despite of the advantages mentioned above, starch-based plastic materials are known to have limitations such as poor processability and limited properties (e.g., weak mechanical properties, large evolution of properties during aging, and high water sensitivity). Formulation development could be the major key to solve these crucial problems. To give some examples, various starch-based blends and biocomposites (i.e., with fillers) have been developed, showing improved performance (Avérous 2004; Avérous and Halley 2009; Kalambur and Rizvi 2006a; Tang and Alavi 2011; Wang et al. 2003; Yu et al. 2006a) compared to the neat matrix. Recently, along with the exponential momentum of the development in polymer nanocomposites (Alexandre and Dubois 2000; Crosby and Lee 2007; Paul and Robeson 2008; Pavlidou and Papaspyrides 2008; Sinha Ray and Okamoto 2003; Sinha Ray and Bousmina 2005; Spitalsky et al. 2010), much attention has been focused on the use of nanosized fillers (at least one dimension in the nanometer range, i.e., 1–100 nm) in producing starch-based nano-biocomposite materials. Their nanosize can generate huge areas at the matrix–filler interface, which largely control the global macrostructures and associated global properties of the corresponding materials. Under certain conditions with the tailored nanofiller and/or organomodification of the nanofiller, nanocomposite materials can exhibit drastic improvement in mechanical behavior, thermal stability, flame retardancy, and gas barrier properties (Alexandre and Dubois 2000). Such property enhancement depends on the nature, geometry, surface area, and surface chemistry of the nanofiller (Sinha Ray and Okamoto 2003).

Unlike most conventional synthetic polymers, starch is a biopolymer directly extracted from biomass, which has unique chemical structure and processing behavior. Therefore, the preparation and properties of starch-based nano-biocomposites are inherently dissimilar to those of other polymer nanocomposite systems. Furthermore, the incorporation of appropriately tuned nanoparticles into starch as a biopolymer may not only enhance the conventional material performance of starch-based materials, but it could also provide new functionalities for new applications due to the resulting novel structures and functional groups. While this chapter aims to provide the basic knowledge of starch and starch-based nano-biocomposites, which should be helpful to those new to this area, it touches some of the most recent international research progress on this topic as well.

2 Starch Characteristics

2.1 Structure of Native Starch

The starch granule provides the main way of storing carbon and energy over long periods in green plants (Pérez et al. 2009). Starch granules are mainly found in seeds, roots, and tubers and display different shapes (spherical, lenticular, and polygonal) depending on their origins such as maize (corn), wheat, potato, and rice. In Fig. 1, an overview of starch granule structure is shown, which presents

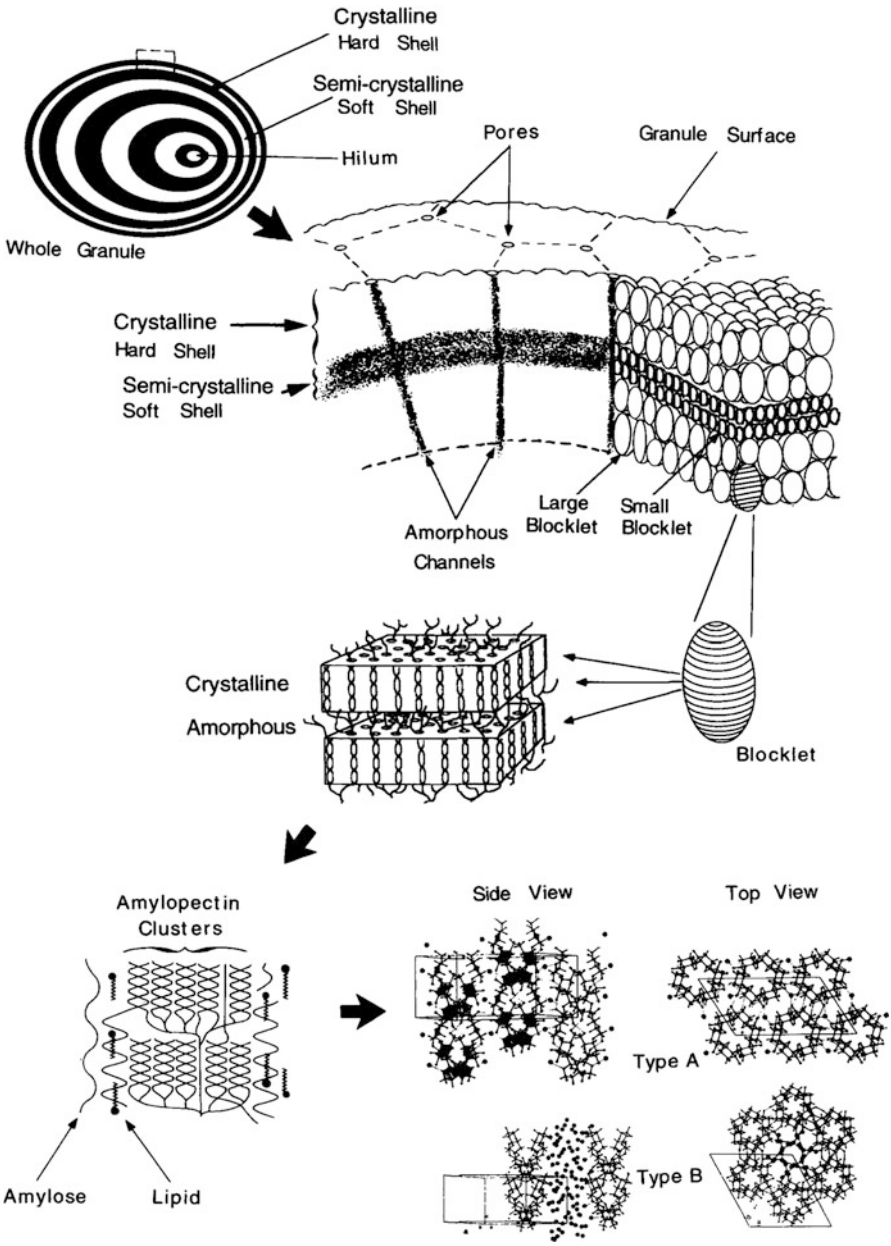

Fig. 1 Overview of starch granule structure. At the lowest level of granule organization (*upper left*), alternating crystalline (*hard*) and semicrystalline (*soft*) shells are shown (*dark and light* colors, respectively). Shells are thinner toward the granule exterior (due to increasing surface area to be added to by constant growth rate) and the hilum is shown off-center. At a higher level of structure, the blocklet structure is shown in association with amorphous radial channels. Blocklet size is smaller in the semicrystalline shells than in the crystalline shells. At the next highest level of

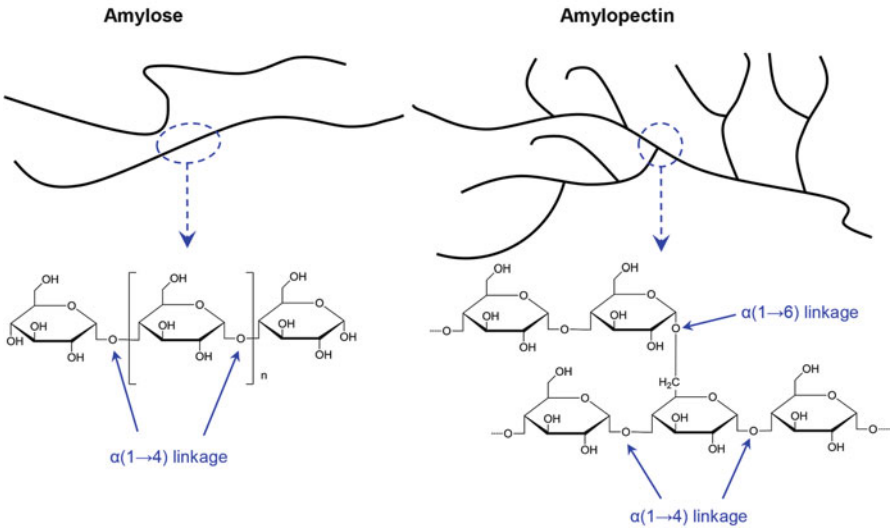

Fig. 2 Chemical structures of amylose and amylopectin molecules of starch

a concentric three-dimensional architecture from the hilum, with a crystallinity varying from 15 % to 45 % depending on the botanical source (Zobel 1988). The starch granule is well known to possess multilevel structures from macro- to molecular scales, i.e., starch granules (<1–100 μm), alternating amorphous and semicrystalline shells (growth rings) (100–400 nm), crystalline and amorphous lamellae (periodicity) (9–10 nm), and macromolecular chains (~nm) (Buléon et al. 1998; Jane 2009; Pérez et al. 2009; Pérez and Bertoft 2010).

In molecular level of structure, starch is a polysaccharide consisting of D-glucose units, referred to as homoglucan or glucopyranose. Figure 2 presents the two major biomacromolecules of starch, i.e., amylose and amylopectin. Amylose is a sparsely branched carbohydrate mainly based on α(1–4) bonds with a molar mass of 10^5–10^6 and can have a degree of polymerization (DP) as high as 600 (Pérez et al. 2009). The number of macromolecular configurations based on α(1–6) links is directly proportional to the amylose molar mass (Tako and Hizukuri 2002). The chains show spiral-shaped single or double helices with a rotation on the α(1–4) link and with six glucoses per turn, where the hydroxyl groups are mainly located toward the exterior of the helices. As a result, the core of the helix is mainly hydrophobic.

Fig. 1 (continued) structure, one blocklet is shown containing several amorphous crystalline lamellae. In the next diagram, amylopectin is shown in the lamellae. The next image is a reminder of the importance of amylose–lipid (and protein) components in the organization of amylopectin chains. At the highest level of order, crystal structures of the starch polymers are shown (Reprinted from Gallant et al. (1997), Copyright (1997), with permission from Elsevier. Redrawn in Gallant et al. (1997) from Imberty et al. (1988) and Imberty and Perez (1988))

On the other hand, amylopectin is a highly multiple-branched polymer with a high molar mass of 10^7–10^9. It is one of the largest natural polymers on earth (Pérez et al. 2009). Amylopectin is based on α(1–4) (around 95 %) and α(1–6) (around 5 %) links, with constituting branching points localized every 22–70 glucose units, generating a highly branched structure with a lot of pending chains of DP ≈ 15, which are mainly responsible for the materials' crystallinity (between 20 % and 45 %). This specific structure has a profound effect on the physical and biological properties (Pérez et al. 2009; Pérez and Bertoft 2010).

Besides, in starch granules, very small amounts of proteins, lipids, and phosphorus are also found depending on the botanical resource (Jane 2009; Pérez and Bertoft 2010). These components can interact with the carbohydrate chains during processing (e.g., Maillard reaction) and then modify the behavior of the starchy materials.

Depending on the source, amylose content of starch can be varied from <1 % to 83 % (Tan et al. 2007). The so-termed waxy starch contains little or no amylose, whereas high-amylose starch contains >50 % amylose. The amylose content has a great impact on the crystalline structure and degree of crystallinity (Tan et al. 2007), as well as on the thermal, rheological, and processing properties (Li et al. 2011a; Liu et al. 2006, 2011d; Wang et al. 2010a; Xie et al. 2009).

2.2 Gelatinization/Melting of Native Starch

When native starch granules are heated in water, their semicrystalline nature and three-dimensional architecture are gradually disrupted, resulting in a phase transition from the ordered granular structure into a disordered state in water, which is known as "gelatinization" (Atwell et al. 1988; Lelievre 1974; Ratnayake et al. 2008). The process of gelatinization is schemed in Fig. 3. Gelatinization is an irreversible process that includes, in a broad sense and in time/temperature sequence, granular swelling, native crystalline melting (loss of birefringence, as observed under polarized light), and molecular solubilization (Russo et al. 2009). Full gelatinization of starch under shearless conditions requires excess water, which Wang et al. (1991) have defined as >63 % for waxy maize starch, for example. If the water concentration is too high, the crystallites in starch might be pulled apart by swelling, leaving none to be melted at higher temperature. If the water concentration is limited, the swelling forces will be much less significant and the steric hindrance is high. Thus, complete gelatinization will not occur in the usual temperature range (Lai and Kokini 1991; Liu et al. 2005, 2006). However, as the temperature increases, starch molecules will become progressively more mobile and eventually the crystalline regions will be destructured (Donovan 1979). The process of gelatinization with low water content could more accurately be defined as the "melting" of starch (Lai and Kokini 1991).

The gelatinization/melting behavior of starch is quite different when shear treatment is applied (Xie et al. 2006). With abundant water, shear can enhance

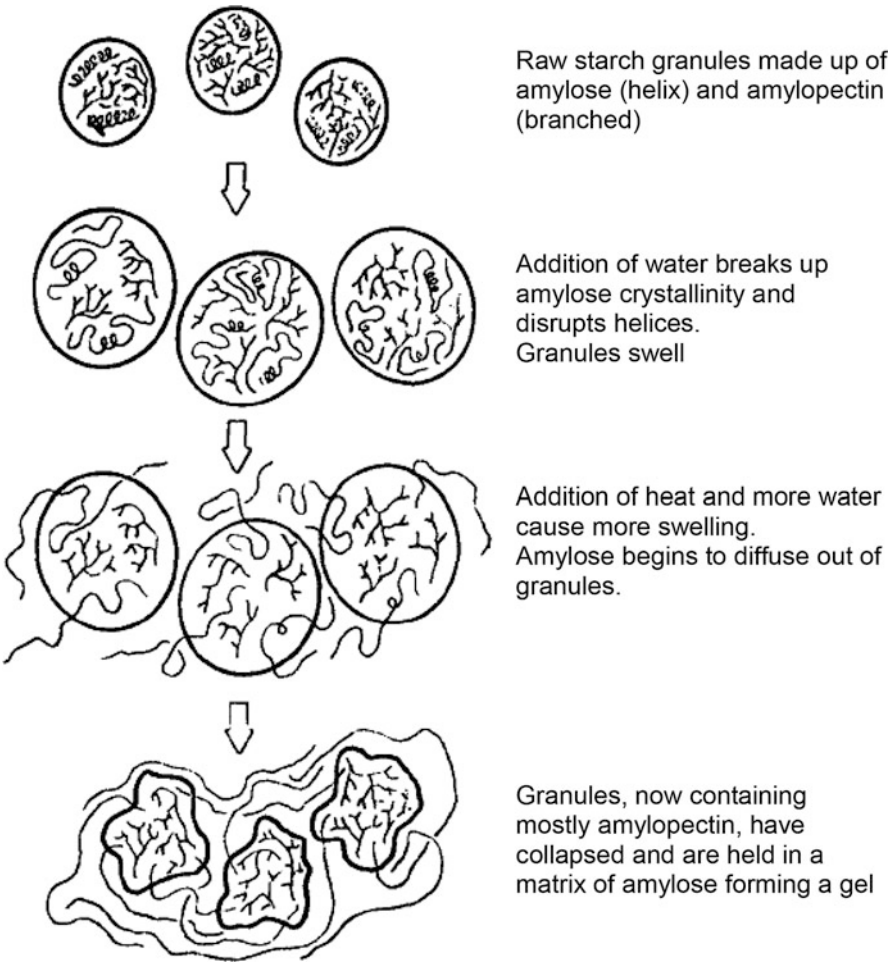

Fig. 3 Mechanism of starch gelatinization (Copyright © 1978 Wiley Periodicals, Inc. Reproduced from Remsen and Clark (1978) with permission)

the destructuration of starch granules (Chen et al. 2007; Yu et al.2006b). When the water content is limited, shear can assist the melting of crystallites (Wang et al. 2010a; Xie et al. 2008; Xue et al. 2008). The significance of such studies is that most processing techniques for starch involve shear treatment. In extrusion processing (which will be discussed below in Sect. 3.1), shear forces can physically destructure starch granules, allowing faster transfer and distribution of water into the material (Burros et al. 1987). Therefore, during extrusion, the loss of the crystallinity is not only caused by the water penetration but also by mechanical disruption due to the intense shear fields within the extruder (Barron et al. 2001; Wang and Zheng 1995; Zheng and Wang 1994; Zheng et al. 1995).

3 Starch-Based Materials

3.1 Processing Techniques

The techniques that have been used to process starch polymers, such as solution casting, internal mixing, extrusion, injection molding, and compression molding, are similar to those widely used for conventional synthetic thermoplastics. Solution casting has been frequently utilized in the literature as a method to prepare starch-based films for research purposes. However, this technique suffers from disadvantages such as low efficiency and the difficulty in scaling up from the lab scale to the industry and not being considered as a "green process" due to the use of toxic organic solvents and the great consumption of energy for solvent evaporation. Alternatively, extrusion processing is more aligned to modern industrial production. A single-screw extruder (SSE) can handle the high viscosity of starch and provide high processing pressure for continuous metering of starch through a die shape. A twin-screw extruder (TSE) has large operational flexibility (individual barrel zone temperature control, multiple feeding/injection, and screw configuration for different degree of mixing/kneading) and is useful to intensive mixing and compounding of other components into starch plastics. Another advantage of TSE is to allow the decoupling of the die flow and the mechanical treatment. In both SSE and TSE, the residence time and specific mechanical energy (SME) input can be controlled, and high-efficiency production can be achieved. Other processing techniques such as film blowing and injection molding are often combined with the extrusion. More details about the specifics of the processing techniques for starch polymers as well as the recent advances in the understanding of starch processing can be found elsewhere (Liu et al. 2009a; Xie et al. 2014).

A schematic of twin-screw extrusion process for the production of TPS is shown below in Fig. 4 (Xie et al. 2012). The native starch granules are introduced, preferably by a powder gravimetric feeder, into the extruder in a hungry way (i.e., the feeding capacity is less than the conveying capacity of the screws at the feeding port). Liquids (typically plasticizer) could be introduced in a following location, preferably using a pressure-injection way to ensure uniform mixing between the solid(s) and the liquid(s). The granules undergo exposure to high temperatures and pressures in the extruder, resulting in the disruption, and gelatinization/melting, of starch granules (Liu et al. 2006). After gelatinization, the TPS will undergo further melting and compression, which may be increased with increased use of high mixing (kneading or reversing) elements in the screw. It is important to note that degradation of starch molecules also occurs with the thermal mechanical treatment in the extruder, with amylopectin molecules suffering more than amylose (Li et al. 2014; Liu et al. 2010b). And the mechanism for the scission of the polymer chains is believed to preferentially take place close to the center of the molecule, causing the size distribution to narrow and converge toward a maximum stable size (Liu et al. 2010b). The TPS is forced through the extrusion line and finally out of a die at the end of the extruder at a controlled rate (possibly with the aid of a melt pump to further control the flow rate).

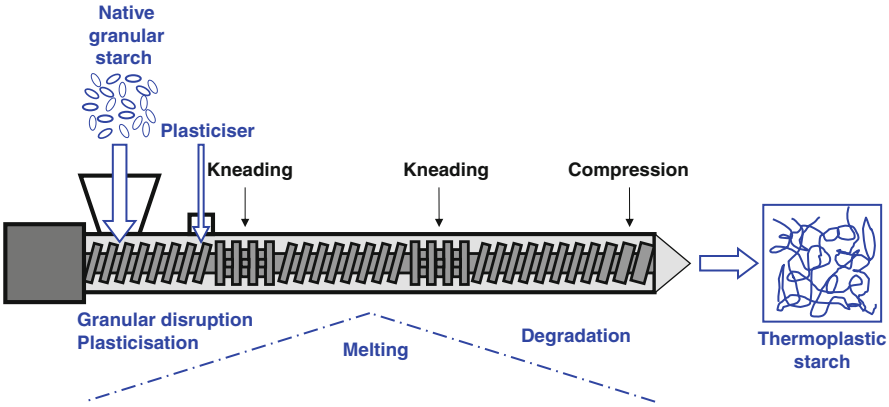

Fig. 4 Schematic representation of starch processing by extrusion (Reprinted from Xie et al. (2012), Copyright (2012), with permission from Elsevier)

3.2 Plasticizers

Water is indispensable for the thermal processing of starch. By reducing the moisture content, the melting temperature of starch would progressively increase, and that of dry starch is often larger than its degradation temperature as extrapolated by Flory Law (Liu et al. 2008; Russell 1987). Water functions by lowering the melting temperature and plasticizing the starch polymers. As a result, in practical processing, water acts as a volatile "plasticizer." Thus, unstable processing or undesirable foaming may be caused due to the evaporation of the water. Furthermore, the final products based on starch containing only water usually have poor mechanical properties especially due to the brittleness, because of its final temperature usually lower than its glass transition temperature (T_g) (Chaudhary et al. 2011b; Enrione et al. 2010; Forssell et al. 1997; Liu et al. 2011b, 2009b, 2010a; Lourdin et al. 1997) and/or resulting from the densification (happening below T_g) or retrogradation (also known as recrystallization, happening above T_g) (Atwell et al. 1988; Bulkin et al. 1987; Gudmundsson 1994; Liu and Thompson 1998). To overcome these issues, nonvolatile (at the processing temperature) plasticizers such as polyols (glycerol, sorbitol, glycol, etc.) (Da Róz et al. 2006; Enrione et al. 2010; Forssell et al. 1997; Gaudin et al. 1999; Kirby et al. 1993; Lin and Tung 2009; Liu et al. 2011d; Lourdin et al. 1997; Mathew and Dufresne 2002a; Nashed et al. 2003; Pushpadass and Hanna 2009; Pushpadass et al. 2009; Qiao et al. 2011; Rodriguez-Gonzalez et al. 2004; Shi et al. 2007a, b; Smits et al. 2003; Tan et al. 2004a, b; van Soest et al. 1994, 1996; Wang et al. 2009a; Yan et al. 2012; Zhang et al. 2008), nitrogen-based compounds (urea, ammonium derived, amines, etc.) (Dai et al. 2009b, 2010a, b; Ma and Yu 2004a, b, c; Ma et al. 2004, 2005, 2006; Shogren et al. 1992; Tung et al. 2011; Wang et al. 2009a; Yang et al. 2006a, b, c, 2007; Zhang et al. 2008; Zheng et al. 2009b), or citric acid (Shi et al. 2007b; Wang et al. 2007; Yu et al. 2005a) are utilized. In particular, citric acid has been regarded as an effective plasticizer for starch as the acidity of citric acid can promote the

fragmentation and disruption of starch granules, as well as the effective interactions with the C–O groups of starch (more than glycerol) and thus the restriction of hydrogen bonds between the hydroxyl groups of starch. Therefore, citric acid can result in a greater degree of the amorphous state and a low degree of recrystallization (or "retrogradation," a term specifically for starch) (Yu et al. 2005a).

3.3 Chemical Modification

Chemical modification of starch by substituting ester or ether groups for the hydroxyls is an effective way to improve the processing and product properties. For example, hydroxypropylated starch displays improved processability (lower viscosity) and mechanical properties (Chaudhary et al. 2008; Lafargue et al. 2007; Vorwerg et al. 2004); and acetylated starches with a degree of substitution of 2–3 are water insoluble and have good mechanical properties (Fringant et al. 1996, 1998; Jarowenko 1986; Mbougueng et al. 2012; Volkert et al. 2010; Yan et al. 2012; Zamudio-Flores et al.2010). However, it should be noted that chemical modification often decreases the polysaccharide molar mass, potentially leading to materials with less mechanical strength, and also the toxic chemical residues may modify the biodegradability and negatively impact the life-cycle assessment (LCA) of the final products.

3.4 Starch-Based Blends and Composites

To improve the performance such as moisture resistibility, mechanical properties, and long-term stability, several starch-based multiphase systems have been developed during the last two decades, such as blends or composites. Starch is often blended with other polymers (mainly biodegradable) such as poly(lactic acid) (PLA), polycaprolactone (PCL), poly(butylene succinate adipate) (PBSA), poly (butylene adipate-*co*-terephthalate) (PBAT), poly(vinyl alcohol) (PVA), and many more, as extensively reviewed in several papers (Avérous 2004; Kalambur and Rizvi 2006a; Liu et al. 2009a; Tang and Alavi 2011; Wang et al. 2003; Yu et al. 2006a).

Starch biocomposites can be produced with the reinforcement by cellulose fibers (potato pulp, bleached leafwood fibers, fibers from bleached eucalyptus pulp, and flax and jute fibers) (Alvarez et al. 2004; Averous and Boquillon 2004; Avérous 2002, 2007; Avérous et al. 2001; Carvalho et al. 2003; Curvelo et al. 2001; Dufresne et al. 2000; Funke et al. 1998; Lawton et al. 2004; Matsui et al. 2004; Romhány et al. 2003; Wollerdorfer and Bader 1998) and lignin fillers (Baumberger et al. 1998a, b; Baumberger 2002). When the fillers are nanoscaled, nanocomposites are obtained. They exhibit drastic modification in the properties of the matrix, like improvement in the mechanical properties and barrier properties, and/or changes in the thermal and electrical conductivity (Alexandre and Dubois 2000). Various kinds of starch-based nano-biocomposites will be discussed in the following sections.

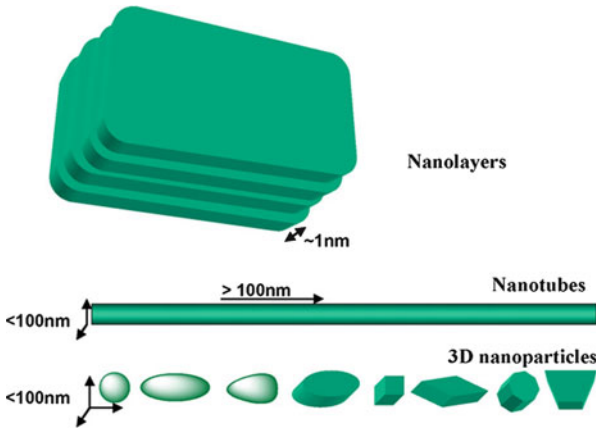

Fig. 5 Various types of nanofillers: nanolayers, nanotubes, and nanoparticles (Reprinted from Kumar et al. (2009), Copyright (2009), with permission from Elsevier)

4 Nanofillers

There are a diversity of nanofillers that can be used for the preparation of nanocomposites. Regarding their shapes, three distinct types of nanofillers can be observed (cf. Fig. 5) (Kumar et al. 2009):

(a) Nanoparticles: When the three dimensions of particulates are in the order of nanometers, they are referred as equiaxed (isodimensional) nanoparticles.
(b) Nanotubes: When two dimensions are in the nanometer scale and the third is larger, forming an elongated structure, they are generally referred as nanotubes, also known as nanowhiskers/nanofibers/nanorods.
(c) Nanolayers: The particulates are characterized by only one dimension in nanometer scale and are present in the form of sheets of one to a few nanometers in thickness and hundred to thousand nanometers in length.

Nevertheless, it should be noted that the term "nanoparticles" is also frequently used in broad sense in the literature to describe a nanofiller regardless of its shape.

The different nanofillers that have been examined for the use in starch-based nano-biocomposites are summarized in Table 1. They have different geometry (size and shape) and surface chemistry; therefore, different kinds of interactions between the nanofiller and the starch matrix and variation in the reinforcing capability are expected. Among them, phyllosilicates, especially montmorillonite (MMT), are most frequently utilized due to their appealing advantages such as wide availability, low cost, and low toxicity. In addition, polysaccharide nanofillers especially cellulose nanowhiskers and starch nanoparticles have also been widely used with one of the advantages in using them being the similar polysaccharide chemical structure of the nanofiller and the matrix, which could benefit the nanofiller–matrix interactions. The use of carbonaceous nanofillers (in particular carbon nanotubes (CNTs)) is also interesting to be incorporated into starch due to their superior reinforcing abilities

Table 1 Overview of the nanofillers used for starch-based nanocomposites

Category/ source	Nanofiller	Dimensional type	Remark
Phyllosilicates			
Clay minerals			
Kaolinite group	Kaolinite	Nanolayer	Non-expandable
	Halloysite	Nanolayer (actually in cylindrical shape)	Non-expandable
Smectite group	Montmorillonite	Nanolayer	Most expandable; most frequently used; natural sodium montmorillonite being a preferable choice due to the matching of polarity with starch
	Hectorite	Nanolayer	Expandable
Sepiolite group	Sepiolite	Nanolayer (actually in needle shape)	–
Synthetic clays	Somasif™ ME 100 fluorohectorite/ fluoromica	Nanolayer	Montmorillonite- or hectorite-type synthetic clay
	Laponite® B, Laponite® RD	Nanolayer	Hectorite-type synthetic clay
Mica group	Muscovite	Nanolayer	Non-expandable
	Paragonite	Nanolayer	Non-expandable
	Illite[a]	Nanolayer	Non-expandable
Polysaccharides			
Cellulose	Nanowhiskers/nanofibrils/ nanofibers from plant cellulose (flax, hemp, ramie, wood, pea hull, cassava bagasse, wheat straw) and microcrystalline cellulose (commercially available)	Nanotube/ nanoparticle	Tend to form aggregates; size, shape, and crystallinity largely dependent on the preparation method and conditions
	Nanorods from tunicin (animal cellulose)	Nanotube	
	Nanofibrils/nanofibers from bacterial cellulose	Nanotube	High crystallinity
Starch	Nanoparticles/nanocrystals from starch (waxy maize and regular maize)	Nanoparticle	Tend to form aggregates
Chitin	Nanoparticles from chitin	Nanoparticle	
Chitosan	Nanoparticles from chitosan	Nanoparticle	
Carbonaceous materials			
	Carbon nanotubes	Nanotube	–
	Graphite oxide	Nanolayer	–
	Carbon black	Nanoparticle	–

(*continued*)

Table 1 (continued)

Category/ source	Nanofiller	Dimensional type	Remark
Metalloid oxides			
	Silicon dioxide (also silica) [SiO_2]	Nanoparticle	–
	Antimony trioxide [Sb_2O_3]	Nanoparticles	–
Metal oxides and chalcogenides			–
	Zinc oxide [ZnO]	Nanoparticle	
	Hydrous zirconium dioxide (also zirconia) [$ZrO_2{\cdot}nH_2O$]	Nanoparticle	–
	Titanium dioxide (also titania) [TiO_2]	Nanoparticle	–
	Cadmium sulfide [CdS]	Nanoparticle	–
	Cadmium selenide [CdSe]	Nanoparticle	–
Metal phosphates			
	α-Zirconium phosphate [$Zr(HPO_4){\cdot}H_2O$]	Nanolayer	–
Layered double hydroxides (LDH)			
	$[M^{II}_{1-x}M^{III}_x(OH)_2]_{intra}[A^{m-}_{x/m}{\cdot}nH_2O]_{inter}$[b]	Nanolayer	–
Nonsilicate minerals			
	Brucite [$Mg(OH)_2$]	Nanolayer	–
	Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]	Nanoparticle	–

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[a]Hydrous mica, considered to belong to the clay–mica group, sometimes categorized to the clay minerals

[b]"M^{II}" and "M^{III}" are metal cations, "A" is the anion, and "intra-" and "inter-" denote the intralayer domain and the interlayer space, respectively

as demonstrated in other polymer systems as well as their potentials for developing nanocomposites with new functionalities (e.g., electrical conductivity and electroactivity), although there have been a limited number of the related reports so far. CNT-reinforced nanocomposites have already shown great potentials in biomedical applications such as sensors, stimulators of bone cells, etc. (Harrison and Atala 2007; Lahiff et al. 2010; Tsai et al. 2007; Wang 2005; Wu and Liao 2007; Xiao and Li 2008), although the toxicity of CNTs appears still controversial (Famá et al. 2011; Valdés et al. 2009). Moreover, despite the long list of nanofillers in Table 1, some only appear in very recent reports, and their potential as nanofillers for reinforcing starch-based materials remains uncertain. For example, unfortunately synthetic nanolayers like LDH and brucite can hardly be intercalated by starch molecules (Wilhelm et al. 2003a), which greatly limited their usability.

Different kinds of starch-based nano-biocomposites could result from the use of a variety of nanofillers, which are discussed in the following sections separately.

5 Starch-Based Nano-biocomposites Reinforced by Phyllosilicates

5.1 Phyllosilicates

Phyllosilicates, or layered silicates, are an important group of minerals that includes the clay minerals, the micas, chlorite, serpentine, talc, etc. They possess some strong advantages such as wide availability, low cost, versatility, eco-friendliness, and low toxicity. They have different structure, texture, and/or morphology. Due to the complexity of the structure, it is no surprise to find the classification of phyllosilicates to be different according to the literature source. Table 1 lists the most commonly used phyllosilicates for starch-based nano-biocomposites in the literature.

Phyllosilicates mainly present three organizational levels depending on the observational scale, i.e., (a) the layer, (b) the primary particle, and (c) the aggregate (cf. Fig. 6) (Chivrac et al. 2009a):

(a) The layer is equivalent to a disk or a platelet with the width varying from 10 nm to 1 μm and the thickness of 1 nm. These layers, especially the widest, are flexible and deformable.
(b) The primary particle is composed of five to ten stacked platelets. The cohesion of the structure is assured by van der Waals and electrostatic attraction forces between the cations and the platelets. The stacking of these particles is perpendicular to the z direction and is disordered in the plane (x, y). The structure thickness is around 10 nm.
(c) The aggregate is the association of primary particles orientated in all the directions. The size of the aggregates varies from 0.1 to 10 μm.

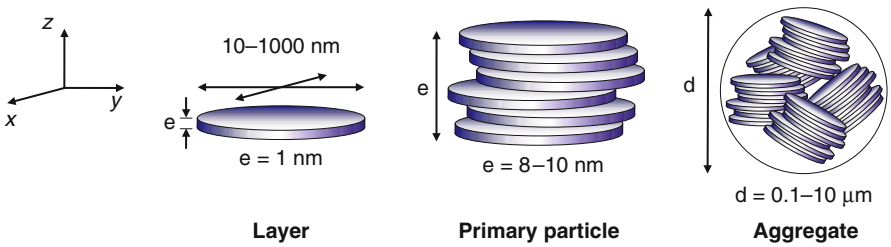


Fig. 6 Phyllosilicate multi-scale structure showing the individual clay platelet, a stack of a few platelets forming the primary particle, and the aggregate structure consisting of associated primary particles with their characteristic dimensions (thickness e and average diameter d) (Reprinted from Chivrac et al. (2009a), Copyright (2009), with permission from Elsevier)

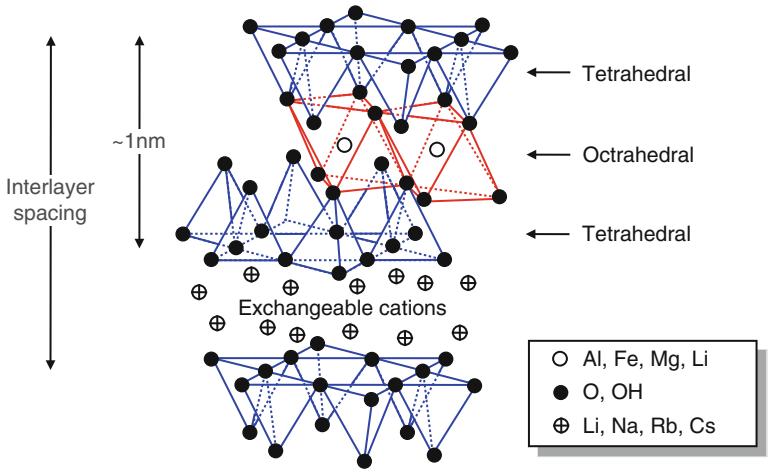


Fig. 7 Structure of 2:1 phyllosilicate (Adapted with permission from Sinha Ray et al. (2003). Copyright (2008) American Chemical Society)

The layer is the result of the condensation of tetrahedral sheets (T) and octahedral sheets (O) (Bergaya et al. 2012). Phyllosilicates are often referred to as 1:1 or 2:1. A 1:1 phyllosilicate would consist of one tetrahedral sheet and one octahedral sheet (T–O), and examples would be kaolinite and serpentine. A 2:1 phyllosilicate consists of an octahedral sheet sandwiched between two tetrahedral sheets (T–O–T), such as the smectite, illite, and chlorite (although chlorite has an external octahedral sheet often referred to as "brucite"). For a 2:1 phyllosilicate, the crystal structure is based on the pyrophyllite structure $Si_4Al_2O_{10}(OH)_2$ and can be described as a crystalline 2:1 phyllosilicate with a central alumina octahedral sheet sandwiched between two silica tetrahedral sheets corresponding to seven atomic layers superposed (cf. Fig. 7) (Hendricks 1942). This structure becomes $(Si_8)(Al_{4-y}Mg_y)O_{20}(OH)_4$, M_y^+ for MMT or $(Si_8)(Al_{6-y}Li_y)O_{20}(OH)_4$, M_y^+ for hectorite. The differences are mainly due to the isomorphic substitutions that take place inside the aluminum oxide layer (Jozja et al. 2003). These substitutions induce a negative charge inside the silicate platelet, which is naturally counterbalanced by inorganic cations (Li^+, Na^+, Ca^{2+}, K^+, Mg^{2+}, etc.) located in the interlayer spacing. The global charge varies depending on the phyllosilicate. For the smectite and the mica families, this charge varies from 0.4 to 1.2 and from two to four per unit cell, respectively. The charge amount is characterized by the cationic exchange capacity (CEC) and corresponds to the amount of monovalent cations necessary to compensate the platelet negative charge, which is usually given in milliequivalent per 100 g (meq/100 g). For instance, the CEC of MMT varies from 70 to 120 meq/100 g depending on their extraction site (Thomas et al. 1999).

The distance observed between two platelets of the primary particle, named interlayer spacing or *d*-spacing (d_{001}), depends on the phyllosilicate type. This value does not entirely depend on the layer crystal structure but also on the type of

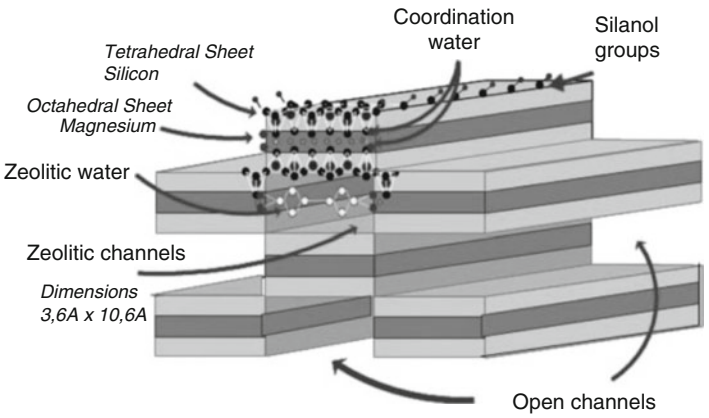


Fig. 8 Structure of sepiolite (Reprinted with permission from Duquesne et al. (2007). Copyright (2007) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim)

the counter-cation and on the hydration state of the phyllosilicate. For instance, the d_{001} equals 1.2 nm in usual conditions, but the value becomes 0.96 nm for anhydrous MMT with sodium as the counterion. This increase is linked to the adsorption of one layer of water molecules between the silicate platelets (Alexandre and Dubois 2000).

Particularly, some phyllosilicates do not display a normal layered structure. Sepiolite is also a phyllosilicate and displays a kind of fibrous structure. In opposition to all the other phyllosilicates mentioned above which have continuous tetrahedral and octahedral sheets, sepiolite has a discontinuous magnesium oxide–hydroxide octahedral sheet between two-dimensional silica tetrahedral sheets that are continuous but exhibit regular inversions of tetrahedron orientation along one crystallographic direction (*b*). In fact, the lath-like fragments of T–O–T structure extend along the *c* axis, being connected by siloxane bridges (Fig. 8) (Bergaya et al. 2012; Duquesne et al. 2007). Some isomorphic substitutions occur inside these central layers creating a negative charge naturally counterbalanced by the presence of inorganic cations (Na^+, Ca^{2+}, etc.). The sepiolite channels are filled both with coordinated water molecules, which are bonded to the Mg^{2+} ions located at the edges of the octahedral sheets, and with zeolitic water, which is associated to the structure by hydrogen bonding. The discontinuity of the silica sheets gives rise to the presence of silanol groups (Si–OH) at the edges of the external surfaces of sepiolite nanoparticles (Chivrac et al. 2010d). Another example is halloysite, which has a structure similar to kaolinite but has a single layer of water molecules existing between the T–O sheets. It has structural constraints in the layers which cause them to curl and forms spheroidal aggregates (Bergaya et al. 2012).

The phyllosilicate multi-scale structure has different porosity levels, which drive its swelling ability. The water absorption occurs, thanks to the intercalated cation hydration, which lowers the attractive forces between the silicate layers (Sposito and Grasso 1999), and also thanks to the water capillarity phenomenon, which takes place in the interparticle and inter-aggregate porosities (Luckham and Rossi 1999; Mering 1946).

For a given pressure, this swelling is characterized by a d_{001} increase until an equilibrium distance (Cases et al. 1992). In general, the smaller the cation and the lower its charge, the higher is the phyllosilicate swelling. For MMT, the swelling decreases depending on the cation chemical type according to the trend $Li^+ > Na^+ > Ca^{2+} > Fe^{2+} > K^+$ (Powell et al. 1997, 1998; Tettenhorst 1962). The potassium cation is a specific case because its size is equal to the dimension of the platelet surface cavity. Thus, the potassium is trapped in these cavities, leading to the reduced hydration ability.

Swelling is typical enough of smectites to have provided their alternative name of "swelling clays" (Bergaya et al. 2012). In contrast, the other natural phyllosilicates lack the ability to swell in water, which greatly limits their application in the development of starch-based nano-biocomposites.

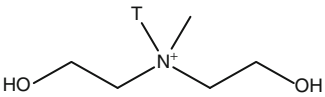
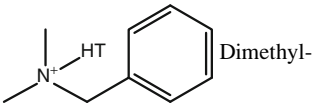
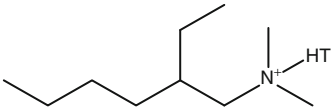
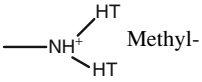
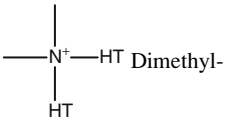
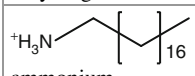
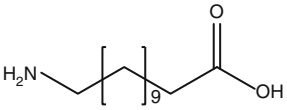
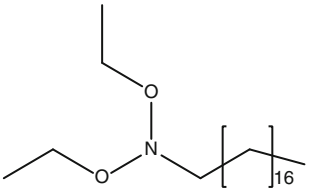
To enhance the intercalation/exfoliation process (which will be discussed in Sect. 5.2) in a polymer matrix, chemical modification of the phyllosilicate surface, with the aim to match the polymer polarity, is often carried out (Alexandre and Dubois 2000; Sinha Ray and Okamoto 2003). Cationic exchange is the most common technique, but other original techniques such as organosilane grafting (Dai and Huang 1999; Ke et al. 2000), the use of ionomers (Lagaly 1999; Shen et al. 2002), and block copolymers adsorption (Fischer et al. 1999) are also used.

The cationic exchange involves the substitution of inorganic cations by organic ones. These cations are often alkylammonium surfactants having at least one long alkyl chain. Phosphonium salts are also interesting silicate modifiers, thanks to their higher thermal stability, but they are not often used (Wilkie et al. 2001). The ionic substitution is performed in water because of the phyllosilicate swelling which facilitates the organic cation insertion between the platelets. Then, the solution is filtered, washed with distilled water (to remove the salt formed during the surfactant adsorption and the surfactant excess), and lyophilized to obtain the organomodified phyllosilicate. In addition to the modification of the phyllosilicate surface polarity, organomodification increases the d_{001}, which will also facilitate the polymer chain intercalation (Lagaly 1986). Using this technique, various organomodified MMTs (OMMTs) and hectorites used for the fabrication of starch-based nano-biocomposites (cf. Table 2) can be produced, which mainly differ in the counter-cation nature and the CEC. It is noteworthy that, in contrast to most synthetic polymers which are hydrophobic, starch possesses the nature of being highly hydrophilic as previously mentioned (cf. Sect. 2.1); however, most conventional organomodifiers increase the hydrophobicity of the phyllosilicate. Consequently, the compatibility with the starch matrix could be reduced using most of the commercial OMMT presented in Table 2. Thus, it is one of the key points to consider for the development of phyllosilicate-reinforced starch-based nano-biocomposites.

5.2 Nanocomposite Structures

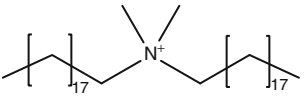
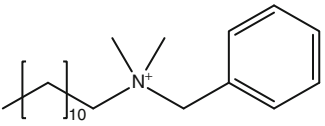
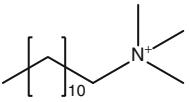
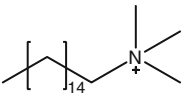
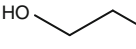
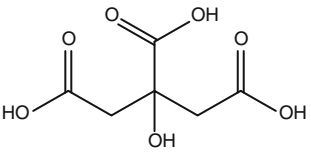
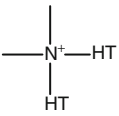
Depending on the processing conditions and the matrix–nanolayer affinity, different structures can be obtained when a phyllosilicate (except sepiolite and halloysite) is associated with a polymer (cf. Fig. 9) (Alexandre and Dubois 2000; Avérous and

Table 2 Unmodified and organomodified smectite group clays used for starch-based nanocomposites with the corresponding chemical structures of counterions and their commercial trade names (Reprinted from Xie et al. (2013), Copyright (2013), with permission from Elsevier)

Clay type	Counter-cation	Name
Montmorillonite (MMT)	Na^+	Natural sodium MMT; MMT–Na^+; Cloisite® Na^+; Dellite® LVF; Dellite® HPS; Nanofil® 757; BH Natural
	 Methyl-tallow-bis-2-hydroxyethyl ammonium	Cloisite® 30B
	 Dimethyl-benzyl-hydrogenated tallow ammonium	Cloisite® 10A; Bentone® 111; Dellite® 43B
	 Dimethyl-hydrogenated tallow-2-ethylhexyl ammonium	Cloisite® 25A
	 Methyl-dihydrogenated tallow ammonium	Cloisite® 93A
	 Dimethyl-dihydrogenated-tallow ammonium	Cloisite® 20A; Cloisite® 15A; Cloisite® 6A; Dellite® 67G; Dellite® 72 T
	 Octadecyl ammonium	Nanomer® I.30E
	 Aminododecanoic acid	Nanofil® 784
	 Stearyl dihydroxyethyl ammonium	Nanofil® 804

(*continued*)

Table 2 (continued)

Clay type	Counter-cation	Name
	 Distearyl dimethyl ammonium chloride	Nanofil® 948
	 Dodecyl benzyl dimethyl ammonium bromide	–
	 trimethyl ammonium	–
	 trimethyl ammonium	–
	Cationic starch	–
	 Ethanolamine	–
	 Citric acid	–
Hectorite	Ca^{2+}	Natural calcium hectorite; Bentone EA-163
	 dihydrogenated-tallow ammonium	Bentone 109

Cloisite® is a trademark of South Clay Products, Inc. (USA); Nanomer® is a trademark of Nanocor, Inc.; Dellite® is a trademark of Laviosa Chimica Mineraria, S.p.A. (Italy); Nanofil® is a trademark of Süd Chemie AG (Germany); BH Natural is a product from Black Hills Bentonite LLC (USA); Bentone® is a trademark of Elementis Specialties (USA); Nanomer® is a trademark of Nanocor, Inc. (USA)

The surface hydrophobicity of Cloisite clays: $Na^+ < 30B < 10A < 25A < 93A < 20A < 15A$

T tallow (≈65 % C18, ≈30 % C16, ≈5 % C14), *HT* hydrogenated tallow

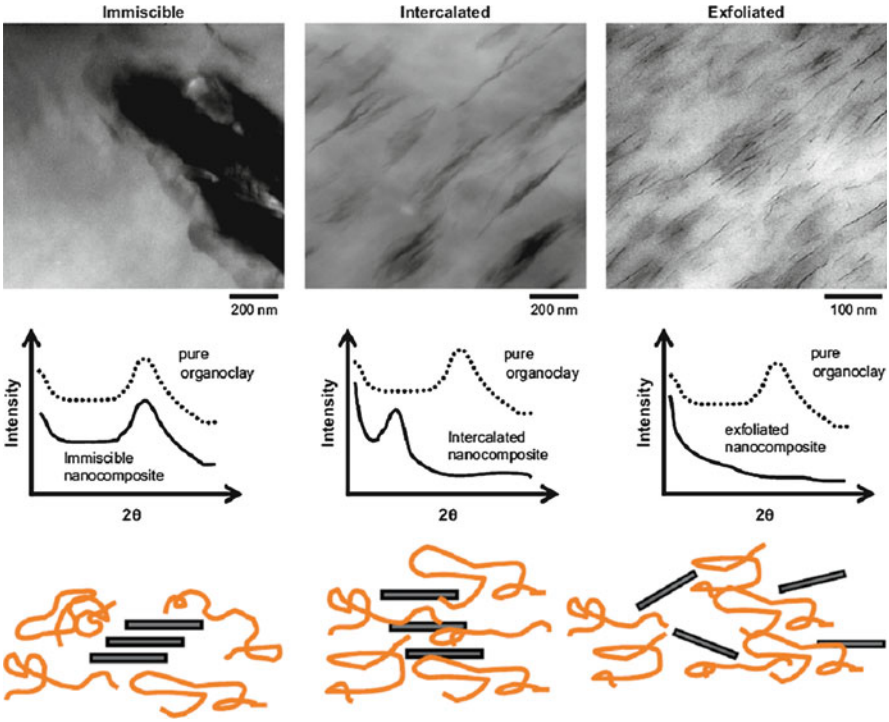


Fig. 9 Illustration of different states of dispersion of phyllosilicate in polymers with the corresponding typical results of XRD and TEM. (In this copyrighted material, the word "organoclay" could be appropriately replaced by "phyllosilicate" in the context of the current chapter) (Reprinted from Paul and Robeson (2008), Copyright (2008), with permission from Elsevier)

Halley 2009; Kumar et al. 2009; Paul and Robeson 2008; Sinha Ray et al. 2003; Sinha Ray and Okamoto 2003; Sinha Ray and Bousmina 2005; Tang et al. 2008b; Vaia and Giannelis 1997):

(a) *Phase-separated composite (microcomposite)*: the polymer chains have not penetrated into the phyllosilicate interlayer spaces, and the silicate layers are mainly stacked and aggregated. The aggregates are micron sized, and the corresponding behavior is close to a conventional composite. (In this case, the nanocomposite designation is actually incorrect.)
(b) *Intercalated nanocomposite*: the polymer diffusion into the phyllosilicate interlayer spaces leads to an increase in the d_{001}. This structure shows regularly alternating silicate layers and polymer chains.
(c) *Exfoliated nanocomposite*: the silicate layers are individually delaminated and homogeneously and fully dispersed into the matrix. (In some papers, this category is further divided into oriented–exfoliated and unoriented–exfoliated systems.)

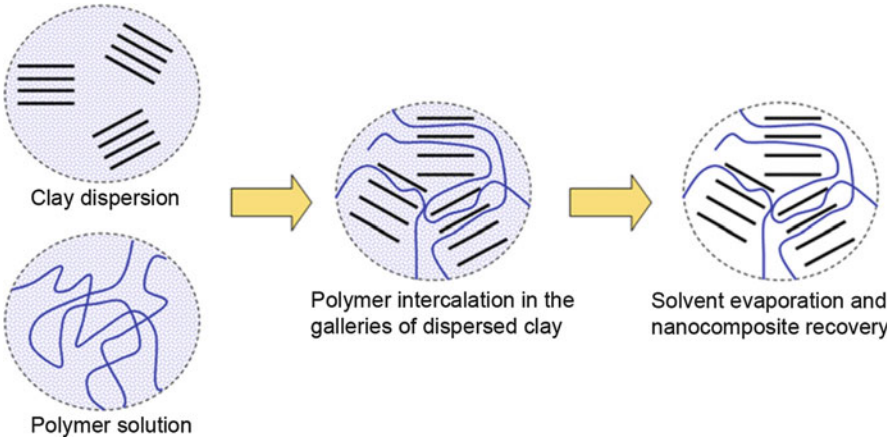


Fig. 10 Schematic representation of polymer–phyllosilicate nanocomposites obtained by intercalation from solution (Reprinted from Pavlidou and Papaspyrides (2008), Copyright (2008), with permission from Elsevier)

5.3 Preparation Techniques

Normally, the incorporation of phyllosilicate nanolayers into a polymer matrix can be carried out with one of the three main techniques, i.e., (a) solution intercalation, (b) in situ intercalative polymerization, or (c) melt intercalation (Alexandre and Dubois 2000; Giannelis 1996; Mallapragada and Narasimhan 2006; Sinha Ray and Okamoto 2003; Sinha Ray and Bousmina 2005). It should be noted that these techniques might not be fully applicable to sepiolite and halloysite due to special structures as previously mentioned.

Solution intercalation process is based on a solvent system in which the polymer is soluble and the phyllosilicate is swellable and dispersible. The swelling of phyllosilicates is crucial for nanocomposite structure development, and thus swellable phyllosilicates (mainly smectite group clays) are preferably used (cf. Table 1). Starch, as the matrix, is first dissolved in an appropriate solvent. Water is often used since starch is usually gelatinized in water. In parallel, the phyllosilicate is swollen in the same or another solvent such as water, chloroform, or toluene to obtain a miscible solution. Sonication may be employed to assist the swelling and dispersion process. When the solution of starch and that of swollen phyllosilicate are mixed, the polymer chains intercalate and may replace the solvent molecules within the interlayer spaces of the aggregated phyllosilicate nanoparticles. Upon solvent removal, the intercalated structure remains, resulting in nanocomposite formation. Figure 10 is a schematic representation of solution intercalation for fabricating polymer–phyllosilicate nanocomposites (Pavlidou and Papaspyrides 2008).

For the preparation of starch–phyllosilicate nano-biocomposites, it is important to minimize the intercalation of the plasticizer (glycerol) in the phyllosilicates

(which will be detailed in Sect. 5.6). Regarding this, the mixing order of different ingredients (starch, nanofiller, and plasticizer) in solution processing could have some effect on the intercalation/exfoliation of silicate layers and thus the properties of the resulting nano-biocomposites (Pandey and Singh 2005). For this sample reason, Chung et al. (2010) introduced a new way to prepare well-dispersed starch–phyllosilicate nano-biocomposites by adding a dilute phyllosilicate dispersion to a solution of starch followed by coprecipitation in ethanol.

In situ polymerization is often used to make conventional nanocomposites. In this case, a phyllosilicate is swollen in a monomer solution, and then the monomer polymerization takes place, leading to a d_{001} increase, till in some cases a fully exfoliated morphology (Sinha Ray and Okamoto 2003). Since starch molecular chains are synthesized during plant growth and then extracted from the vegetal, in situ polymerization is limited to nano-biocomposites with chemically modified starch such as starch graft copolymer, which is synthesized in situ with a phyllosilicate (Al et al. 2008; Luo et al. 2005; Namazi et al. 2009; Wu et al. 2000; Zhou et al. 2011).

Compared with solution intercalation, melt intercalation has strong advantages such as the absence of solvents and the compatibility with current industrial polymer processing techniques, such as extrusion and injection molding. As a result, melt intercalation has often been used and described in the literature to produce starch–phyllosilicate nano-biocomposites.

Melt intercalation involves processing a mixture of a polymer with a phyllosilicate in a melt processing unit (e.g., extruder or internal mixer). During processing, the chains diffuse between the aggregated silicate layers to produce a (nano)structured system that is controlled by the processing conditions such as temperature, shearing, and residence time in the processing equipment. Shearing is necessary to induce platelet delamination from the phyllosilicate tactoids, and extended residence time is needed to allow the polymer chains to diffuse into the interlayer spaces and then to obtain exfoliated morphology (Dennis et al. 2001). Fornes et al. (2001) proposed a stepwise mechanism for describing the phyllosilicate dispersion and exfoliation under shear stress during melt processing (cf. Fig. 11). However, it should be noted that the strong shear and long residence time would also contribute to the degradation of the starch matrix. Therefore, it is necessary to balance the processing parameters to minimize the polymer chain degradation and to obtain a kind of well-exfoliated morphology.

More related to starch–phyllosilicate nano-biocomposites, Zhang et al. (2007) proposed a schematic depicting the main force components operating on a pair of adjacent silicate layers in melt processing (Fig. 12). The van der Waals force and the electrostatic attractive force act against silicate layer exfoliation, while the shearing force and the elastic force arising with starch molecular intercalation favor silicate layer exfoliation (Zhang et al. 2007). Both the molar mass of the polymer and the polar interactions between the phyllosilicate and the polymer could influence the polymer intercalation (Vaia and Giannelis 1997).

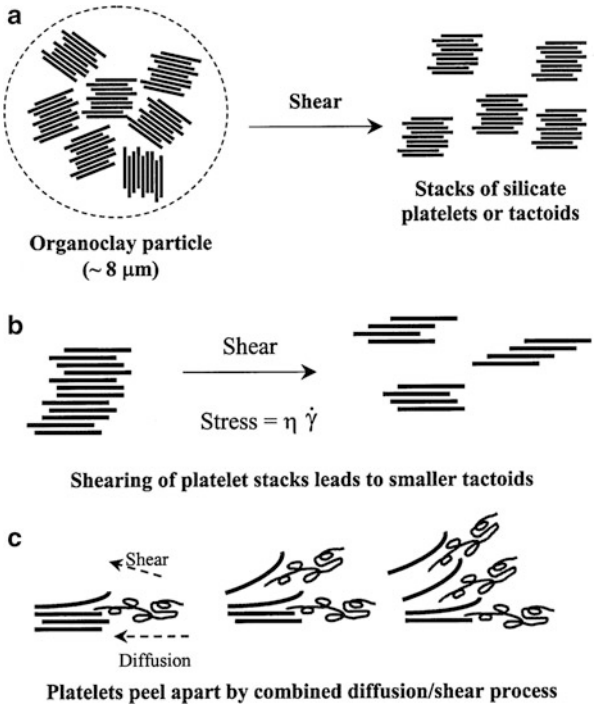


Fig. 11 Stepwise mechanism of phyllosilicate platelet exfoliation in the melt compounding of nanocomposites: (**a**) organoclay particle breakup, (**b**) clay tactoid breakup, and **c** platelet exfoliation. (In this copyrighted material, the word "organoclay" could be more appropriately replaced by "phyllosilicate" in accordance with the context of the current paper) (Reprinted from Fornes et al. (2001), Copyright (2001), with permission from Elsevier)

5.4 Effect of Phyllosilicate Addition

Starch-based systems reinforced by phyllosilicates normally exhibit increases in tensile strength (Chivrac et al. 2008b, 2010d; Chung et al. 2010; Cyras et al. 2008; Dai et al. 2009a; de Carvalho et al. 2001; Gao et al. 2012; Hassan Nejad et al. 2011; He et al. 2012; Huang et al. 2004, 2005a, b, 2006; Huang and Yu 2006; Ibrahim 2011; Kvien et al. 2007; Lilichenko et al. 2008; Ma et al. 2007b; Majdzadeh-Ardakani et al. 2010; Maksimov et al. 2009; Mondragón et al. 2008; Müller et al. 2011; Park et al. 2002, 2003; Qiao et al. 2005; Ren et al. 2009; Tang et al. 2008b, 2008c; Wang et al. 2009b, 2010b; Xie et al. 2011), Young's modulus (Chaudhary 2008; Chen and Evans 2005; Chivrac et al. 2009b, 2010c, d; Chung et al. 2010; Cyras et al. 2008; de Carvalho et al. 2001; Hassan Nejad et al. 2011; He et al. 2012; Huang et al. 2004, 2005a, b; Huang and Yu 2006; Lilichenko et al. 2008; Ma et al. 2007b; Majdzadeh-Ardakani et al. 2010; Maksimov et al. 2009; Mondragón et al. 2008; Müller et al. 2011; Nejad et al. 2010; Pandey and Singh 2005; Ren et al. 2009; Wang et al. 2010b; Wilhelm et al. 2003b), storage modulus (measured by dynamic mechanical analysis [DMA]) (Kvien et al. 2007; Park et al. 2003; Wilhelm et al. 2003a, b), T_g (Chivrac et al. 2010a, c; Huang et al. 2006; Kvien et al. 2007; Nejad et al. 2010; Park et al. 2003; Wang et al. 2009d; Wilhelm et al. 2003b; Xiong et al. 2008; Xu et al. 2005), thermal stability (Chiou et al. 2007; Chivrac et al. 2010d; Cyras et al. 2008; Dai et al. 2009a;

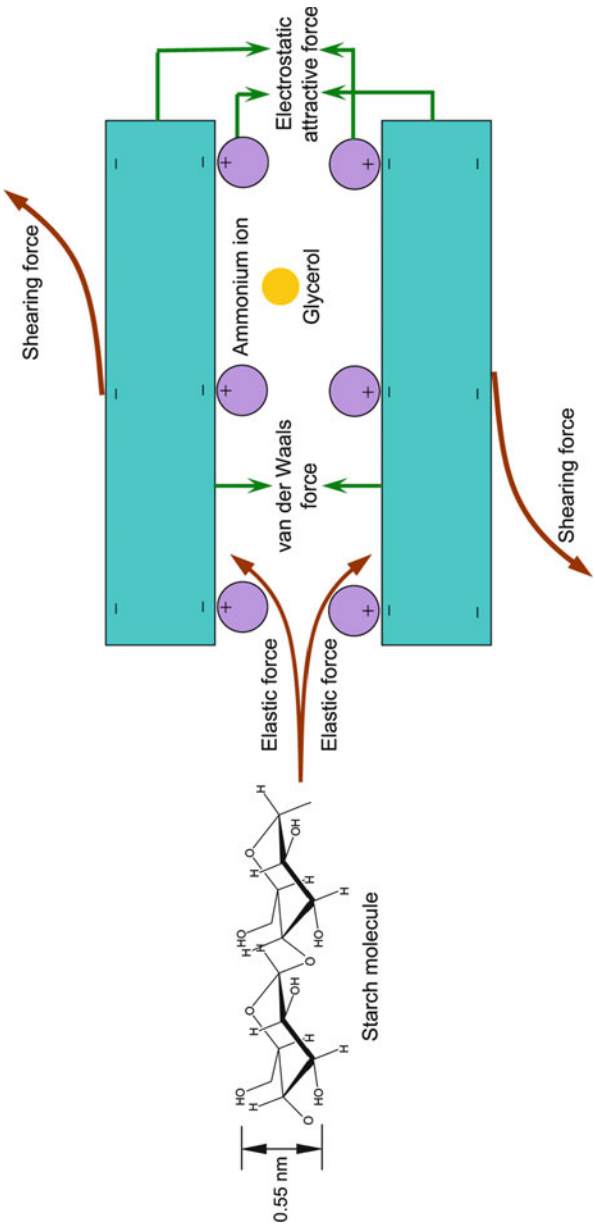


Fig. 12 Schematic representation of the intercalation process between starch and organomodified phyllosilicate (Reprinted from Zhang et al. (2007), Copyright (2007), with permission from Elsevier)

Huang et al. 2004, 2005a, b; Huang and Yu 2006; Ma et al. 2007b; Namazi et al. 2009; Pandey and Singh 2005; Park et al. 2002, 2003; Wang et al. 2009d; Wilhelm et al. 2003a, b; Xu et al. 2005; Zhou et al. 2011), moisture resistance (i.e., moisture uptake, water vapor permeability [WVP], etc.) (Chiou et al. 2006, 2007; Chivrac et al. 2010a; Cyras et al. 2008; Dai et al. 2009a; de Carvalho et al. 2001; Gao et al. 2012; Huang et al. 2004, 2005a; Huang and Yu 2006; Ibrahim 2011; Lilichenko et al. 2008; Maksimov et al. 2009; Müller et al. 2011; Pandey and Singh 2005; Park et al. 2002, 2003; Tang et al. 2008b, c; Wang et al. 2009b, 2010b), oxygen barrier property (Zeppa et al. 2009), and biodegradation rate (Magalhães and Andrade 2009), generally meaning improved performance, even though the elongation at break was observed to be reduced in most studies (Chivrac et al. 2008b, 2010c; Chung et al. 2010; Cyras et al. 2008; Dai et al. 2009a; de Carvalho et al. 2001; Hassan Nejad et al. 2011; Huang et al. 2004, 2005a, b; Ibrahim 2011; Ma et al. 2007b; Majdzadeh-Ardakani et al. 2010; Maksimov et al. 2009; Pandey and Singh 2005; Park et al. 2002, 2003; Qiao et al. 2005; Ren et al. 2009; Tang et al. 2008b, c; Wang et al. 2009b; Wilhelm et al. 2003b). While these changes could usually be ascribed to the structural reasons such as (a) the homogeneous dispersion of silicate layers in the starch matrix (Chivrac et al. 2010d; Chung et al. 2010; de Carvalho et al. 2001; Huang et al. 2005a, 2006 Huang and Yu 2006; Ibrahim 2011; Kampeerapappun et al. 2007; Kvien et al. 2007; Ma et al. 2007b; Majdzadeh-Ardakani et al. 2010; Nejad et al. 2010; Wang et al. 2009b, 2010b; Zhang et al. 2007), (b) the strong interactions (typically by hydrogen bonding) between the silicate nanofiller and the starch matrix (Chivrac et al. 2008b, 2010d; Cyras et al. 2008; Dai et al. 2009a; Huang et al. 2004; Ma et al. 2007b; Majdzadeh-Ardakani et al. 2010; Park et al. 2003; Ren et al. 2009; Tang et al. 2008c; Wang et al. 2009b, 2010b), and (c) the high aspect (width-to-thickness) ratio and thus the vast exposed surface of the silicate layers (Cyras et al. 2008; Huang and Yu 2006; Ibrahim 2011), the detailed mechanisms regarding the changes in different properties are summarized below from the literature:

- Glass transition: an increase in the T_g can be a result of the restriction of chain mobility due to nanofiller–polysaccharide interactions (Kvien et al. 2007; Xu et al. 2005).
- Mechanical properties: increases in Young's modulus, storage modulus (DMA), and tensile strength can be ascribed to (a) the facilitation of stress transfer from the starch matrix to the nanofiller as a result of the strong nanofiller–matrix interactions (Chivrac et al. 2008a; Chung et al. 2010; Tang et al. 2008c); (b) the formation of a physical cross-linking network as a result of the high nanofiller–matrix interactions, which strengthens the material through mechanical percolation (Park et al. 2003; Ren et al. 2009); and (c) the stretching resistance of the oriented backbones of the chains in the interlayer spaces (Cyras et al. 2008). Besides, a decrease in the elongation at break can be attributed to the decreased flexibility of the starch molecules in the presence of the nanofiller (Cyras et al. 2008; Ren et al. 2009; Tang et al. 2008c).

- Moisture resistance: the homogeneous dispersion of high-aspect-ratio silicate layers (a) introduces tortuous and thus longer pathways through the starch polymer matrix for the diffusion of water molecules (Cyras et al. 2008; Dai et al. 2009a; Ibrahim 2011; Lilichenko et al. 2008; Maksimov et al. 2009; Mondragón et al. 2008; Park et al. 2003; Tang et al. 2008c; Wang et al. 2009b, 2010b) and also (b) causes the shielding of the exposed water-sensitive hydroxyl groups of the starch (Müller et al. 2011; Wang et al. 2009b, 2010b), both of which contribute to a reduction in the moisture sensitivity.
- Oxygen barrier property: a higher oxygen barrier can be ascribed to the silicate layers inducing a higher tortuosity and longer diffusion pathways for oxygen molecules (Lilichenko et al. 2008; Maksimov et al. 2009; Zeppa et al. 2009).
- Thermal stability: improvement in the thermal stability can be due to several concomitant phenomena such as (a) the higher thermal stability of the inorganic phase (phyllosilicate) compared to the organic one (starch), (b) the phyllosilicate acting as a heat barrier which enhances the overall stability (Cyras et al. 2008; Ibrahim 2011), (c) the increase in the tortuosity of the diffusion pathways for oxygen and the combustion (pyrolysis) gas (Alexandre and Dubois 2000; Chiou et al. 2007; Chivrac et al. 2010d; Cyras et al. 2008; Namazi et al. 2009; Xu et al. 2005), and (d) the reorganization of the starch structure with less exposed hydroxyl groups and thus less susceptibility to degradation (Pandey and Singh 2005; Wang et al. 2010b; Wilhelm et al. 2003b).

In spite of the changes in the properties mentioned above, increased elongation at break (Chivrac et al. 2010d; Chung et al. 2010; Huang and Yu 2006; Huang et al. 2006; Kvien et al. 2007; Pandey and Singh 2005), decreased thermal stability (Magalhães and Andrade 2010), unchanged thermal stability (Qiao et al. 2005), decreased T_g (de Carvalho et al. 2001; Gao et al. 2012), and unchanged moisture resistance (Chivrac et al. 2010c) have also been observed in certain cases. Majdzadeh-Ardakani et al. (2010) found that the elongation at break first decreased with the phyllosilicate addition level of up to 6 %, while further increasing the nanofiller content causes an increase in the elongation at break. Some authors reported that the phyllosilicate addition reduced the melting temperature of glycerol-plasticized starch-based nano-biocomposites (Liu et al. 2011c), but increased that of sorbitol-plasticized starch-based nano-biocomposites (Chaudhary et al. 2011a; Kvien et al. 2007). Furthermore, breaking energy could either be increased (Huang et al. 2004, 2005a, b; Huang and Yu 2006; Ma et al. 2007b; Wang et al. 2010b) or decreased (Chivrac et al. 2010c; Huang et al. 2004) with the addition of a phyllosilicate. To address these discrepancies, it is useful to consider the crystalline structures in the materials as influenced by the nanofillers. Crystallinity and/or crystal size could affect the biodegradation (Magalhães and Andrade 2009), mechanical properties (Chivrac et al. 2010c, d; Chung et al. 2010; Majdzadeh-Ardakani et al. 2010), and moisture resistance (Cyras et al. 2008). Most typically, a higher crystallinity could embrittle nanocomposite materials with lower elongation at break values (Chivrac et al. 2010c, 2010d; Majdzadeh-Ardakani et al. 2010). Nevertheless, the literature shows that the recrystallization of

a starch-based material could either be restrained (Chung et al. 2010; Huang et al. 2004, 2005a; Liu et al. 2011c; Magalhães and Andrade 2009, 2010; Ren et al. 2009; Wang et al. 2009b; Zhang et al. 2007), unchanged (Zeppa et al. 2009), or enhanced (Chaudhary 2008; Müller et al. 2011) with the addition of a phyllosilicate.

5.5 Effects of Phyllosilicate Type and Content

Table 2 highlights various natural and organomodified MMT nanofillers that have been utilized in starch-based nano-biocomposite systems. It is important to note that the surface hydrophobicity of Cloisite MMT nanofillers follows the order $Na^+ <$ 30B $<$ 10A $<$ 25A $<$ 93A $<$ 20A $<$ 15A $<$ 6A. Although OMMT could provide much greater d_{001} than natural sodium MMT (MMT–Na^+ or Cloisite Na^+) to possibly facilitate starch molecular intercalation, it has been demonstrated that the structure of the resulting composites more highly depends on the hydrophilicity of MMT (Zhang et al. 2007). It has been shown that the incorporation of hydrophobic OMMT nanofillers such as Cloisite 15A, Cloisite 6A, Cloisite 10A, Nanomer I.30E, etc. (cf. Table 2 for designations and corresponding chemical structures) led to the formation of microcomposites (Chiou et al. 2005; Park et al. 2002; Tang et al. 2008c), as evidenced by the unchanged values of the d_{001}. When Cloisite 30B, a more hydrophilic OMMT, was utilized, higher d_{001} values were obtained, with a slight d_{001} shift and a strong decrease in the diffraction peak intensity, corresponding to higher dispersion (Chen and Evans 2005; Chiou et al. 2005, 2006; Park et al. 2003). Exfoliated nanocomposites have also been produced with MMT–Na^+ due to the more hydrophilic character of the Na^+-based nanofiller, which makes it more compatible with plasticized starch (Chen and Evans 2005; Chen et al. 2005; Chiou et al. 2005, 2006, 2007; Cyras et al. 2008; Huang et al. 2004; Pandey and Singh 2005; Park et al. 2002, 2003; Tang et al. 2008c; Zeppa et al. 2009; Zhang et al. 2007). Uniform dispersion of the MMT in the plasticized starch can be achieved in this case due to the polar interactions, especially hydrogen bonds formed between the hydroxyl groups of the MMT and the hydroxyl groups of the starch molecules (Huang et al. 2004; Park et al. 2002, 2003; Tang et al. 2008c).

Chiou et al. (2005) investigated the rheology during the solution processing of starch biocomposites filled by different MMT nanofillers. They demonstrated that the paste with Cloisite Na^+ displayed a larger increase in the rheological elastic modulus (G') at the temperature reaching 95 °C due to an intercalation process during the starch gelatinization, while the samples with the more hydrophobic MMT nanofillers had comparable modulus values to the neat starch sample (Chiou et al. 2005). More importantly, the high compatibility of MMT–Na^+ with a starch matrix and corresponding good dispersion could result in improved properties (as previously mentioned in Sect. 5.4) compared with those of other starch–OMMT hybrids (Park et al. 2002, 2003). In addition, these last authors also showed that MMT–Na^+ could shift the T_g of the starch-rich phase and the β-transition of the glycerol-rich phase of the starch matrix to higher temperatures (from 7 °C to 12 °C and from −64 to −53 °C, respectively), whereas the two relaxation temperatures were decreased for the other

starch–OMMT hybrids (Park et al. 2002, 2003). These results were clearly attributed to the matching of the surface polarity of and the interactions between the Cloisite Na^+ and the starch (Park et al. 2002). Moreover, Zhang et al. (2007) showed that while a starch-based biocomposite reinforced by MMT–Na^+ has a higher thermal decomposition temperature, that reinforced by Cloisite 93A displayed a reduced thermal stability, which was ascribed to the earlier decomposition of the organic alkylammonium on the clay. However, Magalhães et al. (Magalhães and Andrade 2010) proposed that the catalytic effect of acidic sites of MMT may also reduce the thermal stability of starch.

Though OMMT is normally less capable than natural MMT–Na^+ to bring about the nanostructure of starch-based biocomposites, some other reasons may account for the desired property improvement of starch–OMMT hybrids. For example, by water absorbance tests, Chiou et al. (2006) found that, while a MMT–Na^+ sample containing the most dispersed (exfoliated) nanoclays had the lowest water uptake, a Cloisite 30B sample also absorbed less water than the MMT–Na^+ sample containing intercalated clay. This might be due to Cloisite 30B being more hydrophobic than MMT–Na^+ (Chiou et al. 2006). Actually, for understanding the moisture sensitivity of a nanocomposite, it could be better to consider the water uptake capacity of each component (nanofiller, matrix, and plasticizer), especially when a hydrophilic nanofiller like MMT–Na^+ is used (Mondragón et al. 2008; Zeppa et al. 2009). However, proper organomodification of MMT may display even better reinforcing ability than natural MMT–Na^+. As reported by Qiao et al. (2005), the use of OMMT based on trimethyl dodecyl ammonium with acetylated starch matrix could result in a nano-biocomposite with higher tensile strength and storage modulus than the sample reinforced by MMT–Na^+. This was attributed to the higher dispersion and d_{001} of the OMMT in the acetylated starch matrix (Qiao et al. 2005). Remarkably, Chivrac et al. (2008b, 2009b, 2010a, b, c; de Carvalho et al. 2001) used cationic starch as a new phyllosilicate organomodifier to better match the polarity of a starch matrix and thus to facilitate phyllosilicate exfoliation process. The morphological analyses (X-ray diffraction [XRD] and transmission electron microscopy [TEM]) showed that MMT organomodified by cationic starch (OMMT–CS) (which can be prepared either in solution with ultrasonication or in a mixer with strong shear treatment, with the latter resulting in greater delamination of the clay platelets) allowed the preparation of well-exfoliated nano-biocomposites, compared to natural MMT–Na^+, which only led to the formation of intercalated nano-biocomposites (Chivrac et al. 2008b). As a result, the OMMT–CS could lead to greater stiffness without affecting the elongation at break. Such a behavior is not observed for most other starch-based nano-biocomposites reinforced by MMT–Na^+ (Chivrac et al. 2008b, 2009b).

Although Tang et al. (2008c) reported that the MMT content did not have any significant effect on the occurrence of intercalation or exfoliation as observed by XRD, it is worth noting that there can be an optimized level of MMT addition for the greatest improvement in properties such as mechanical properties (Huang and Yu 2006; Huang et al. 2006; Kampeerapappun et al. 2007; Mondragón et al. 2008; Park et al. 2003; Tang et al. 2008c), moisture barrier property (Chiou et al. 2007; Mondragón et al. 2008), and thermal stabilities (Park et al. 2003). A higher content of MMT might contribute to aggregates and stacks of MMT in a starch matrix

(Chung et al. 2010; Huang et al. 2004; Kampeerapappun et al. 2007; Majdzadeh-Ardakani et al. 2010; Park et al. 2003) and also to lower plasticization of the starch phase (Wang et al. 2009b).

Other clays (natural or synthetic) such as hectorite (Chen and Evans 2005; Kvien et al. 2007; Wilhelm et al. 2003a, b) and kaolinite (Chen and Evans 2005; de Carvalho et al. 2001; Wilhelm et al. 2003a) have also been incorporated into starch. As a non-swelling clay, kaolinite can hardly generate intercalation/exfoliation, and thus increases in the tensile strength and Young's modulus and a decrease in the WVP could be, though observed, limited (de Carvalho et al. 2001). A recent study reported that kaolinite could be intercalated by dimethyl sulfoxide (DMSO) which is an aprotic polar molecule, and thus the dispersion of kaolinite could be enhanced (Mbey et al. 2012). However, DMSO as a toxic solvent may cause health and environmental problems. Although natural hectorite might perform better than kaolinite for the formation of a nanocomposite structure (as shown by the increasing d_{001}) (Chen and Evans 2005; Wilhelm et al. 2003a), organomodified hectorite (Bentone 109) could only result in conventional composite like kaolinite (Chen and Evans 2005). Again, this can be explained by the dominant role of the hydrophilicity of a phyllosilicate in determining the structure of the resulting biocomposite. In addition, the CEC of nanofillers may also account for the alteration of some properties (Dean et al. 2007). For example, the CEC of MMT–Na^+ is 92 meq/100 g, whereas the CEC of fluoromica (FHT–Na^+) is only 70–80 meq/100 g. A larger CEC could contribute to a stronger water retention property which could influence the mechanical properties (Dean et al. 2007).

Chivrac et al. (2010d) initiated the use of sepiolite to reinforce starch-based materials and found the reinforcing effect of sepiolite was even better than that of MMT, as evidenced by the higher Young's modulus, elongation at break, and breaking tensile strength values, which was ascribed to the stronger interactions established between the sepiolite nanofiller and the polysaccharide chains. Nevertheless, when sepiolite was modified by cationic starch for better interactions with starch, the thermal stability of the nano-biocomposites could be deteriorated, due to the fast thermal decomposition of cationic starch (Chivrac et al. 2010d).

Halloysite, a hydrated kaolinite, has recently been tried to be incorporated into starch, but the mechanical strength of the resulting materials was not high enough to apply for packaging (Xie et al. 2011). This is because halloysite nanotubes are easily aggregated owing to its large specific areas and polar functional groups, and this causes halloysite nanotubes poorly dispersed in a polymer matrix. It was reported that this problem could be solved by ball-milling treatment together with polyethylene glycol (PEG) as the dispersing agent and water–ethanol mixture as the medium. PEG also improved the compatibility between the nanofiller and the starch phase, resulting improved tensile strength and Young's modulus (He et al. 2012).

5.6 Effect of Plasticizers/Additives

Glycerol has been a widely used plasticizer in the preparation of starch–phyllosilicate nano-biocomposites (Bagdi et al. 2006; Chen and Evans 2005;

Chiou et al. 2007; Chivrac et al. 2008a, b, 2009b, 2010a, b, c, d; Chung et al. 2010; Cyras et al. 2008; de Carvalho et al. 2001; Huang et al. 2004; Ibrahim 2011; Kampeerapappun et al. 2007; Lilichenko et al. 2008; Liu et al. 2011c; Magalhães and Andrade 2009, 2010; Majdzadeh-Ardakani et al. 2010; Maksimov et al. 2009; Mondragón et al. 2008; Müller et al. 2011; Pandey and Singh 2005; Park et al. 2002, 2003; Tang et al. 2008b, c; Wang et al. 2009b, d; Wilhelm et al. 2003b; Zeppa et al. 2009; Zhang et al. 2007). It has been demonstrated in the previous studies (Cyras et al. 2008; Park et al. 2002) that, because of the strong polar interactions between the hydroxyl groups from the starch polymer chains, from the glycerol, and from the silicate layers, glycerol and/or starch chains can enter into silicate interlayers to form intercalated starch–MMT nano-biocomposites. However, it was highlighted by Wilhelm et al. (2003a, b) that, for high glycerol-to-clay ratio, an intercalated structure could be formed in such systems with the d_{001} increasing to about 18.5 Å, a very well-known value which was attributed to the intercalation of glycerol instead of starch molecules, whereas a total exfoliation could be obtained in the absence of the plasticizer. Such a similar phenomenon has also been reported elsewhere with glycerol (Chivrac et al. 2010c; Zhang et al. 2007) and also with sorbitol (Chivrac et al. 2010c; Ma et al. 2007b). Such materials, with a very limited polymer chain intercalation, must rather be considered as conventional microcomposites (Chivrac et al. 2010c). Furthermore, the trapping of the plasticizer in the interlayer spaces could also induce a lower plasticization effect on the starch phase (Chivrac et al. 2010c; Müller et al. 2011).

Pandey and Singh (2005) examined the effect of mixing order on the intercalation of MMT–Na^+ in samples containing maize starch, water, and glycerol. They found that the nanofiller dispersion became highly heterogeneous and the product became more brittle when the starch was plasticized with the glycerol before filling with the clay due to the formation of a bulky structure resulting from electrostatic interactions between the starch and the plasticizer. However, if the glycerol was added after mixing the clay into the starch matrix, best dispersion and thus best mechanical properties could be obtained. This study demonstrated that glycerol and starch both have the tendency to penetrate into silicate interlayers, with glycerol being favored by its smaller size (Pandey and Singh 2005). The effect of glycerol content on the nanostructure of starch–MMT biocomposites has also been reported. In a study on MMT–Na^+-reinforced wheat starch–based biocomposites prepared by twin-screw extrusion, Chiou et al. (2007) showed that the addition of 5 wt % glycerol produced the mostly exfoliated nanoclay, whereas adding 10 or 15 wt % glycerol produced the intercalated nanoclay. Similarly, Tang et al. (2008b) reported that the degree of clay exfoliation increased as the glycerol decreased from 20 % to 5 %. As a result, the film with 5 % glycerol exhibited the lowest WVP (0.41 g mm/kPa h m^2), highest T_g (53.8 °C), and highest tensile strength (35 MPa), but a low elongation at break value (2.15 %). From these studies, it was proposed that samples containing higher glycerol concentrations had an increase in the starch–glycerol interactions, which competed with the interactions with the nanoclay surfaces (Chiou et al. 2007; Majdzadeh-Ardakani et al. 2010; Tang et al. 2008b). To address this issue, Chung et al. (2010) introduced a novel method

to prevent the intercalation of glycerol into silicate interlayer spaces: starch and clay were first well dispersed in a water solution at very low concentrations, which was then coprecipitated by ethanol, followed by hot-pressing with glycerol. This method was reported to lead to good dispersion of the clay in the starch matrix (Chung et al. 2010).

Chiou et al. (2007) proposed that the hydrophilic nature of glycerol could negate the improved water resistance of starch-based nano-biocomposites containing exfoliated MMT. This issue has been extensively addressed by Chivrac et al. (2010a), who investigated the effect of plasticizer (glycerol) content on the structure of starch-based nano-biocomposite materials and on their moisture barrier properties (permeability, water sorption, and diffusivity). It was shown that the relatively high plasticizer content (23 wt % glycerol) could induce a phase separation, with plasticizer-rich and starch-rich phases, resulting in the nanoclay being preferentially located in the starch-rich domains (Chivrac et al. 2010a). As a result, a preferential pathway for water transfer was more likely to be created in the very hydrophilic glycerol-rich domains where the nanoclay platelets were almost totally absent. Thus, even if exfoliated morphology was achieved, the heterogeneous clay distribution and phase separation phenomenon explained the lack of improvement and even the decline in the moisture barrier property for these glycerol-plasticized starch-based nano-biocomposites (Chivrac et al. 2010a).

Other plasticizers such as sorbitol (Chivrac et al. 2010c; Kvien et al. 2007; Ma et al. 2007b), citric acid (Wang et al. 2009b), urea (Chen et al. 2005; Huang and Yu 2006; Huang et al. 2006; Ren et al. 2009; Tang et al. 2008b; Wang et al. 2010b; Zeppa et al. 2009), formamide (Huang et al. 2004, 2005a, b, 2006; Ma et al. 2007b; Tang et al. 2008b; Wang et al. 2010b), *N*-(2-hydroxyethyl)formamide (Dai et al. 2009a), ethanolamine (Huang et al. 2005a, b; Huang and Yu 2006; Ren et al. 2009; Zeppa et al. 2009), or their combinations have been used in the preparation of starch–phyllosilicate nano-biocomposites and have been proved to be effective in enhancing the dispersion and exfoliation of silicate layers. Also, 1-allyl-3-methylimidazolium chloride, a hydrophilic ionic liquid (IL), which can be considered as a plasticizer, has been experimented for the preparation of starch-based nano-biocomposites with promising results (Wang et al. 2009c). Nevertheless, some systems mentioned above are highly eco-toxic and cannot be used to develop safe "green" materials. However, the use of these latter plasticizers may avoid the disturbance of small polyols in the intercalation/exfoliation of silicate layers, as mentioned before. As a result, the starch-based nano-biocomposites plasticized by these plasticizers usually have improved properties. For example, Tang et al. (Tang et al. 2008b, c) demonstrated that a formamide- or urea-plasticized starch–MMT nano-biocomposite film exhibited a lower WVP, higher T_g, and higher tensile strength than a glycerol-plasticized nano-biocomposite film. Particularly, Chen et al. (2005) reported that the use of urea as the plasticizer enhanced the dispersion of ammonium-treated MMT in a starch matrix, making exfoliated starch–clay nano-biocomposites possible. This is due to the $-NH_2$ groups

from urea, which could develop strong interactions with the quaternary ammonium from the organoclay, providing better dispersion of the organoclay in the matrix (Chen et al. 2005).

Nevertheless, the strong plasticizer–nanoclay interactions might play a negative effect on the exfoliation in nanohybrid materials. In a study on wheat starch–based nano-biocomposites reinforced by OMMT–CS, Chivrac et al. (2010c) reported that a kind of exfoliated morphology was obtained with glycerol as the plasticizer, while an intercalated/exfoliated structure could be formed with Polysorb®, a glycerol and sorbitol mixture, or with only sorbitol. The sorbitol-plasticized starch-based nano-biocomposite displayed a slight decrease in the mechanical properties corresponding to a more aggregated structure compared with the glycerol-plasticized sample (Chivrac et al. 2010c).

For producing more desirable starch-based nano-biocomposites, phyllosilicates can be pretreated with glycerol (Wang et al. 2009b, d), ethanolamine (Huang et al. 2005a, b; Huang and Yu 2006), citric acid (Huang et al. 2006; Majdzadeh-Ardakani et al. 2010), urea (Wang et al. 2010b), 1-allyl-3-methylimidazolium chloride (Wang et al. 2009c), etc. before the compounding step. This pretreatment can increase the *d*-spacing and destruct the stacked layered structure of a phyllosilicate during a preprocessing procedure. As reported by Huang et al. (2005a, b, 2006; Huang and Yu 2006), when MMT–Na^+ was treated with citric acid or ethanolamine, the XRD peak of the MMT–Na^+ (001) crystal plane moved to a lower value, which indicates that the distances between the layers of the MMT–Na^+ widened, and the citric acid or ethanolamine had intercalated into the layers of the MMT–Na^+. This made it easy for the MMT–Na^+ to interact with plasticized starch to achieve total dispersion and exfoliation, as indicated by the disappearance of the diffraction peak d_{001} (Huang et al. 2005a, b, 2006; Huang and Yu 2006). A two-step processing method was recently utilized for the preparation of starch-based nano-biocomposites reinforced by plasticizer-modified MMT–Na^+ (Ma et al. 2007b; Wang et al. 2009b, d, 2010b; Zhang et al. 2007). In the first modification step, the plasticizer enlarged the *d*-spacing and destructed the stacked multilayer structure of the MMT–Na^+ effectively either by using high-speed emulsifying machine (Wang et al. 2009b, d) or by using a SSE (Ma et al. 2007b; Wang et al. 2010b; Zhang et al. 2007), and the resulting MMTs were propitious to form an intercalated or exfoliated nano-biocomposite in the melt extrusion processing in a second step. Nonetheless, the possible competition between the starch and the plasticizer for the intercalation might decrease the plasticization of starch, because the intense interactions (the hydrogen bonding and the ion–dipole) existed in these multiphase nano-biocomposites (Wang et al. 2009b, 2010b).

Alternatively, chitosan, an abundant polysaccharide, has been focused as a new eco-friendly compatibilizer to promote MMT platelet exfoliation in solution (Chung et al. 2010; Kampeerapappun et al. 2007). Unfortunately, the intercalation of chitosan was not observed since the molar mass of chitosan was too high (Kampeerapappun et al. 2007). However, it was assumed that this polycation can act as a compatibilizing agent leading to fewer clay aggregates and improved mechanical properties (Kampeerapappun et al. 2007).

5.7 Effects of Starch Type, Amylose Content, and Chemical Modification

The amylose content of starch or starch type was also reported to have a structural impact on starch-based biocomposites, even though the results were not consistent (Mondragón et al. 2008; Tang et al. 2008c). Mondragón et al. (2008) compared regular maize starch, high-amylose maize starch (maize starch with 70 % amylose), and waxy maize starch (almost pure amylopectin) and concluded that the plasticized waxy starch molecules were the easiest of them all to form an intercalated/exfoliated structure. The structural features corresponded with the mechanical properties as the tensile strength and Young's modulus of the nano-biocomposites tended to increase with the MMT content and experienced an incremental improvement following the order high-amylose < normal < waxy maize starch (Mondragón et al. 2008). In contrast, Tang et al. (2008c) reported that a regular maize starch–based film presented better barrier and mechanical properties than either a waxy starch– or high-amylose starch–based nano-biocomposite film. Besides, the WVP, tensile strength, and elongation at break of the films did not change significantly as the amylose content increased to >50 %. These results should be ascribed to complicated reasons including the degree of gelatinization and the starch crystallinity. Moreover, Chiou et al. (2005) reported that amylose, leached from the granules during the gelatinization when the temperature was higher than 95 °C, was intercalated into the MMT–Na^+ interlayer spaces, resulting in a large increase in the elastic modulus during rheological measurement. In addition, MMT–Na^+ samples containing wheat and normal maize starch had comparable elastic modulus values during the gelatinization, while both potato and waxy maize starch samples had elastic modulus values that decreased rapidly at higher temperatures. The results of the potato starch sample could be explained in part by a higher swelling capacity, while the waxy maize starch results could be explained by the lack of amylose resulting in fewer physical cross-links between the leached amylose, the starch granule, and the nanoclay (Chiou et al. 2005).

To seek better performance, various chemically modified starches have also been experimented to develop nano-biocomposites (Gao et al. 2012; Hassan Nejad et al. 2011; Kvien et al. 2007; Namazi et al. 2009; Nejad et al. 2010; Qiao et al. 2005; Wilhelm et al. 2003a; Xu et al. 2005). Chemical modification can result in starch derivatives with varied properties such as the molecular chain length and hydrophilicity, which could affect the interactions with a phyllosilicate. For example, Wilhelm et al. (2003a) showed that the substitution of an unmodified starch matrix by an oxidized starch gave rise to the d_{001} value of the nano-biocomposite, indicating that the short oxidized starch chains were easier to intercalate into the silicate interlayer spaces. They also found that the glycerol (the plasticizer) intercalation was minimized in the oxidized starch film where the intercalation of the oxidized starch chains was preferred (Wilhelm et al. 2003a). On the other hand, by replacing some hydroxyl groups of starch with less hydrophilic functional groups such as acetate groups, the polarity matching between the starch and a specific phyllosilicate can differ (Gao et al. 2012; Hassan Nejad et al. 2011; Namazi

et al. 2009; Nejad et al. 2010). For instance, Nejad et al. (2011, 2010) reported that OMMT (Dellite 67G or Dellite 43B), compared with MMT–Na^+ (Dellite LVF), matched better with hydrophobic starch derivatives (starch acetate, starch propionate, and starch propionate acetate laurate) as the matrices. As a result, very good dispersion and partially exfoliated structures were achieved.

5.8 Effects of Preparation Techniques and Processing Conditions

Namazi et al. (2009) compared solution intercalation and in situ polymerization methods for preparing starch-*g*-PCL nano-biocomposites reinforced by Cloisite 15A. Their result showed that, even though the d_{001} could be varied by the clay addition level and swelling/process time, the diffusion and intercalation of copolymer into the interlayer spaces were generally better by solution intercalation than by in situ intercalative method (Namazi et al. 2009).

Chiou et al. (2006) examined the effects of moisture content, temperature, and screw speed in a twin-screw extrusion process of wheat starch–based biocomposites reinforced by MMT–Na^+ and Cloisite 30B. They found that the moisture content was the major factor affecting the MMT–Na^+ dispersion; an increase in the water content led to intercalation and eventual exfoliation (for the sample with 47 wt % moisture) of the nanoclay. The results were explained by the greater degree of gelatinization at a higher moisture content, which allowed more leaching of hydrophilic amylose and amylopectin molecules from the granules to penetrate into the MMT–Na^+ interlayer spaces (Chiou et al. 2006). However, the other processing parameters like temperature and screw speed had little effect on the MMT–Na^+ dispersion (Chiou et al. 2006). For the samples based on Cloisite 30B, only an increase in the temperature produced slight intercalation of the nanoclay, while changes in the moisture content and screw speed did not produce intercalation. This was ascribed to the incompatibility of starch with the rather hydrophobic Cloisite 30B (Chiou et al. 2006). For both Cloisite Na^+ and 30B samples, intercalation did not depend on the SME (Chiou et al. 2006).

Dean et al. (2007) investigated the effects of the levels of phyllosilicates (1–3.2 %) and plasticizer (13–20 %), types of phyllosilicates (MMT–Na^+ and sodium fluorohectorite), dispersion methodologies, and processing conditions on the degree of intercalation/exfoliation of the starch-based nano-biocomposites. Three different mixing regimes were studied, i.e., (a) dry blending of the components prior to extrusion, (b) conventional mixing of the phyllosilicate in solution prior to high-speed mixing with starch and extrusion, and (c) ultrasonic treatment of the phyllosilicate in solution prior to high-speed mixing with starch and extrusion. It was shown that the use of ultrasonics was only advantageous in terms of phyllosilicate dispersion at medium nanofiller concentrations for the MMT–Na^+ samples and at higher nanofiller concentrations for the sodium fluorohectorite samples due to the difference in the cationic exchange capacity. When the levels of the phyllosilicate, water, and starch were optimized, an exfoliated structure could be produced via conventional standard mixing (Dean et al. 2007). This seems in

contrast with a recent study (Majdzadeh-Ardakani et al. 2010) where starch-based nano-biocomposites were reinforced by either MMT–Na^+, Cloisite 30B, or citric acid–modified MMT–Na^+ and were prepared via a solution casting method. The authors of this publication (Majdzadeh-Ardakani et al. 2010) reported that a combined mechanical and ultrasonic mixing mode led to the most dispersion of the silicate layers in the nano-biocomposites and thus the highest Young modulus, irrespective of the clay type, compared to a process involving only one mixing mode, either mechanical or ultrasonic. This is due to the contribution of both dispersive (the breakup of the silicate agglomerates to individual silicate layers, provided by the ultrasonic device) and distributive (a spatial uniformity of all the components in system, provided by the mechanical mixer) mixing mechanisms (Majdzadeh-Ardakani et al. 2010).

5.9 Effect of Blending with Other Polymers

In order to produce starch-based nano-biocomposites with better properties, starch has been blended with different biodegradable and nonbiodegradable polymers, including PLA (Arroyo et al. 2010; Lee and Hanna 2008, 2009; Lee et al. 2007b, 2008a, b), PCL (Ikeo et al. 2006; Kalambur and Rizvi 2005, 2006b, 2004; Perez et al. 2008, 2007, 2008a, b; Vertuccio et al. 2009), PVA (Dean et al. 2008, 2011; Majdzadeh-Ardakani and Nazari 2010), PBAT (Nayak 2010; Raquez et al. 2011), PBSA (Bocchini et al. 2010), and some trademarked polyesters (Ikeo et al. 2006; McGlashan and Halley 2003; Park et al. 2007). In addition, starch–natural rubber nano-biocomposites have also been studied (Mondragón et al. 2009). Some of these studies (Nayak 2010; Perez et al. 2008, 2007; Pérez et al. 2008a, b; Raquez et al. 2011), though also quite interesting, will less be discussed in the present paper since they involved nano-biocomposites of which the matrix contained starch as a minor component (e.g., Mater-Bi® Z, containing 18 % starch, 75 % PCL, and 7 % additives).

McGlashan and Halley (2003) successfully prepared starch–polyester nanocomposite films by melt extrusion and film blowing. They found that the addition of MMT to the matrix could make the processing more stable: the die and die lip temperature could be lowered without detrimentally affecting the film blowing process (McGlashan and Halley 2003). The reason could be the exfoliated MMT acting as a barrier for plasticizer migration and evaporation. This also contributed to the products with greater stability with storing time (McGlashan and Halley 2003). Ikeo et al. (Vertuccio et al. 2009) suggested that the addition of MMT–Na^+ could improve the compatibility between starch and PCL. Besides, Dean et al. (2011) suggested there was a "nanostabilization" effect of MMT–Na^+ in starch–PVA blends, meaning the starch recrystallization could be disrupted by the nanofiller, which reduced the rate of embrittlement over time, in agreement with a previous study (McGlashan and Halley 2003).

In some studies (Arroyo et al. 2010; Avella et al. 2005; Bocchini et al. 2010; Dean et al. 2008; Majdzadeh-Ardakani and Nazari 2010; McGlashan and Halley 2003), systematic investigations were carried out to find out the effects of the

second polymer content, the nanofiller type and content, and some other parameters on the properties of the nanocomposites and also to find out the optimized applicable products. Nanocomposites based on starch blends are also expected to show improved characteristics such as mechanical properties (increases in the storage modulus (Bocchini et al. 2010; Vertuccio et al. 2009), Young's modulus (Arroyo et al. 2010; Avella et al. 2005; Dean et al. 2008, 2011; Ikeo et al. 2006; McGlashan and Halley 2003; Mondragón et al. 2009; Pérez et al. 2007), and tensile strength (Arroyo et al. 2010; Avella et al. 2005; Dean et al. 2008, 2011; Ikeo et al. 2006; McGlashan and Halley 2003; Mondragón et al. 2009; Pérez et al. 2007)). However, it is quite significant to note that the incorporation of another polymer (usually being relatively hydrophobic) would modify the hydrophilic/hydrophobic balance of the material. As a result, MMT–Na^+ would probably not match anymore the polarity of the blend. In the study by McGlashan and Halley (2003), it was proposed that the organic constituents of the OMMT consisted of alcohols and hydrogenated tallow could be more thermodynamically compatible with the polyester in the matrix. Lee and co-workers (Lee and Hanna 2008, 2009; Lee et al. 2007b, 2008a, b) carried out a series of studies on nanocomposite foams based on tapioca starch and PLA by melt intercalation using different MMTs. Their results showed that Cloisite 30B, instead of MMT–Na^+, could result in the greatest extent of intercalation (Lee et al. 2007b). The greatest enlargement in the d_{001} of the Cloisite 30B in the nanocomposite was caused by the strong hydrogen bonding between the hydroxyl groups of the matrix and the hydroxyl groups of the Cloisite 30B organomodifier (Lee et al. 2007b). Therefore, the functional properties of the nanocomposite filled with Cloisite 30B were generally better than those of the Cloisite Na^+ counterpart at a nanoclay addition level of 3 wt % (Lee et al. 2007b). Similarly, in another study by Bocchini et al. (2010) who experimented with systems based on starch and PBSA with different ratios and filled with either MMT–Na^+ or Cloisite 30B as the nanofiller, only Cloisite 30B could result in an exfoliated structure because of the high affinity between the Cloisite 30B and the PBSA. Majdzadeh-Ardakani and Nazari (2010) compared starch–PVA nanocomposites reinforced by either MMT–Na^+, Cloisite 30B, or citric acid–modified MMT. The results showed that the citric acid–modified MMT led to better mechanical properties in comparison with the MMT–Na^+ and Cloisite 30B because of the strong interactions between the citric acid and the starch–PVA polymer chains (Majdzadeh-Ardakani and Nazari 2010).

Despite of the above discussion, the properties can be not only dependent on the degree of intercalation/exfoliation. Dean et al. (2008) prepared a series of plasticized starch–PVA nanocomposites reinforced by MMT–Na^+ which exhibited intercalated and highly exfoliated structures and investigated the key interactions in these products. Their results showed that the relative concentration of the PVA and MMT–Na^+ could be directly correlated to the change in the d_{001} (Dean et al. 2008). Although good dispersion of the nanoclay platelets was important in improving the mechanical properties in these nanocomposites, the interfacial interactions between the nanofiller and the matrix played also a key role. The more agglomerated biocomposites containing both the MMT–Na^+ and PVA showed significant

increases in the tensile strength (up to 67 % increase) and Young's modulus (up to 85 % increase) as compared to the better dispersed biocomposites without PVA. The improvement in the properties could be attributed to both the interfacial interactions and the disruption of the starch retrogradation (recrystallization process) (Dean et al. 2008).

Starch is known to often show problems of compatibility with other polymers, which can also be an issue in materials based on nano-biocomposites. In a study on starch–PLA nano-biocomposites, Arroyo et al. (2010) found that the MMT–Na^+ was preferentially located in the starch phase or at the blend interface, which could reduce the interactions between the plasticized starch and PLA phases in the compatibilized blends resulting in lower stress transfer from the PLA matrix to the plasticized starch dispersed phase. In another study on starch–natural rubber nano-biocomposites, Mondragón et al. (2009) revealed that the MMT–Na^+ nanolayers were mainly dispersed in the natural rubber domains forming a well-ordered intercalated structure. This could be ascribed to the reasons related to the high molar mass and the matching polarity of the natural rubber (Mondragón et al. 2009). Of course, some studies have addressed the compatibility issue, and methods such as high-energy ball milling (for starch–PCL nano-biocomposites (Vertuccio et al. 2009)) and reactive processing (for starch–PCL nano-biocomposites (Ikeo et al. 2006; Kalambur and Rizvi 2005, 2006b, 2004) and starch–PBAT nano-biocomposites (Nayak 2010; Raquez et al. 2011)) have been employed, which showed to be quite effective.

5.10 Toward Some Applications

There have been some studies on starch graft copolymer–phyllosilicate superabsorbent nano-biocomposites with excellent water absorbency and retention properties (Al et al. 2008; Luo et al. 2005; Wu et al. 2000; Zhou et al. 2011), which can be applied in industrial, agricultural, and horticultural applications. The graft copolymer could either be starch-*graft*-polyacrylamide (Wu et al. 2000), starch-*graft*-acrylic acid (Al et al. 2008), or starch-*graft*-poly[acrylamide-*co*-(acrylic acid)] (Luo et al. 2005; Zhou et al. 2011). The addition of a low-cost phyllosilicate which is abundant of –OH groups into the starch matrix is expected to improve the morphological homogeneity, water absorbing property, and gel strength because the cross-linking network can be improved with homogeneous dispersion of MMT (Luo et al. 2005). Wu et al. (2000) compared the water absorbency values of starch-*g*-polyacrylamide nano-biocomposites filled with either bentonite (MMT), kaolinite, or sericite (mica) and found that the best result was obtained with kaolinite which had the right hydration and distension to form the most suitable cross-linking density. On the other hand, several studies (Al et al. 2008; Luo et al. 2005; Zhou et al. 2011) indicated that increasing the phyllosilicate content or phyllosilicate-to-monomer ratio would first increase and then decrease the water absorbency of the nano-biocomposites, which was also ascribed to the reasons related to cross-linking density. When the cross-linking density was high, the space in the network would

become small with an adverse effect to absorb water (Al et al. 2008; Luo et al. 2005; Wu et al. 2000; Zhou et al. 2011). Furthermore, Al et al. (2008) showed that this kind of materials could also be useful in the removal of a dye (safranine T) from an aqueous solution.

Xu et al. (2005) prepared starch acetate–based nano-biocomposite foams reinforced by different OMMTs by melt extrusion and found the nano-biocomposite foams showed a decrease in the cell size. The incorporation of OMMT also significantly decreased the compressibility of the nano-biocomposite foams but did not substantially affect their spring index (Xu et al. 2005). Chen et al. (2005) prepared novel starch-based nano-biocomposite foams with ammonium-treated MMT as the nanofiller and urea as the plasticizer. Spontaneously formed regular foam structure with 84 % porosity was obtained due to the ammonium surfactant of the clay which produces ammonia gas acting as an internal blowing agent (Chen et al. 2005).

Wang et al. (2009c) prepared starch-based nano-biocomposites filled with MMT, with the use of IL both as the plasticizer for the starch and the modifier for the MMT. They found that the MMT and water contents exerted an important influence on the conductance of the nano-biocomposite films, with the maximum conductance being $10^{-0.3}$ S/cm with the MMT content of 9 wt % and the moisture content of 23 wt % (Wang et al. 2009c), which was higher than alkali metal–doped plasticized starch (Ma et al. 2007a). This makes IL-plasticized starch-based nano-biocomposites to be a promising alternative for the development of new solid electrolytes, which had a wide variety of potential applications such as antistatic plastics, electronic shielding, biosensor, environmentally sensitive membranes, etc. (Wang et al. 2009c). Studies (Wang and Zhao 2006; Zhao et al. 2008) have been carried out to investigate the electrorheological activity of a novel ternary kaolinite–dimethyl sulfoxide–carboxymethyl starch (CMS) nano-biocomposite as an electrorheological fluid (which could produce an instantaneous, reversible change in rheological properties when exposed to an electric filed in the flow direction), which was influenced by the degrees of intercalation and substitution.

6 Starch-Based Nano-biocomposites Reinforced by Cellulose Nanowhiskers/Nanoparticles

6.1 Cellulose Nanowhiskers

Cellulose, which is the most abundant biopolymer on earth and can be obtained from various sources such as plants, animals, and bacteria, is a linearly condensed polymer consisting of β-1,4-linked D-anhydroglucopyranose units (cf. Fig. 13). Depending on the source, the DP ranges from 2,500 to 15,000 (Kumar et al. 2009). Often associated with hemicellulose and lignin, cellulose is the main constituent of wood, flax, ramie, hemp, or cotton (Chivrac et al. 2009a).

Since polysaccharides such as cellulose normally have a semicrystalline structure, the most common method to prepare polysaccharide nanofillers is by acid hydrolysis, which can remove the amorphous regions and leave the highly

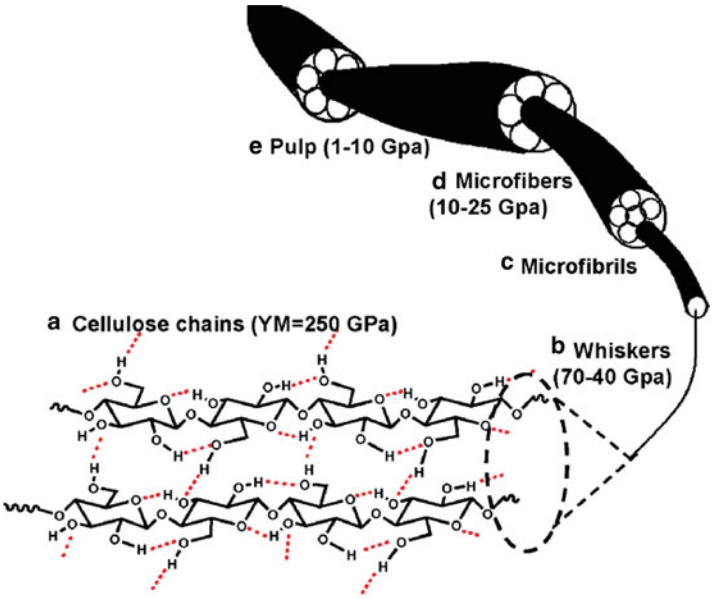


Fig. 13 Schematic representation of cellulose chemical structure and fibrillar organization (Reprinted from Kumar et al. (2009), Copyright (2009), with permission from Elsevier)

crystalline parts of the material. The resulting nanocrystals occur as rodlike particles or whiskers, whose dimensions depend on the nature of the polysaccharide (Dufresne 2008).

CNWs (also cellulose nanofibers, which are typically cellulose nanocrystals; cf. Fig. 13) can be isolated from biomass like flax (Cao et al. 2008a), hemp (Cao et al. 2008b), ramie (Lu et al. 2006), peal hull (Chen et al. 2009a, b), cassava bagasse (Teixeira et al. 2009), and tunicate (a sea animal) (Angles and Dufresne 2000, 2001; Mathew and Dufresne 2002b; Mathew et al. 2008), or from microcrystalline cellulose (Chang et al. 2010c; Kvien et al. 2007), through acid hydrolysis with a concentrated mineral acid (typically sulfuric acid) under strictly controlled conditions of time and temperature (Angles and Dufresne 2000; Azizi Samir et al. 2004, 2005; Bondeson et al. 2006; Cao et al. 2008a, b; Chen et al. 2009a, b; Dong et al. 1996; Lu et al. 2006; Mathew and Dufresne 2002b; Teixeira et al. 2009). Acid action results in a decrease of the amorphous parts by removing the polysaccharide material closely bonded to the crystallite surface and breaking down portions of glucose chains in most accessible, nanocrystalline regions and by acid hydrolysis of cellulosic materials. Generally, the final geometrical characteristics depend on the cellulose origin and the acid hydrolysis process conditions such as time, temperature, and purity of the materials (Kumar et al. 2009). Tunicin (animal cellulose) nanowhiskers have a comparatively high aspect ratio (*L*/*D*: 50–200), which could enhance the interfacial phenomena between this nanofiller and a polymer matrix (Xie et al. 2013).

Chen et al. (2009b) investigated the effect of the hydrolysis time on the length (L), diameter (D), and L/D of the resulting CNWs from pea hull fiber. The authors showed that the chemical removal of hemicellulose and lignin from the original pea hull fiber was mostly complete in the first 4 h of acid hydrolysis and that 8 h of hydrolysis was long enough to remove most of the amorphous regions in the pea hull fiber, when the CNWs showed the highest L/D value (36). However, if the hydrolysis time was too long (particularly 24 h), the crystalline regions could be destroyed (Chen et al. 2009b).

Though direct acid hydrolysis is frequently used to prepare CNWs, other methods have been employed to produce this kind of nanofiller as well, as some examples listed below:

- The precipitation of a NaOH/urea/H_2O solution of microcrystalline cellulose by dropwise addition of an ethanol/HCl aqueous solution; particularly, the resulting material is in the form of particles (Cai and Zhang 2005; Chang et al. 2010c).
- The disintegration of the plant fiber cell wall by mild enzymatic hydrolysis combined with mechanical shearing and high-pressure homogenization (Mohanty et al. 2005; Paakko et al. 2007; Svagan et al. 2009).
- The extraction from wheat straw using steam explosion, acidic treatment, and high shear mechanical treatment (Kaushik et al. 2010).
- The production by *Acetobacter* bacteria; enzyme hydrolysis may be applied further (Grande et al. 2008, 2009; Wan et al. 2009; Woehl et al. 2010).

It is worth noting that, regardless of the preparation method, a high crystallinity of the nanofiller is generally encouraging because the higher the crystallinity, the higher the Young modulus of the nanofiller (Kumar et al. 2009) (cf. Fig. 13), which is beneficial to mechanical property improvement in the resulting nano-biocomposites. Complementary treatments in addition to the traditional hydrolysis may further decrease the contents of hemicellulose and lignin, disintegrate the bundles, and increase the crystallinity of CNWs. In a study by Kaushik et al. (2010), for example, the crystallinity increased from 54 % for untreated wheat straw to 67 % for the chemically treated sample and to 80 % for the CNWs treated both chemically and mechanically. Nevertheless, nanoparticles or nanowhiskers were not fully crystalline in some studies (Cai and Zhang 2005; Chang et al. 2010c).

Though much less frequently, commercially available CNWs (e.g., Celish, Daicel Chemical Industries Co. Ltd., Japan) have also been used in some studies to develop starch-based nano-biocomposites (Sreekala et al. 2008).

6.2 Preparation Techniques

A solution casting method has been mostly used to prepare starch-based nano-biocomposite films, which were then conditioned at specific temperature and relative humidity conditions. For the predominant use of the solution casting method, two reasons might account:

– Cellulose nanowhiskers/nanoparticles tend to aggregate due to the association by strong hydrogen bonding in the presence of high density of the –OH groups (Cao et al. 2008a, b; Kaushik et al. 2010).
– The nanofiller is prepared in aqueous condition, with the resulting dispersion (without either sedimentation or flocculation, as a consequence of charge repulsion due to the surface sulfate groups created during the sulfuric acid treatment) easy to be incorporated into a starch solution (Angles and Dufresne 2000; Cao et al. 2008a, b; Chen et al. 2009a, b; Kvien et al. 2007; Lu et al. 2006; Mathew and Dufresne 2002b).

In a solution for preparing starch-based nano-biocomposites, the key point is to fully gelatinize starch and to break nanofiller aggregates and create a homogeneous dispersion state. For the dispersion of nanowhiskers/nanoparticles in a starch matrix, some additional treatments such as ultrasonication and homogenization might be helpful (Chang et al. 2010c; Kaushik et al. 2010).

As far as bacterial cellulose nanowhiskers (BCNWs) are concerned, some special preparation methods were practiced when this kind of nanofiller is used as reinforcement. In a study by Wan et al. (2009), pellicles of BCNWs were incorporated into glycerol-plasticized starch via a solution impregnation method (Wan et al. 2009). Grande et al. (2008, 2009) developed a bioinspired bottom-up technique to produce self-assembled nano-biocomposites of cellulose synthesized by *Acetobacter* bacteria and native starch. As a final step, in order to produce starch–BCNW nano-biocomposite sheets, the BCNW–starch gels were hot-pressed and the starch phase underwent a "second gelatinization" (Grande et al. 2008, 2009). This technique took advantages of the way some bacteria "extrude" cellulose nanofibers and of the transport process that occurs during gelatinization of starch. The interesting solution preparation method could result in nano-biocomposites with a coherent morphology (Grande et al. 2008, 2009).

Contrary to most studies where the preparation processes were carried out in solution conditions, Teixeira et al. (2009) used a batch mixer equipped with roller rotors to hot melt (140 °C) a mixture of starch, the plasticizer, stearic acid, and the nanofiller with low moisture content (previously adjusted to 20 %), which was then hot-pressed at 140 °C into sheets. This preparation technique is promising since it is close to the way that the thermal processing of plasticized starch-based materials is normally carried out.

6.3 Effect of Cellulose Nanowhisker Addition

With homogeneous dispersion of CNWs within a starch matrix, property enhancement could often be observed. According to the different reports, the mechanical properties (tensile strength and Young's modulus), thermal property (T_g), and moisture resistance generally show improvement (Angles and Dufresne 2000, 2001; Cao et al. 2008a, b; Chang et al. 2010c; Chen et al. 2009a, b; Grande et al. 2009; Kaushik et al. 2010; Kvien et al. 2007; Lu et al. 2006; Mathew and Dufresne 2002b; Mathew et al. 2008; Svagan et al. 2009; Teixeira et al. 2009;

Wan et al. 2009; Woehl et al. 2010). The improvement in different properties can be linked to the morphology of nano-biocomposites which showed not only good dispersion of nanofillers in the matrix but also good adhesion between the nanofiller and the matrix. This can be explained by the chemical similarity between starch and cellulose and the hydrogen-bonding interactions existing at the nanofiller–matrix interface (Cao et al. 2008a; Chen et al. 2009b; Lu et al. 2006). Specifically, the main reasons for such improvements are summarized below:

- Mechanical properties: improvement in the mechanical properties (but usually at the expense of the elongation at break) can be benefited by (a) the formation of a rigid network of the CNWs connected by hydrogen bonds, the mutual entanglement between the nanofiller and the matrix, and the good bonding and thus the efficient stress transfer from the matrix to the nanofiller (Kaushik et al. 2010; Siqueira et al. 2008) and by (b) the increase in the overall crystallinity of the system resulting from the nucleating effect of the CNWs (Mathew and Dufresne 2002b; Mathew et al. 2008). (The latter point (b) has been in dispute since the hindrance, by the CNW dispersion and CNW–matrix interfacial adhesion, of the lateral rearrangement of the starch polymer chains, and hence the hindrance of the recrystallization, was observed in some studies (Kaushik et al. 2010).)
- Glass transition: an increase in the T_g of the starch-rich domains can be ascribed to (a) the occurrence of intermolecular interactions between the starch and the stiff CNWs, which restricted the mobility of the amorphous starch polymer chains in contact with the CNW surface (Cao et al. 2008b; Chen et al. 2009a; Lu et al. 2006); to (b) the increased crystallinity upon the CNW addition, which also restricted the mobility of the amorphous starch polymer chains (Mathew and Dufresne 2002b; Mathew et al. 2008); and to (c) the relocation of the plasticizer(s) (including water) from the starch matrix to the CNW surfaces (detailed in Sect. 6.5), which decreases the plasticization effect on the amorphous regions of the bulk starch matrix (Angles and Dufresne 2000, 2001).
- Moisture resistance: the reasons for the improvement include (a) the less hydrophilic nature of cellulose and the geometrical impedance created by the CNWs, (b) the constraint of the starch swelling due to the presence of the CNW network (initial moisture absorption may also contribute to the establishment of strong hydrogen bonds), (c) the resistance of the diffusion of water molecules along the nanofiller–matrix interface due to the strong adhesion between them (Angles and Dufresne 2000; Luo et al. 2006; Mathew and Dufresne 2002b; Sreekala et al. 2008; Svagan et al. 2009; Wan et al. 2009), and (d) the decrease in the mobility of the amylopectin chains, resulting from an increase in the T_g or the crystallinity (Angles and Dufresne 2000).

It is noteworthy that a high level of nanofiller addition is not necessarily good because of the aggregation which results in the reduction in the matrix homogeneity and cohesion. This could affect the properties in an inverse trend (Chen et al. 2009a). Moreover, one controlling factor which needs to be emphasized here is the moisture content (usually related to the relative humidity during post-processing conditioning).

The moisture plays a key role in controlling the abovementioned properties of the nano-biocomposites by assisting in the formation of the hydrogen bonding between the CNWs and by (together with the other plasticizers) increasing the mobility of the starch chains which is favorable either for its recrystallization during the conditioning process or for the decrease in its T_g (Angles and Dufresne 2000, 2001; Mathew and Dufresne 2002b; Mathew et al. 2008).

In addition to the properties mentioned above, Chen et al. (2009a, b) reported that nano-biocomposites filled with CNWs showed transparency (tested by light transmittance in the wavelength range of 200–800 nm) very close to or even slightly higher than that of the pure starch matrix, which was attributed to the nanosize and the homogeneous dispersion of the nanofiller.

In the meantime, the addition of CNWs to a starch matrix could probably result in a decrease in the thermal stability (as observed by the thermal decomposition temperature) of the nano-biocomposites (Chen et al. 2009a, b; Kaushik et al. 2010). The reason might be that, for the CNWs prepared by sulfuric acid treatment, the presence of acid sulfate groups decreased the thermal stability of the cellulose by dehydration; thus, the decrease in the thermal decomposition temperature of the CNWs also decreased the thermal stability of the starch matrix by the incorporation of this nanofiller (Chen et al. 2009a, b; Roman and Winter 2004; Teixeira et al. 2009). Interestingly, such a phenomenon also occurred when hydrochloric acid was used for the acid hydrolysis of cellulose; however, this point was not reasonably explained by the authors (Kaushik et al. 2010). In contrast in another study (Chang et al. 2010c), when a non–acid hydrolysis method was used, the nano-biocomposites showed improved thermal stability.

6.4 Effect of Nanofiller Preparation

Compared to microcomposites based on plasticized starch and common cellulose fibers, the global behaviors of CNW-reinforced materials are primarily driven by the nanofiller–matrix interface. Chen et al. (2009a, b) systematically investigated the effect of the acid hydrolysis time (t) during the CNW preparation on the structure and performance of pea starch–based nano-biocomposites reinforced by pea hull fiber–derived nanowhiskers. Firstly, they found that the starch–CNW nano-biocomposites ($t = 4$–24 h) generally exhibited much better properties, i.e., the higher tensile strength, elongation at break, Young's modulus, T_g, transparency, and moisture resistance except for the thermal stability (the lower thermal decomposition temperature, due to the reason already discussed before), than the microcomposites obtained with the native pea hull fibers (without acid hydrolysis) (Chen et al. 2009a). These were related to the smooth and compact morphology of the starch–pea hull fiber nanowhisker films, while the starch–pea hull fiber composite film showed cracks, holes, and fiber-like aggregates (even though the CNWs could also show aggregates at high content) (Chen et al. 2009a). Secondly, they discovered that, though there were no apparent differences in the results of Fourier transform infrared spectroscopy (FTIR) and XRD of the nano-biocomposites filled with CNWs obtained at different

hydrolysis times ($t = 4$–24 h), the morphological results revealed the smoother cross section of the 8 h hydrolyzed CNW nano-biocomposite sample than those of the other nano-biocomposite films (Chen et al. 2009b). The more homogeneous dispersion of the CNWs within the starch matrix and the fewer outstanding particle-like CNWs on the cross section of the sample indicated that, in this nano-biocomposite sample, the CNWs (obtained after 8 h of acid hydrolysis) were wrapped more tightly by the starch matrix (Chen et al. 2009b). It was suggested that this stronger adhesion between the CNWs and the starch matrix resulted from the highest aspect ratio of the CNWs after 8 h hydrolysis and contributed to the superior properties of the nano-biocomposite such as transparency, tensile strength, and elongation at break (Chen et al. 2009b).

Woehl et al. (2010) studied the effect of enzyme hydrolysis of bacterial cellulose (to prepare CNWs) on the structure and mechanical properties of the resulting starch-based nano-biocomposites. Their results showed that hydrolysis for 60 min decreased the DP of the cellulose without changing its crystallinity index or promoting a significant mass loss. However, the nano-biocomposites reinforced by such CNWs displayed the most improved mechanical properties than those with CNWs hydrolyzed for either shorter or longer time (Woehl et al. 2010). The authors proposed two mechanisms for the enzyme-treated CNWs, i.e., (a) the elimination of less organized regions between the fibers that entangled them to each other in the original material, thus allowing much better dispersion of the reinforcing agent into the starch matrix, and (b) the reduction of defects in the surface of the fibers that could act as crack propagators (Woehl et al. 2010). However, they also mentioned that longer hydrolysis time could lead to gradual hydrolysis and weakening of the crystalline regions of the CNWs, resulting in worsening mechanical properties (Woehl et al. 2010), which is in agreement with the study by Chen et al. (2009b).

A noteworthy fact is that the preparation of CNWs may also result in non-cellulose components, which could be, together with the CNWs, incorporated into a starch matrix and affect the properties of the resulting nano-biocomposites. Teixeira et al. (2009) prepared CNWs from cassava bagasse (mainly composed of water (70–80 wt %), residual starch, and cellulose fibers) by acid hydrolysis and found that sugars (mainly glucose and sucrose) could be originated from starch hydrolysis during the acid extraction. For glycerol-plasticized nano-biocomposites, these sugars caused considerable reduction in the T_g of the starch-rich domains and inhibited the formation of V_H-type crystalline structure, as agreed by the increased elongation at break (Teixeira et al. 2009). This is because the chemical similarity between the starch and the sugars (such as glucose) could favor the interactions between them, reducing the specific interactions between the starch matrix and the CNWs, which resulted in very high mobility of the starch chains (Teixeira et al. 2009).

6.5 Plasticizer Relocation and Transcrystallization Phenomena

Dufresne and co-workers (Angles and Dufresne 2000, 2001) have undertaken a series of studies on plasticized waxy maize starch–based nano-biocomposites reinforced by tunicin nanowhiskers. The results indicated that, though the strong

interactions between CNWs such as hydrogen bonding (which could be affected by the moisture content) could lead to a rigid network, which was responsible for the improved mechanical performance, some effects could tamper the reinforcement induced by the CNWs (Angles and Dufresne 2000, 2001):

- The accumulation of the plasticizer in the vicinity of the cellulose/amylopectin interfacial zone (because of the stronger interactions of either the water or glycerol with the cellulose than with the starch, as demonstrated by the contact angle measurements), enhanced in moist conditions.
- The coating of the CNWs by a soft plasticizer-rich interphase as a result of the previous effect.
- Especially in highly moist conditions, the abovementioned plasticizer accumulation, and the presence of the CNWs as a nucleating agent, could improve the ability of the amylopectin chains to crystallize, leading to the formation of a highly oriented layer, i.e., transcrystalline zone, around the CNWs.

These effects could then interfere with the inter-CNW hydrogen-bonding forces and hinder the stress transfer at the nanofiller–matrix interface and thus compromise the mechanical properties of the ensuing nano-biocomposites (Angles and Dufresne 2000, 2001).

However, by the substitution of sorbitol for glycerol as the plasticizer, no evidence of the preferential migration of the plasticizer toward the cellulose and the transcrystallization phenomenon of the amylopectin on the CNW surfaces could be observed; instead, only a single glass transition was observed for the nano-biocomposites (Mathew and Dufresne 2002b; Mathew et al. 2008). In this case, strong interactions among the CNWs, starch matrix, sorbitol, and water and a rigid three-dimensional network of the CNWs were formed, benefiting the mechanical enhancement (Mathew and Dufresne 2002b; Mathew et al. 2008).

While Dufresne and co-workers (Angles and Dufresne 2000, 2001) used waxy maize starch as the matrix and tunicin nanowhiskers as the nanofiller in their systems, it would be probable that the plasticizer relocation and transcrystallization phenomena also happen in other CNW-reinforced systems, as one example demonstrated by Teixeira et al. (2009).

7 Starch-Based Nano-biocomposites Reinforced by Starch Nanoparticles

7.1 Starch Nanoparticles

As previously presented in Sect. 2.1, starch has multilevel structures. A review by Le Corre et al. (2010) has summarized starch SNPs prepared by different methods, mainly (a) starch nanocrystals resulting from the disruption of amorphous domains from semicrystalline granules by acid hydrolysis and (b) starch nanoparticles produced from gelatinized starch.

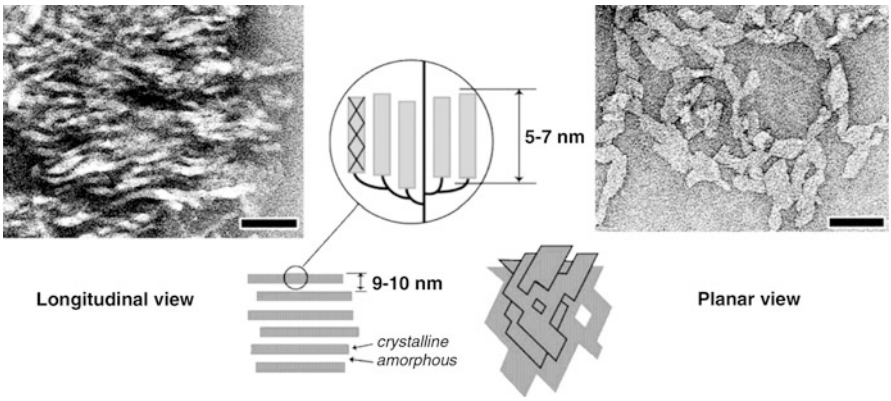


Fig. 14 TEM images of starch nanocrystals: longitudinal and planar views (Reprinted with permission from Putaux et al. (2003). Copyright 2003 American Chemical Society)

Starch nanocrystals can be obtained by acid (normally sulfuric acid) hydrolysis of native starch granules by strictly controlling the temperature, acid concentration, starch concentrations, hydrolysis duration, and stirring speed (Angellier et al. 2004; García et al. 2009; Putaux et al. 2003; Viguié et al. 2007; Zeng et al. 2011). Figure 14 shows one of the first observations of starch nanocrystals by TEM with longitudinal and planar views. The starch nanocrystals prepared by acid hydrolysis have a strong drawback since they gelatinize in hot water which could be a problem when preparing plasticized starch nano-biocomposites.

In the second method, SNPs were prepared by dropwise delivering ethanol as the precipitant into a gelatinized starch solution with constant stirring, followed by centrifugation, washing with ethanol, and oven-drying. The resulting SNPs free from water were further modified by citric acid in a dry preparation technique. The so-formed amorphous citric acid–modified SNPs could not be swelled or gelatinized in hot water because of the cross-linking induced by the citric acid (Ma et al. 2008c). The reaction between the SNPs and the citric acid decreased the aggregation of the SNPs, and the size of the SNPs was reduced (Ma et al. 2008c).

7.2 Preparation Techniques

Similarly as for those reinforced with cellulose nanowhiskers/nanoparticles, solution casting is most commonly used to prepare starch-based nano-biocomposite films reinforced with SNP, which were then conditioned at specific temperature and relative humidity conditions. The reasons for this could be:

– SNPs tend to aggregate due to the association by strong hydrogen bonding in the presence of high density of the –OH groups (Angellier et al. 2006; García et al. 2009, 2011; Viguié et al. 2007).

- The structure of SNPs may be destroyed (gelatinized) at high temperature (e.g., during extrusion), which tampers the reinforcing ability (Angellier et al. 2006; Viguié et al. 2007).
- The nanofiller is prepared in aqueous condition, with the resulting dispersion (without either sedimentation or flocculation, as a consequence of charge repulsion due to the surface sulfate groups created during the sulfuric acid treatment) easy to be incorporated into a starch solution (Angellier et al. 2006; García et al. 2009, 2011; Viguié et al. 2007).

In a solution for preparing starch-based nano-biocomposites, the key point is to fully gelatinize starch and to break SNP aggregates and create a homogeneous dispersion state. SNPs may be added to a gelatinized starch solution at reduced temperature to avoid gelatinization (Angellier et al. 2006; Viguié et al. 2007).

7.3 Effect of Starch Nanoparticle Addition

The nano-biocomposites generally showed increased values of the strength at break, Young's modulus (Angellier et al. 2006; Ma et al. 2008c; Viguié et al. 2007), and T_g (of the starch-rich domains) (Angellier et al. 2006; Ma et al. 2008c; Viguié et al. 2007) and decreased values of the WVP (García et al. 2009; Ma et al. 2008c), indicating improved performance. However, some unfavorable property changes were also observed, e.g., higher water uptake percentage (García et al. 2009) and lower thermal decomposition temperature (García et al. 2009, 2011).

Compared with CNWs, SNPs should have strong affinity with a starch matrix due to the same chemical structure. As a result, good interfacial interactions (nanofiller–nanofiller and nanofiller–matrix) by hydrogen bonding and hence a strong reinforcing effect of SNPs could be expected (Angellier et al. 2006; Ma et al. 2008c; Viguié et al. 2007). Besides, SNPs, acting as a nucleating agent, could facilitate the recrystallization process of the starch polymer chains at the interface, as demonstrated by the increases in the melting temperature and in the melting enthalpy, indicating the increase in the size of the crystalline domains and in the global crystallinity, respectively. However, at high nanofiller content, SNPs seem to hinder the formation of crystalline domains possibly because of an increase in the viscosity in the nano-biocomposite (Viguié et al. 2007). Starting from these points, the mechanisms accounting for all the property changes are summarized below:

- Mechanical properties: increases in Young's modulus and strength at break and a decrease in the elongation at break result from (a) the good interfacial interactions and the strong reinforcing effect of the SNPs (Angellier et al. 2006; Ma et al. 2008c; Viguié et al. 2007) and (b) the increased crystallinity in the starch matrix (Angellier et al. 2006; Viguié et al. 2007).
- Glass transition: an increase in the T_g (of the starch-rich domains), indicating the restraint of the starch polymer chain mobility and the reduction in the free

volume, was due to the presence of the SNPs (a) which function as physical joints, bringing the adjacent starch polymer chains closer and strengthening the intermolecular interactions (Angellier et al. 2006; Ma et al. 2008c; Viguié et al. 2007), and (b) which increase the crystallinity in the starch matrix (Angellier et al. 2006; Viguié et al. 2007).

- Moisture resistance: a reduction in the WVP is ascribed to (a) the less hydrophilic nature of the SNPs (especially citric acid–modified SNPs, of which the hydrophilic –OH groups were substituted by hydrophobic ester groups (Ma et al. 2008c)) and to (b) the tortuous pathways introduced by the SNPs for water molecules to pass through (García et al. 2009; Ma et al. 2008c).
- Thermal stability: a decrease in the thermal decomposition temperature is attributed to (a) the sulfate groups on the acid-hydrolyzed SNPs (García et al. 2009, 2011; Roman and Winter 2004), the reason similar to that for the nano-biocomposites reinforced by CNWs as discussed before; another reason might be (b) the strong interactions between the SNPs and the glycerol (García et al. 2009, 2011), which will be discussed in detail hereafter.

7.4 Effect of Plasticizer

Regarding some property deterioration and result discrepancy in the literature, it is believed that a starch–SNP nano-biocomposite is a complex system governed by more than the nanofiller. One major factor affecting the structure and properties of the nano-biocomposites could be the plasticizer.

By comparing the data of starch–CNW nano-biocomposites and those of starch–SNP nano-biocomposites reported in the literature, it would be interesting to note that the reinforcing effect of waxy maize SNPs in plasticized starch generally is higher than that of tunicin nanowhiskers (Angellier et al. 2006; Angles and Dufresne 2001), even though tunicin nanowhiskers have a higher aspect ratio than SNPs. Nevertheless, this should not be surprising by understanding that starch–tunicin nanowhisker nano-biocomposites have the problems of the plasticizer relocation and transcrystallization (cf. Sect. 6.5), which undermine the interactions between the tunicin nanowhiskers and the starch matrix. It should be reasonable to think that these phenomena are quite limited for starch–SNP nano-biocomposites because of the same chemical nature of the filler and matrix and thus the same affinity of plasticizers such as glycerol and water for both components (Angellier et al. 2006). As a result, Angellier et al. (2006) suggested that strong interactions between the SNPs and between the nanofiller and matrix could be established. The same study (Angellier et al. 2006) also proposed that the same chemical nature of the nanofiller and matrix possibly results in "crystallization" (co-crystallization?) occurring at the nanofiller–matrix interface, which was favorable for mechanical property improvement. These could possibly explain the results from this group of people (Angellier et al. 2006; Viguié et al. 2007) showing that, for waxy maize starch–waxy maize SNP nano-biocomposites, the relative reinforcing effect of the SNPs was more significant when the plasticizer content

(glycerol or sorbitol) was high. Nonetheless, Viguié et al. (2007) from the same research group had to admit a possible "transcrystallization" phenomenon occurring for starch–SNP nano-biocomposites plasticized by sorbitol regarding their DMA results. Anyway, the authors also suggested that there was an increase in the crystallinity (double helices) during aging, which enhanced the mechanical stiffness (though intramolecular crystallites could result in a reduction of intermolecular interactions and in the cohesion of the matrix and thereby result in cracks) (Angellier et al. 2006; Viguié et al. 2007).

Despite the same chemical nature of plasticized starch and SNPs, García et al. (2009) suggested that the large number of –OH groups on the surfaces of SNPs, which were mainly the crystalline zones of hydrolyzed waxy starch, led to more association of the SNPs with glycerol molecules through hydrogen bonding than with the cassava starch matrix. This contributed to the cassava starch matrix having more –OH groups available to interact with moisture, as compared to the unfilled film. As a result, an increase in the water uptake with the SNP addition was observed (García et al. 2009). In another study where waxy maize starch was used as the matrix, García et al. (2011) suggested that the SNPs were mainly located in the glycerol-rich domains of the matrix. They compared a nano-biocomposite without glycerol plasticization and the plasticized one and found the former displayed a rather smooth and homogeneous surface associated with a brittle fracture, while the latter showed a nanometric fibrillar structure, as shown in the images of scanning electron microscopy (SEM). The peculiar structure was referred as "nanothreads" which resulted from the association of the aggregated SNPs through the glycerol. They proposed that the nanothreads were formed by the SNPs, glycerol, and transcrystallized amylopectin. The nanothreads which have high concentration of –OH groups become a preferential path for water vapor diffusion, resulting in an increase in the WVP (García et al. 2011).

8 Starch-Based Nano-biocomposites Reinforced by Chitin/Chitosan Nanoparticles

8.1 Chitin/Chitosan Nanowhiskers/Nanoparticles

Chitin is the second most abundant polysaccharide produced in nature after cellulose, appearing as ordered crystalline microfibrils forming structural components in the exoskeleton of arthropods or in the cell walls of fungi and yeast, but also being produced by a number of other living organisms in the lower plant and animal kingdoms, serving in many functions where reinforcement and strength are required (Rinaudo 2006). It is an acetylated polysaccharide composed of *N*-acetyl-D-glucosamine units linked by β(1→4) linkages (cf. Fig. 15). Chitosan is obtained from chitin by deacetylation. Interestingly, chitosan shows antibacterial activity (Dutta et al. 2009).

Chitin nanowhiskers of slender parallelepiped rods can be made from different chitin sources such as crab shells (Gopalan Nair et al. 2003), shrimp shells

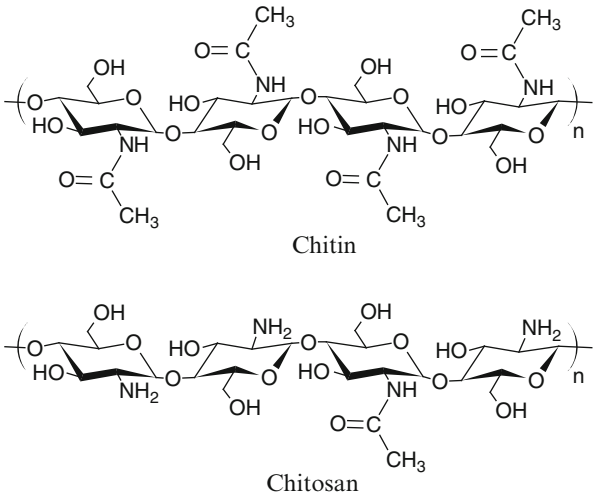


Fig. 15 Chemical structures of chitin and chitosan

(Sriupayo et al. 2005), squid pens (Paillet and Dufresne 2001), and tubes of *Tevnia jerichonana* (Saito et al. 1997) and *Riftia pachyptila* tube worms (Morin and Dufresne 2002). They are obtained by deproteinization in a boiling alkaline (KOH) solution and then hydrolyzing the sample with a boiling HCl solution with vigorous stirring. Besides, Chang et al. (2010b) introduced a modified method to prepare chitin nanoparticles, in which two identical acidic treatments were applied followed by repeated disruption/dispersion processes with sonication. The chitin nanoparticles obtained had a low crystallinity because acid hydrolysis converted some of the crystalline regions into amorphous parts (Chang et al. 2010b). The use of chitin nanoparticles has been reported in starch-based nano-biocomposites, whereas chitin nanowhiskers have hardly been used in this case so far.

Besides, for transforming chitosan into a nanofiller, a very simple and mild method based on ionotropic gelation between chitosan and sodium tripolyphosphate was used: chitosan was dissolved into an acetic acid solution, followed by the dropwise addition of sodium tripolyphosphate into the solution with vigorous stirring and sonication (Chang et al. 2010a; Shu and Zhu 2000; Tsai et al. 2008). This method involves physical cross-linking by electrostatic interactions (instead of chemical cross-linking), which avoids possible toxicity of reagents and other undesirable effects (Shu and Zhu 2000).

8.2 Nanocomposites Reinforced by Chitin/Chitosan Nanoparticles

The use of chitin and chitosan nanoparticles in starch-based nano-biocomposites has been recently described. Chang et al. (2010b) investigated chitin nanoparticle–reinforced starch-based nano-biocomposites and found that the chitin nanoparticles could be uniformly dispersed in the starch matrix at low loading

levels (e.g., 2 wt %), while, at a higher level (5 wt %), conglomeration of the chitin nanoparticles occurred. However, the tensile strength, storage modulus (DMA), T_g, and WVP of the starch–chitin nanoparticle nano-biocomposites showed improvement with the addition of the nanofiller at loading levels up to 5 wt %, due to the good interfacial interactions between the nanofiller and the starch matrix (Chang et al. 2010b). The same group of people (Chang et al. 2010a) carried out another similar study, whereas chitosan nanoparticles instead were used as the nanofiller. Similarly, the nanofiller addition resulted in increases in the tensile strength, storage modulus (DMA), T_g, WVP, and thermal decomposition temperature. The mechanisms of the property improvement in both studies are similar to those for starch–SNP nano-biocomposites. It should be noted that the aggregation of the nanoparticles at high content could deteriorate the reinforcing effect of the nanofiller, as demonstrated by the decrease in the tensile strength when the content of chitosan nanoparticles was higher than 6 wt % (Chang et al. 2010a).

9 Starch-Based Nano-biocomposites Reinforced by Carbon Nanotubes

9.1 Carbon Nanotubes

CNTs are tubular derivatives of fullerenes but exhibit properties quite different from those of the closed cage fullerenes such as C_{60}, C_{70}, C_{76}, etc. CNTs can be visualized as a sheet of graphite that has been rolled into a tube. Unlike diamond, where a three-dimensional diamond cubic crystal structure is formed with each carbon atom having four nearest neighbors arranged in a tetrahedron, graphite is formed as a two-dimensional sheet of carbon atoms arranged in a hexagonal array. In this case, each carbon is covalently bonded to three neighboring carbon atoms through sp^2 hybridization in such a way to form a seamless shell. "Rolling" sheets of graphite into cylinders forms CNTs (Falvo et al. 1997; Kumar et al. 2009; Nicole 2007; Schadler 2003; Wilder et al. 1998). The properties of nanotubes depend on the atomic arrangement (how the sheets of graphite are "rolled"), the diameter and length of the tubes, and the morphology or nanostructure. This rolling can be from one (single-wall carbon nanotubes (SWCNTs)) or more (multiwall carbon nanotubes (MWCNTs)) cylindrical shells of graphitic sheets, with the latter being simply composed of concentric SWCNTs held together with relatively weak van der Waals forces (Kis and Zettl 2008; Kumar et al. 2009; Thostenson et al. 2001). The special topologies are responsible for the unique and interesting properties of CNTs. Due to their high mechanical strength, capillary properties, and remarkable electronic structures, a wide range of potential uses have been reported within the field of materials science research (Kumar et al. 2009).

Though CNTs have already been widely used for other polymer nanocomposite systems (Kumar et al. 2009; Spitalsky et al. 2010), the use of CNTs as the nanofiller to develop starch-based nano-biocomposites has just been initiated, and mostly MWCNTs were involved (Cao et al. 2007; Famá et al. 2011; Liu et al. 2011e;

Ma et al. 2008d) (with the only exception that Bonnet et al. (2007) used SWCNTs in their initial study). This may be due to the lower price and more abundance of MWCNTs than SWCNTs. Besides, MWCNTs also exhibit high aspect ratio (as high as 1,000) and excellent mechanical (with Young's modulus as high as 1 TPa), thermal, and electrical properties (Ajayan 1999; Curran et al. 1998; Qi et al. 2003).

The effectiveness of utilizing CNTs for nanocomposites strongly depends on two main factors: (a) homogeneous dispersion of nanotubes throughout the matrix without destroying the integrity of them and (b) adequate interfacial adhesion between the phases (Kumar et al. 2009; Ruan et al. 2003). These are difficult to achieve especially in the fabrication of starch-based nano-biocomposites regarding the highly hydrophilic nature of starch. For these purposes, chemical modifications such as surface modification with the aid of surfactants, functionalization of endcaps, and functionalization of sidewalls can be helpful (Cao et al. 2007; Kumar et al. 2009). However, care must be taken since these types of procedures might destroy the extended networks on the CNT surfaces, diminishing their mechanical and electronic properties (Famá et al. 2011). The modification methods for CNTs to be incorporated into a starch matrix mainly include:

- The treatment by a surfactant (e.g., sodium dodecyl sulfate (Ma et al. 2008d) and sodium dodecyl benzene sulfonate (Bonnet et al. 2007))
- The carboxylation by strong acids (e.g., sulfuric acid and nitric acid) (Cao et al. 2007; Liu et al. 2011e)
- The wrapping by an aqueous solution of a starch–iodine complex (Famá et al. 2011)

The last method is especially interesting regarding starch-based nanocomposites. This method was initiated by Star et al. (2002), in which the helical conformation of amylose was initially formed by complexation with iodine (or bromide (Casey et al. 2005)), and then a subsequent treatment with SWCNTs displaced the iodine molecules inside the helix. The other methods for the wrapping of CNTs with starch (either amylose or amylopectin) have also been reported (Fu et al. 2007; Kim et al. 2003; Lii et al. 2003; Stobinski et al. 2003; Yang et al. 2008). While the original purpose of these studies was just to improve the dispersion stability of CNTs in aqueous solutions, the described methods may be of great interest for the preparation of starch–CNT nanocomposites since it could be a very smart solution to address the two main issues mentioned earlier, namely, the homogeneous dispersion of nanotubes and a good interfacial adhesion between the phases.

While it is relatively easy to prepare modified CNTs in a non-covalent way (e.g., the wrapping with starch and the modification by a surfactant), the covalent modification of CNT surfaces seems more promising since strong interactions between the CNTs and the starch are expected for the dispersion of the CNTs into a starch matrix and the enhancement of the nanofiller–matrix interface. The successful grafting of different polysaccharides such as chitosan (Wu et al. 2007), cellulose acetate (Ke 2010), and starch (Yan et al. 2011) onto the CNT surfaces has already been reported, although these kinds of modified CNTs have never been

tried into starch-based nano-biocomposites. Starch-grafted MWCNTs, prepared by covalently grafting starch onto carboxylated MWCNTs (Yan et al. 2011), are especially interesting in regard to the same chemical nature of the grafts and the starch matrix. Nevertheless, one should bear in mind that covalent sidewall functionalization usually destroys the extended networks on the CNT surfaces, diminishing their mechanical and electronic properties (Famá et al. 2011).

9.2 Preparation Techniques

Starch–CNT nano-biocomposites were mostly prepared by a convenient solution process assisted by sonication and strong stirring, which are necessary to achieve dispersion of CNTs in a solvent. However, care should be taken since the sequence of adding ingredients might affect the nanofiller dispersion, the gelatinization/plasticization, and thus the final structure of the nano-biocomposite. Summarized from the literature, three ways of solution preparation process were practiced:

(a) The nanofiller was first dispersed in a water solution (with surfactant or starch–iodine complex) and treated with sonication, and then starch and glycerol were added to the same solution with strong stirring and increased temperature for gelatinization, followed by casting (Bonnet et al. 2007; Famá et al. 2012, 2011; Liu et al. 2011e; Ma et al. 2008d).
(b) The nanofiller was dispersed in water with sonication; in parallel, starch and glycerol were dispersed in water with increased temperature and strong stirring for gelatinization. After that, the two dispersions were mixed with stirring, followed by casting (Cao et al. 2007).

By the first method, there might be an anti-gelatinization/plasticization effect of the nanofiller, which deteriorates the cohesion of the starch matrix and thus the final properties (Liu et al. 2011e). For better gelatinization of starch, the second method could be better.

9.3 Effect of Carbon Nanotube Addition

The incorporation of MWCNTs generally increased the ultimate tensile strength (Cao et al. 2007; Famá et al. 2011; Ma et al. 2008d), Young's modulus (Cao et al. 2007; Famá et al. 2011; Ma et al. 2008d), T_g (Cao et al. 2007; Famá et al. 2012, 2011), and thermal decomposition temperature (Liu et al. 2011e) and decreased water sensitivity (Cao et al. 2007; Famá et al. 2012), showing improved performance. Homogeneous dispersion of the nanofiller in the matrix and strong interactions between the nanofiller and the matrix can account for the property enhancement (Cao et al. 2007; Famá et al. 2012, 2011; Liu et al. 2011e; Ma et al. 2008d), which relies on the proper MWCNT modification. Specially, the reasons for the variation in the different properties by the incorporation of MWCNTs are summarized below:

- Glass transition: a shift in the T_g (of the starch-rich phase) to higher temperature can be attributed to the occurrence of interactions between the starch and the stiff MWCNTs, which reduced the flexibility of the starch molecular chains in contact with the MWCNT surfaces (Cao et al. 2007). Moreover, a depression in the loss tangent (tan δ) in DMA indicates the reduction in the number of the mobile chains during the relaxation process (Famá et al. 2012; Rao and Pochan 2006).
- Thermal melting: a reduction in the melting enthalpy could reflect the restraint of the starch recrystallization, with the reason being the good dispersion of the MWCNTs and the formation of MWCNT–starch interactions spatially preventing the starch molecules from moving, interacting, and crystallizing again (Cao et al. 2007; Ma et al. 2008d).
- Mechanical properties: higher values of the tensile strength and Young's modulus can be ascribed to (a) the formation of an isotropic, three-dimensional nanotube network by the MWCNT interactions, which inhibits the crack propagation (Ma et al. 2008d); (b) the interfacial adhesion between the MWCNTs and the starch matrix, which allows the effective stress transfer from the matrix to the nanofiller (Ma et al. 2008d); and (c) the increase in the T_g, which contributes to the increase in the stiffness (Famá et al. 2012, 2011).
- Thermal stability: there are several reasons for an increase in the thermal decomposition temperature: (a) MWCNTs are more stable than starch, and (b) a barrier effect of the nanotubes and their formed aggregates hinders the diffusion of the degradation products from the bulk of the polymer into the gas phase (Chatterjee and Deopura 2006; Liu et al. 2011e; Yang et al. 2005).
- Moisture sensitivity: a reduction in moisture sensitivity can be associated with (a) the nanofiller–matrix interactions which suppress the swelling of the starch matrix when submitted to a highly moist atmosphere (Cao et al. 2007), (b) the relatively low water sensitivity of MWCNTs which reduces the moisture sensitivity of the whole nano-biocomposite system (Cao et al. 2007), and (c) the increase in the T_g (for the starch-rich phase) which significantly decreases the free volume where the water diffusion occurs (Famá et al. 2012; Pinnavaia and Beall 2000).

While a small amount of MWCNT addition can result in significant improvement in the properties, a high loading level of MWCNTs may have some counter-effect. For example, for starch-based nano-biocomposites reinforced with MWCNTs treated by a surfactant, Ma et al. (Ma et al. 2008d) found that the maximum value of the tensile strength occurred at the 3.8 % level of MWCNT addition, while, in another study by Liu et al. (2011e), the optimized addition level of carboxylated MWCNTs was 1.5 %. Noting that starch was added to the MWCNT solution during the preparation of nano-biocomposites, it was suggested that high content of the MWCNTs could not only deteriorate the plasticization of starch which destructed the efficient load transfer from the starch matrix to the ultra-strong MWCNTs (as demonstrated by SEM and FTIR) but also destructed the continuity of the starch matrix (Liu et al. 2011e).

It is worth noting that there have been discrepancies over the trends of the property changes with the MWCNT addition. For example, while it is no surprise to observe a decrease in the toughness or elongation at break with the loading of a nanofiller like MWCNTs into a starch matrix, which was attributed to the spatial restraint of the slippage movement of the starch molecules with good dispersion of the MWCNTs, yielding the increasingly brittle samples (Ma et al. 2008d), there have also been some exciting reports showing that MWCNTs could enhance the toughness (Famá et al. 2011) or elongation at break (Cao et al. 2007; Famá et al. 2011; Liu et al. 2011e) in addition to the tensile strength and Young's modulus. Again, this is a consequence of the homogeneous dispersion of the MWCNTs in the starch matrix and the really strong interfacial adhesion, resulting from the proper nanofiller modification, which will be further detailed.

9.4 Effect of Nanofiller Modification

The effects of two kinds of modification of MWCNTs, namely, carboxylation and starch–iodine complex wrapping, are discussed here regarding their effect on the nanofiller dispersion and interface adhesion and thus the property changes.

For the carboxylated MWCNTs, the acid-treatment process can incorporate various polar groups (carboxylic, carbonyl, and hydroxyl groups), which can improve the hydrophilicity and reduce the agglomeration of the MWCNTs (Cao et al. 2007; Liu et al. 2011e). Interestingly, Liu et al. (2011e) showed that, compared with water, glycerol containing much hydroxyl groups could dramatically increase the dispersion of these MWCNTs in a solution and restrain their agglomeration (Liu et al. 2011e). As a result, the hydrogen-bonding interactions and thus the compatibility between the acid-treated MWCNTs and a starch matrix could be enhanced, resulting in improvement in the mechanical performance of starch–MWCNT nano-biocomposite films (Cao et al. 2007). It was shown that the tensile strength and Young's modulus did not come at the expense of the elongation at break (which usually exists in conventional filled polymer systems), which increased from 30 % to 42 % with the MWCNT addition level up to 1 % and then slightly decreased with higher MWCNT content (which might be due to some degree of aggregation of the MWCNTs) (Cao et al. 2007). Furthermore, the carboxyl groups on the surface of the MWCNTs could increase the interfacial interactions between the MWCNTs and the starch matrix, which improved the degradation activation energy (Liu et al. 2011e).

Very recently, Famá et al. (2012, 2011) experimented with starch-based nano-biocomposites reinforced by MWCNTs wrapped with starch–iodine complex, where the starch used to wrap the MWCNTs was the same as the one used as the matrix. The SEM results showed that, because of the wrapping, the failure around an MWCNT agglomerate occurred within the matrix rather than between the MWCNTs and their starch coating, indicating strong MWCNT–starch adhesion (Famá et al. 2011). As a consequence, the efficient load transfer from the matrix to

the nanofiller was attained, and the high tensile strength and elongation at break of the MWCNTs were transferred to the biocomposites (Famá et al. 2011). Therefore, the good interfacial adhesion obtained avoided the formation of holes and kept a high deformation, resulting in an increase in the toughness (defined as the work done to break a sample which is determined by integrating the stress–strain curve) with the nanofiller loading (Famá et al. 2011). This work is remarkable since very small content of MWCNTs, i.e., 0.055 %, could result in great changes in the material performance as a result of the exceptionally well-dispersed MWCNT and optimized interfacial adhesion (Famá et al. 2011).

9.5 Electrical Conductivity

Besides the properties discussed above, the addition of CNTs into a starch matrix gives the resulting nano-biocomposite electrical conductivity (Liu et al. 2011e; Ma et al. 2008d). For this purpose, an amorphous state of the starch matrix should be advantageous because the recrystallization of starch could reduce the good dispersion of the MWCNTs in the matrix (Ma et al. 2008d). On the other hand, the electrical conductivity can be influenced by the water content and the MWCNT content of the nano-biocomposites (Liu et al. 2011e; Ma et al. 2008d). As demonstrated in a study by Ma et al. (2008d), higher water content would result in an increase in the electrical conductivity in a very sensitive way, with the conductivity (y) versus water content (x) relationship being described with a second-order polynomial ($y = B_2x^2 + B_1x + B_0$); an increase in the MWCNT content decreased the sensitivity of conductivity of the nano-biocomposites to water (both the monomial coefficient B_1 and the binomial coefficient B_2 approached more to zero), until an electrical percolation threshold was reached when the effect of water content was eliminated. If the water content was fixed at 0 %, the electrical conductivity firstly observed a gradual increase with increasing the MWCNT content and then a stepwise increase when a specific level (3.8 wt %) of MWCNT content was reached (Liu et al. 2011e; Ma et al. 2008d). The reasons accounting for these phenomena are:

(a) Although water is advantageous to improve the conductivity of a starch matrix (Meyer 1998) by improving the movement of the starch polymer chains (Van Soest and Knooren 1997), the introduction of MWCNTs and good dispersion of the MWCNTs in the matrix spatially restrained the movement of the starch polymer chains even at high water content (Ma et al. 2008d).
(b) While the gradual increase in the conductivity with the increase in the MWCNT content at a low MWCNT addition level (<2.85 wt %) was due to the formation of a conductive network through hopping and tunneling processes (Lee et al. 2007a), the creation of an interconnected structure of the MWCNTs allows a very high percentage of electrons to flow through the sample at an applied electric field at the MWCNT content higher than a specific value (Ma et al. 2008d).

10 Starch-Based Nano-biocomposites Reinforced by Graphite/Graphite Oxide

10.1 Graphite and Graphite Oxide

Graphite is composed of layers of graphene which has a structure of one-atom-thick two-dimensional individual sheet composed of sp^2-hybridized carbon (Geim and Novoselov 2007). It combines the lower price and the layered structure of phyllosilicates with the superior thermal and electrical properties of CNTs. As a result, graphite can also be used to produce nanocomposites with competitive multifunctional properties (Jiang et al. 2010; Kim and Macosko 2009; Wei et al. 2009). While as-prepared graphite cannot be dispersed in water or organic solvent, which makes the fabrication of nanocomposites difficult, graphite oxide (GO) is hydrophilic and can form strong physical interactions with a polymer like starch due to its various oxygen functional groups including hydroxyls, epoxides, carbonyls, and carboxyls (Li et al. 2011b). Therefore, GO can be regarded as a promising nanofiller for the development of high-performance starch-based nano-biocomposites. On the other hand, because of the layered structure of GO just like that of a phyllosilicate, a GO-reinforced polymer nanocomposite is expected to have an intercalated or exfoliated structure, and the mechanisms for discussing phyllosilicate-reinforced polymer nanocomposites might also be useful to explain the property variation of GO-reinforced nanocomposites.

10.2 Nanocomposites Reinforced by Graphite Oxide

Li et al. (2011b) pioneered the work on starch-based nano-biocomposites reinforced by GO, of which the content was up to 2 wt %. GO was first dispersed in a water solution and treated with sonication, and then starch and glycerol were added to the same solution with strong stirring and increased temperature for gelatinization, followed by casting (Li et al. 2011b). They found that hydrogen bonding formed between the GO and the starch matrix, as revealed by FTIR, and that the nanofiller was well dispersed (exfoliated) when the GO loading was low, whereas aggregation occurred when the GO loading was high, as demonstrated by XRD and atomic force microscopy (AFM) (Li et al. 2011b). With increasing the GO loading level, the tensile strength, Young' modulus, and thermal decomposition temperature were continuously increased, the ultraviolet (UV) transmittance and elongation at break were decreased, and the moisture uptake percentage first decreased to a lowest value and then slightly increased (Li et al. 2011b). For the improvement in the mechanical properties, the good dispersion of the GO within the starch matrix and the strong interfacial interactions between the GO and the matrix were responsible for the stress transfer from the matrix to the nanofiller, although they also restrained the slippage movement of the starch molecules, which resulted in the decrease in the elongation at break (Li et al. 2011b). For the variation in water resistance, the layered structure of the nanofiller provided tortuous pathways, and the strong nanofiller–matrix interactions reduced the diffusion of water molecules in the materials (Li et al. 2011b).

11 Starch-Based Nano-biocomposites Reinforced by Carbon Black

11.1 Carbon Black

CB is a very fine powdered form of amorphous elemental carbon with a high surface area-to-volume ratio. It plays an important role in the improvement in the mechanical and/or electrical properties of high-performance polymeric materials (typically rubber), due to the formation of a physically bonded flexible filler network and strong polymer–filler couplings, which refer to a high surface activity and specific surface of the filler particles (Donnet et al. 1993; Kluüppel et al. 2007; Kraus 1965; Payne 1962, 1963, 1964, 1965a, b). As the most widely used conductive filler for polymers, a high-structural CB (original millimeter-sized CB particles) consists of many primary nanoparticles fused together in a grapelike aggregate (Norman 1970). Due to the high surface tension, flocculation in a quiescent melt can be formed by dispersing the CB aggregates during processing, which promotes the formation of a conductive network (Böhm and Nguyen 1995). As demonstrated in other polymer systems, this process depends on the compatibility between CB and the matrix, which is related to the similarity of the surface tension of them (Miyasaka et al. 1982; Sumita et al. 1991), and the flocculation process, which relies on the viscosity of the matrix (Breuer et al. 1997; Tchoudakov et al. 1996; Yu et al. 2005b). In Sect. 11.2, the development of novel starch–CB nano-biocomposites by different processing techniques will be discussed.

11.2 Nanocomposites Reinforced by Carbon Black

Ma et al. (2008a) compared two preparation methods, i.e., melt extrusion and solution casting with microwave radiation, on the properties of starch-based nano-biocomposites reinforced by CB. It was revealed that the samples prepared by solution method contained the CB in good dispersion, whereas isolated agglomerates of the CB particles existed in those prepared by melt extrusion. As a consequence, the former approach shows better tensile strength, WVP, and electrical conductivity. It was suggested that the CB particles could act as physical cross-linking points of the starch molecules, which resulted in the increased tensile strength, although the dispersion of the CB particles in the matrix also spatially restrains the slippage movement of the starch molecules, resulting in a decrease in the elongation at break (Ma et al. 2008a). A decrease in the WVP arises from the longer and tortuous diffusive pathways with the dispersion of the CB in the matrix (Ma et al. 2008a). Moreover, the CB could form an electrical conductance network in the matrix (Ma et al. 2008a). The same research group (Ma et al. 2008b) also investigated the effect of the glycerol content on the samples prepared by solution casting with microwave irradiation and found that lower glycerol content (and thus lower viscosity) facilitated the flocculation of the CB during the solution process and thus a better electrical conductive network in the biocomposite.

12 Nanocomposites Reinforced by Metalloid Oxides, Metal Oxides, and Metal Chalcogenides

Metalloid oxides (e.g., silicon dioxide [SiO_2] and antimony trioxide [Sb_2O_3]), metal oxides (e.g., zinc oxide [ZnO], titanium dioxide [TiO_2], and hydrous zirconium dioxide [$ZrO_2{\cdot}nH_2O$]), and metal chalcogenides (e.g., cadmium sulfide [CdS] and cadmium selenide [CdSe]) are grouped together in this section because of their similar chemical categories, preparation methods, and nanofiller reinforcement mechanisms. Novel applications are expected for starch-based nano-biocomposites reinforced by this type of nanofillers. Particularly, metal oxides and chalcogenides are normally semiconductor materials. The incorporation of such a filler into a polymer matrix can result in nanocomposites to be used as components for photovoltaic solar cells, light-emitting diodes, photodiodes, and gas sensors (Godovsky 2000). In addition, Sb_2O_3 is an excellent UV filter (Tigau et al. 2005). Thus, nanocomposites filled with Sb_2O_3 might find applications in UV light-emitting devices and solar cell technology (Chang et al. 2009; Zheng et al. 2009a). Furthermore, TiO_2, particularly in the anatase form, shows a photocatalytic activity under UV light and is suitable for developing nanocomposites for environmental purification (Yun et al. 2011).

The paragraphs below will firstly provide general discussion on starch-based nano-biocomposites reinforced by different metalloid oxides, metal oxides, and metal chalcogenides. Then, nano-biocomposites based on starch blended with PVA, and reinforced by SiO_2, will be separately discussed regarding the particular intermolecular interaction and network structure in such a multiphase system.

12.1 Nanofillers and Preparation Techniques

In order to fabricate a starch-based nano-biocomposite reinforced by a metalloid oxide, metal oxide, or metal chalcogenide, only solution methods have been used, which are listed below:

(a) The nanoparticles (SiO_2 (Wu et al. 2009b; Xiong et al. 2008) and TiO_2 (Yun et al. 2011)) are directly added into a starch matrix dispersion.
(b) The nanoparticles (ZnO (Ma et al. 2009; Yu et al. 2009) and Sb_2O_3 (Chang et al. 2009; Zheng et al. 2009a)) are firstly synthesized with a stabilizing template, and then the resulting encapsulated nanoparticles are incorporated into a starch dispersion.
(c) The nanoparticles (SiO_2 (Tang et al. 2008a; Yao et al. 2011), TiO_2 (Liao and Wu 2008), CdS (Radhakrishnan et al. 2007), and CdSe (Zhao et al. 2008)) are directly synthesized in a starch dispersion, which acts not only as the stabilizer but also as the matrix of the resulting nano-biocomposite (sol–gel method).

The first method has only been applied to nano-SiO_2, which is a kind of amorphous powder with a molecular structure that is a three-dimensional net, the

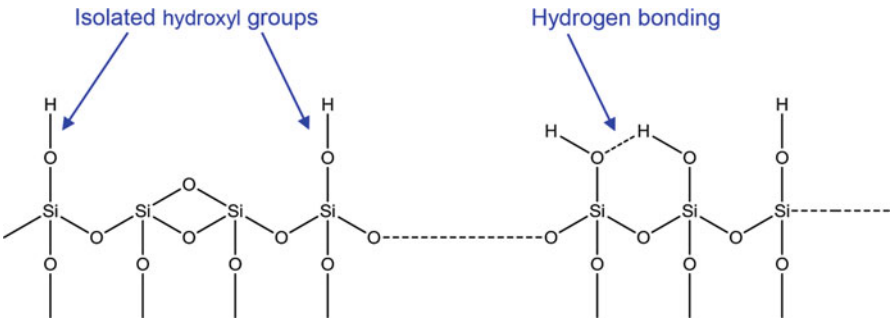


Fig. 16 Structure of nano-SiO_2

structure of which is shown in Fig. 16. Nano-SiO_2 deviates from a stable silicon–oxygen structure for the lack of oxygen in its surface. Its molecular formula is SiO_{2-x}, in which x ranges from 0.4 to 0.8 (Tang et al. 2009; Xiong et al. 2008). Because of its small size (30–60 nm), large specific surface area (>400 m^2/g), high surface energy, as well as a lot of unsaturated chemical bonds and hydroxyl groups on the surface, nano-SiO_2 is easy to disperse into a polymer matrix (Tang et al. 2009; Wu et al. 2009b; Xiong et al. 2008). However, the direct addition of nano-SiO_2 into a starch slurry normally requires intensive sonication and/or shearing; otherwise, large aggregates of the nanoparticles will remain, resulting in less improved or even deteriorated properties (Frost et al. 2011; Wu et al. 2009b). A better solution to achieve good dispersion of SiO_2 nanoparticles in a starch matrix is by using the third method (Tang et al. 2008a; Yao et al. 2011). It is worth noting that the sol–gel method has recently been extended to a reactive extrusion (REX) process for producing starch–PVA nano-biocomposites (Frost et al. 2011), which will be detailed in Sect. 12.3.

The nanoparticles of metal oxides and chalcogenides are mainly prepared by the second and third methods. For the nanoparticle synthesis in the second method, polysaccharides such as native starch (Rodriguez et al. 2008), soluble starch (Chairam et al. 2009; Li et al. 2007; Ma et al. 2009; Radhakrishnan et al. 2007; Vigneshwaran et al. 2006; Wei et al. 2004), and CMC (Chang et al. 2009; Liu et al. 2011a; Yu et al. 2009; Zheng et al. 2009a) have been shown to be good stabilizers. Polysaccharides can form complexes with divalent metal ions due to their high number of coordinating functional groups (hydroxyl and glucoside groups) (Taubert and Wegner 2002) and present dynamic supramolecular associations facilitated by inter- and intramolecular hydrogen bonding, which can act as templates for metal nanoparticle growth (Raveendran et al. 2003). As a result, metal ions can preferentially be associated with a polysaccharide, of which the nucleating effect can promote the initial crystal growth of the metal oxide, improve the stability of the nanoparticles in water, and prevent the aggregation of the nanoparticles (Chang et al. 2009; Ma et al. 2009; Yu et al. 2009; Zheng et al. 2009a). After synthesis, there is normally strong binding in the ZnO nanoparticles, although no obvious formation of covalent bonds occurs between

the stabilizer (soluble starch or CMC) and the ZnO (Ma et al. 2009; Yu et al. 2009). In contrast, for the CMC-stabilized $ZrO_2 \cdot nH_2O$, interaction exists between the ZrO_2 and the C–O groups of the CMC (Liu et al. 2011a; Pawlak and Mucha 2003). On the other hand, by using the third method, the average size (3.6–5 nm) of CdS nanoparticles can be dependent on the initial concentration of cadmium acetate during the sample preparation (Radhakrishnan et al. 2007).

12.2 Effects of the Addition of Metalloid Oxides, Metal Oxides, and Chalcogenides

As reported in the literature, uniform dispersion of a nanofiller in a starch matrix and strong interfacial adhesion through hydrogen bonding between the nanoparticles and the matrix could normally be achieved, especially for polysaccharide-encapsulated metal nanoparticles due to the similar chemical structures of the stabilizer and the matrix (Chang et al. 2009; Liu et al. 2011a; Ma et al. 2009; Yu et al. 2009; Zheng et al. 2009a). As a result, improvement in the performance could be normally observed, even though some aspects of property deterioration have also been reported. The details are summarized below:

- Mechanical properties: increases in the tensile strength (Chang et al. 2009; Liu et al. 2011a; Ma et al. 2009; Wu et al. 2009b; Yu et al. 2009; Yun et al. 2011; Zheng et al. 2009a), Young's modulus (Ma et al. 2009; Yu et al. 2009), and the storage modulus (DMA) (Ma et al. 2009; Yu et al. 2009) and a decrease in the elongation at break (Chang et al. 2009; Liu et al. 2011a; Zheng et al. 2009a) have been reported. These could be ascribed to the decreased mobility of the polymer chains in the presence of the nanofiller, which has strong interactions with the matrix (ZnO (Ma et al. 2009; Yu et al. 2009), $ZrO_2 \cdot nH_2O$ (Liu et al. 2011a)).
- Glass transition: an increase in the T_g (Ma et al. 2009; Yu et al. 2009) could result from the reduced free volume with the presence of the nanoparticles which act as physical joints (ZnO (Ma et al. 2009; Yu et al. 2009)).
- Water resistance: a decrease in the WVP with the addition of ZnO (Ma et al. 2009; Yu et al. 2009), ZrO_2 (Liu et al. 2011a), or Sb_2O_3 (Chang et al. 2009) nanoparticles was reported, which was ascribed to the introduction of the nanoparticles providing fewer and more tortuous pathways for water molecules to diffuse. However, Radhakrishnan et al. (2007) observed an increase in the water uptake of starch–CdS nano-biocomposites at specific relative humidity and attributed this phenomenon to the higher exposed hydroxyl groups in the presence of the nanoparticles which broke the intermolecular interactions of the starch matrix.
- Melting/crystallinity: lower melting temperature but higher melting enthalpy was observed for starch–CdS nano-biocomposites by Radhakrishnan et al. (2007), who proposed that, though the CdS nanoparticles have a nucleating effect for the starch chain recrystallization (facilitated by the water diffusion), smaller crystals and broader melting crystal size distribution were also generated.

- Thermal stability: a decrease in the thermal decomposition temperature but an increase in the residual weight at the end of thermal decomposition was observed for nano-biocomposites reinforced by $ZrO_2 \cdot nH_2O$ (Liu et al. 2011a), CdS (Radhakrishnan et al. 2007), CdSe (Božanić et al. 2009), and Sb_2O_3 (Chang et al. 2009). While Liu et al. (2011a) and Chang et al. (2009) ascribed this to the poor thermal stability of the CMC in the nanoparticles, Radhakrishnan et al. (2007) and Božanić et al. (2009) explained that the increased hydroxyl groups (due to the presence of the nanoparticles which broke the intermolecular interactions of the starch matrix) could result in thermal condensation between them, which could consequently direct the reaction toward carbonization instead of the formation of volatile components (Zhang et al. 2002),
- UV–visible light absorbance/transmittance: compared to the pure starch matrix, nano-biocomposites reinforced by ZnO (Ma et al. 2009; Yu et al. 2009) and Sb_2O_3 (Chang et al. 2009) displayed increased UV–Vis absorbance due to the quantum confinement effect of the nanofiller, and both the starch–CdS (Radhakrishnan et al. 2007) and starch–CdSe (Božanić et al. 2009) nano-biocomposite films showed improved light transparency.

A higher addition level of nanofiller may result in the agglomeration of nanoparticles (Chang et al. 2009; Liu et al. 2011a; Ma et al. 2009; Yu et al. 2009; Zheng et al. 2009a), which is unfavorable for the performance improvement of the nano-biocomposites. In a study on the starch–SiO_2 nano-biocomposites for textile applications, for example, Wu et al. (2009b) found that the best tensile strength and wear resistance could be achieved when the nano-SiO_2 addition level was 4 % and 3 %, respectively, with the reasons being the even distribution of the nanoparticles and the strong nanofiller–matrix interactions at these levels, while the lubricating effect of the rigid nanofiller could also contribute to improved abrasion resistance.

12.3 Starch–Poly(vinyl Alcohol)–Silicon Dioxide Nanocomposites

There have been a series of studies (Frost et al. 2011; Tang et al. 2009, 2008a; Xiong et al. 2008; Yao et al. 2011) on SiO_2-reinforced nanocomposites in which starch and PVA were the matrix. This may improve the mechanical properties and water resistance of starch–PVA films which are still lower than those of conventional polymers (Tang and Alavi 2011).

Xiong et al. (2008) utilized a solution casting method to prepare this kind of nanocomposites. It was found that the addition of nano-SiO_2 resulted in the formation of hydrogen bonds in the hybrid materials, whereas the intermolecular hydrogen bonding of the starch was decreased. Meanwhile, chemical bonds of C–O–Si were also formed in the nanocomposites. These contributed to the increased miscibility and compatibility between the starch and the PVA. In addition, the addition of the nano-SiO_2 had no influence on the crystalline type of the films, but decreased the crystallinity because of the disturbance of the parallel arrangement of the starch–PVA chains. As a result of the structural changes, the

nanofiller played a key role as cross-linking points and restricted the movement of the chains. Therefore, the tensile strength, elongation at break, and light transmittance were increased and the water absorption was decreased. Nevertheless, biodegradation as a whole was not affected though there was a lower degradation rate at the early stage. Following this study, Tang et al. (2009) further investigated the effect of the nano-SiO_2 content (1–5 wt %, on the basis of starch and PVA) on the changes in the properties of the nanocomposites. They found that 3 wt % was the optimized level of the nano-SiO_2 for the best tensile strength and the lowest water absorption. The reduced tensile strength with the higher nano-SiO_2 content was due to the intensification of the elastic collision of the nanoparticles which showed aggregation and had phase separation with the matrix. In addition, the high surface energy and plenty of free hydroxyl groups of the nano-SiO_2, which is beneficial to interact with water, might have a counter-effect on moisture resistance when the nanofiller content was high (Tang et al. 2009).

Tang et al. (2008a) and Yao et al. (2011) used a sol–gel method to fabricate starch–PVA–SiO_2 composites, in which tetraethyl orthosilicate (TEOS) was used as the precursor to obtain SiO_2 in situ in the polymer solution. A network structure in the composites was formed by combining the SiO_2 with the starch–PVA with hydrogen and C–O–Si bonding (Tang et al. 2008a; Yao et al. 2011). The trends of changes in the properties (mechanical properties, water sensitivity, light transmittance, and biodegradation) with the SiO_2 content (Tang et al. 2008a; Yao et al. 2011) were similar to those in the previous studies (Tang et al. 2009; Xiong et al. 2008). Using the same principle, Frost et al. (2011) made the first trial of using a continuous REX process to produce films of such composites, where the TEOS-to-SiO_2 conversion efficiency was up to 41.3 % and the actual SiO_2 content was up to 1.24 wt %. The resulting films had spherical SiO_2 agglomerates of size ranging from 20 to <1 μm, which were responsible for the increased tensile strength, Young's modulus, and storage modulus (DMA).

In addition to product property improvement, it is noteworthy that SiO_2 can also be used to adjust the viscosity of a polymer and thus to improve the miscibility of this polymer with starch in a melt blending process (Bélard et al. 2009). More details are given in a recent review paper (Xie et al. 2012).

13 Nanocomposites Reinforced by Layered Double Hydroxides

The LDH structure is referred to as the natural hydrotalcite and described by the ideal formula $[M^{II}_{1-x}M^{III}_{x}(OH)]_{intra}[A^{m-}_{x/m} \cdot nH_2O]_{inter}$, where "$M^{II}$" and "$M^{III}$" are metal cations, "A" is the anion, and "intra" and "inter" denote the intralayer domain and the interlayer space, respectively (Leroux and Besse 2001). The structure consists of brucite-like layers constituted of edge-sharing $M(OH)_6$ octahedra (cf. Fig. 17) (Hofmeister and Platen 1992; Leroux and Besse 2001). Partial "M^{II}" and "M^{III}" substitution induces a positive charge for the layers, balanced with the presence of the interlayered anions (Leroux and Besse 2001).

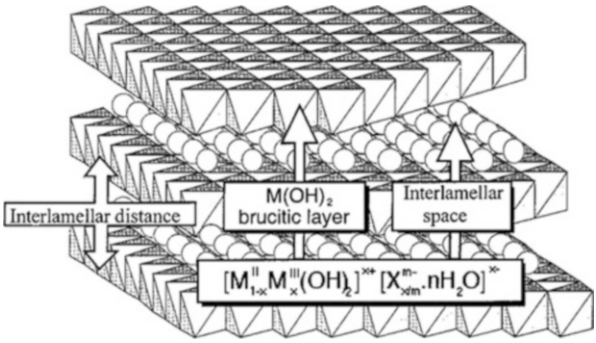


Fig. 17 Scheme of the typical LDH structure (Reprinted with permission from Leroux and Besse (2001). Copyright 2001 American Chemical Society)

Chung and Lai (2010) compared an LDH synthesized in a starch matrix (unmodified maize starch or acid-modified maize starch), which involved fast LDH nuclei precipitation followed by a hydrothermal treatment that simultaneously leached the starch molecules from the granules and aged the LDH nuclei, with one synthesized without the starch matrix. They found that the presence of starch during the synthesis effectively inhibited the stacking of the clay sheets, with the crystal size corresponding to approximately seven brucite-like layers (Chung and Lai 2010). However, no intercalated structure in the starch–LDH biocomposites was indicated, with the reason being ascribed to the rigid structure of the polysaccharide chains (Chung and Lai 2010). The LDH tended to present aggregated morphology in the unmodified maize starch, whereas almost individual dispersion of the LDH was observed in the acid-modified starch. This is due to the lower viscosity of the acid-modified starch. Unfortunately, even the acid-modified starch–LDH biocomposites only showed an increase in Young's modulus, whereas the crystallinity, tensile strength, moisture sensitivity, and transparency were hardly affected. These results could be possibly attributed to the poor interaction between the LDH and the matrix (Chung and Lai 2010).

Alternatively, Wu et al. (2011) used carboxymethyl cellulose sodium (CMC) as a stabilizer to synthesize an LDH, of which the size and number of the stacked sheets were similar to that in the study by Chung and Lai (2010). However, starch-based biocomposites reinforced by this LDH at a low loading level (6 wt %) were reported to show obviously improved mechanical properties (increased tensile strength) and water resistance (decreased WVP) (Wu et al. 2011). There are two reasons for the improvement: (a) because polysaccharides could form complexes with metal ions due to the high number of coordinating functional groups (hydroxyl and glucoside groups) (Taubert and Wegner 2002), strong associations between the metal ions and the CMC occurred for the nucleation and initial crystal growth of the LDH, and thus the LDH was successfully encapsulated by the CMC and (b) the hydrophilic CMC component and the smaller size of each LDH stack allowed the LDH to be well dispersed in the starch matrix and good interactions between the nanofiller and the matrix were formed because of the CMC component (Wu et al. 2011). However, the biocomposites displayed a decrease in the thermal decomposition temperature because the weak thermal stability of the CMC could

weaken the interactions between the LDH filler and the starch matrix and facilitate the decomposition of the starch (Wu et al. 2011). Furthermore, high LDH content (8 wt %) could result in the agglomeration of the nanofiller in the matrix and thus reduce the mechanical properties and WVP (Wu et al. 2011).

14 Nanocomposites Reinforced by α-Zirconium Phosphate

Synthetic α-ZrP (i.e., $Zr(HPO_4)_2 \cdot H_2O$) exhibits similar structural characteristics to natural MMT clay but has advantages such as high purity and ion exchange capacity and ease of intercalation and exfoliation (Clearfield et al. 1972; Clearfield and Berman 1981; Sun et al. 2005; Wu et al. 2009a). In addition, the particle size and aspect ratio can be manipulated by varying the reaction conditions (Wu et al. 2009a). Wu et al. (2009a) investigated starch-based nano-biocomposite films reinforced by α-ZrP at different loading levels. The results from XRD and SEM indicated that the plasticized starch and the α-ZrP interacted and formed strong hydrogen bonds, resulting in improved compatibility. Compared with the neat plasticized starch, the nano-biocomposite films showed increases in the tensile strength and elongation at break, decreases in the crystallinity and moisture uptake, and a slight decrease in the transparency. The improvement in the mechanical properties was due to the much enhanced nanofiller–matrix interaction since α-ZrP has huge surface area The decrease in the moisture sensitivity was attributed to the decreased number of –OH groups available for the interaction with migrating water molecules. Nevertheless, higher content of the nanofiller could contribute to an adverse effect on the improvement in the properties due to the phase separation and the aggregation of the nanofiller, but not as much to reach back the values of the pure starch film. Besides, the maximum thermal decomposition temperatures of the nano-biocomposite films decreased with an increase in the α-ZrP content, which could be ascribed to the increase in the acidity of the α-ZrP with the increase in the temperature, which induces the decomposition of the glycoside bonds (Wu et al. 2009a).

15 Nanocomposites Reinforced by Hydroxyapatite

The use of HA is mainly for making biomaterials for biomedical applications such as clinical orthopedics because HA has the chemical and crystallographic similarity to the inorganic component of natural bone and has excellent biocompatibility, bioactivity, and osteoconductivity (Murugan and Ramakrishna 2004). The successful use of injection molding to produce HA-reinforced starch–EVA nanocomposites with high mechanical performance for temporary tissue replacement applications has been demonstrated (Reis et al. 1997). Besides, like metalloid oxides, metal oxides, and metal chalcogenides, rodlike nano-HA crystals can be synthesized with controlled shape and size using soluble starch as a template (via an in situ biomimetic process) (Meskinfam et al. 2011; Sadjadi et al. 2010). The bioactivity and biocompatibility of the resulting biocomposites were verified (Meskinfam et al. 2011;

Sadjadi et al. 2010). Sundaram et al. (2008) reported the fabrication of a porous scaffold biomaterial made from nano-HA, gelatin, and starch displaying the appropriate enhanced mechanical properties for bone repair and regeneration.

16 Conclusion

Starch-based nano-biocomposites are novel multiphase systems that can be tuneable via the type, geometry (size and shape), and surface chemistry of the nanofiller, the type/amylose content and chemical modification of the starch, the type and content of the plasticizer(s) and additive(s), and the processing steps and conditions.

A wide variety of nanofillers have been examined with starch matrix. Phyllosilicates have been mostly utilized to develop starch-based nano-biocomposites due to their advantages such as wide availability, low cost, and high aspect ratio and thus vast exposed surface area. In this group of nanofiller, smectite group clays especially MMT are most popular due to their swelling nature and large availability. In addition, polysaccharide nanofillers (typically CNWs and SNPs) represent the second most popular group of nanofillers to realize starch-based nano-biocomposites because of their abundance in nature, the biological sources, and the chemical similarity to starch. Unfortunately, the preparation of these biobased nanoparticles is time consuming and involves acid hydrolysis in multiple steps which is not eco-friendly. Furthermore, studies have also been carried out for incorporating many other nanofillers such as carbonaceous nanofillers (typically CNTs), metalloid oxides (e.g., SiO_2 and Sb_2O_3), metal oxides (e.g., ZnO, TiO_2, and $ZrO_2{\cdot}nH_2O$), and metal chalcogenides (e.g., CdS, CdSe). One of the advantages in utilizing such nanofillers is that they can provide new functionalities to starch-based materials in addition to the general reinforcement. Unfortunately, some of these last nanoparticles (e.g., the CNTs) may have a detrimental impact on the human health and the environment (Helland et al. 2007; Klaine et al. 2008; Lam et al. 2006; Oberdörster et al. 2007). Finally, some much less frequently employed nanofillers are LDH, α-ZrP, and HA.

With the incorporation of the nanofiller, starch-based materials generally show improvement in some of their properties such as mechanical properties (typically tensile strength, Young's modulus, and storage modulus), T_g (of the starch-rich domains), thermal stability, moisture resistance, gas/solvent barrier property, and biodegradation rate. The improvement can be fundamentally ascribed to the homogeneous dispersion of the nanofiller in the matrix and the strong interface adhesion, which can contribute to the formation of a rigid nanofiller network and influence the molecular and crystalline structures in the matrix. Regarding the dispersion, intercalation or even exfoliation is desired for the nano-biocomposites reinforced by layered nanofillers, which can be assisted by some pretreatment/activation methods. To achieve good nanofiller dispersion and strong interface adhesion, the compatibility between the nanofiller and the matrix is the key point to address, although intensive processing means such as shearing, stirring, and sonication

could also help. When phyllosilicates are used, the best nanofiller could be those containing Na^+ or cationic starch as the counter-cation due to better polarity matching with the starch matrix. Besides, while polysaccharide nanofillers normally do not have starch compatibility issues, some modifications have to be used for many other types of nanofillers to increase the compatibility with starch which has a hydrophilic nature. In all these systems, the interactions between the nanofiller and the matrix are usually achieved by hydrogen bonding. However, the nanofiller–matrix interactions can be affected by more factors such as the plasticizer(s)/additive(s), the starch chemical modification, the presence of other polymer(s) in the matrix, and the processing conditions. In spite of the discussion above, some property changes could not be exclusively attributed to the dispersion of the nanofiller in the matrix and the nanofiller–matrix interactions. Typical examples are the moisture resistance, which could be counter-affected by the hydrophilicity of the plasticizer(s) and the nanofiller, and the thermal stability, which could be counter-affected by the functional groups or the modifying/stabilizing polymer of the nanofiller. Furthermore, the nanofiller plays an important role in determining the properties of the nano-biocomposite. The extent of improvement can be related to the aspect ratio/surface area, chemistry, and mechanical properties of the nanofiller, which may have a relationship with its mixing preparation and modification of final structure and properties. A typical example is that the geometry (size and shape) and the crystallinity of cellulose nanowhiskers are highly dependent on the preparation method and conditions, influencing the final performance properties of the nano-biocomposites.

Nevertheless, how the nanofiller affected the crystalline structure and crystallinity of the starch matrix has not been unambiguously elucidated across the literature. Several reasons might account for this: (a) the crystalline structure and crystallinity of starch-based materials can be highly affected by the formulation (e.g., the amylose content of the starch and the type and content of the plasticizer), the processing conditions (e.g., temperature, pressure, shearing, and orientation), and the storing conditions (e.g., time, temperature, and relative humidity); (b) phase separation of the plasticizer, the starch, and/or the nanofiller may exist in the system, with the different domains showing different recrystallization/anti-crystallization behaviors; and (c) effective means are lacking in the accurate thermal and structural characterization of starch. It is significant to note that some of these reasons may also account for the discrepancies in some of the other results such as T_g and moisture resistance.

With improved properties that are comparable to those of traditional petroleum-based polymers such as polyethylene and polypropylene, starch-based nano-biocomposites can be designed into various products such as blown/casting films, extruded sheets, expanded foams, and thermoformed shapes. These can greatly enhance and widen the current applications of starch-based materials. In addition to the general reinforcement, some nanofillers have been reported to provide new functionalities to starch-based materials, such as superabsorption, electroactivity, electroconductivity, electrorheological behavior, UV shielding, drug releasing, scaffolding (in tissue engineering), and environmental purification. Consequently,

starch-based nano-biocomposites have been or are expected to be applied in a wide range of fields such as packaging, agriculture, medicine, and electronics. Furthermore, the renewable resource and inherent environmental friendliness of such materials can justify its wide use for a sustainable future.

In the future research, it is still very important to test new nanofillers to be incorporated into starch for developing promising nano-biocomposites with excellent performance and new functionalities to be competitive in the materials world. Specifically, maintaining homogeneous dispersion of the nanofiller in the matrix and having strong interface adhesion are the key prerequisites. Good dispersion of the nanofiller results in a greater contact area; otherwise, the filler is not a nanofiller (but micron-sized aggregates) and the composite can only be regarded as microcomposite instead of nanocomposite. As pointed out by Schaefer and Justice (Schaefer and Justice 2007), the current nanocomposites are often not "nano enough," resulting in the elusive realization of anticipated properties. They discovered that large-scale disorder is ubiquitous in nanocomposites regardless of the level of dispersion, leading to substantial reduction of mechanical properties (modulus) compared to predictions based on idealized filler morphology (Schaefer and Justice 2007). This problem should also be addressed when developing starch-based nano-biocomposites since heterogeneous dispersion of the nanofiller and phase separation unfortunately also existed in some of the studies examined in the current review. While the manipulation of chemistry might help to some extent, the future research should also address the importance in using processing techniques like extrusion, film blowing, and injection/compression molding, which are more aligned to the efficient industrial production. With such processing, intensive thermomechanical treatment is normally involved, which could possibly play the dominant role in controlling the morphology and structure and thus the performance of starch-based nano-biocomposites. Thus more research is needed regarding how to achieve a well-dispersed structure without adding a detrimental effect to the final properties by starch molecular degradation under thermomechanical treatment.

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Chemically Modified Natural Polysaccharides to Form Gels

50

John Garner and Kinam Park

Contents

Abstract

Polysaccharides have been utilized for a wide variety of industrial, cosmetic, food, and medical applications. The presence of functional groups on polysaccharides has been exploited for chemical modification to prepare polymers with unique properties. Various polysaccharides form hydrogels through physical or chemical cross-linking, and many of them possess environmentally responsive properties, known as smart hydrogels. Polysaccharide-based smart hydrogels are ideal for biomedical and pharmaceutical applications due to their inherent biocompatibility, degradability, and environment sensitivity, such as pH, temperature, and specific biomolecules.

Keywords

Polysaccharides • Chemical modification • "Smart" hydrogels • Chemically cross-linked • Physically cross-linked

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1 Overview on Polysaccharides

Polysaccharides are polymeric carbohydrates in which a large number of carbohydrate repeating units are linked together by glycosidic bonds. In nature these are typically used for energy storage (e.g., starch and glycogen) or for mechanical structure (e.g., cellulose and chitin). Polysaccharides can be divided into homopolysaccharides (homoglycans) having the monosaccharide repeating unit and heteropolysaccharides (heteroglycans or hemicelluloses) having different types of repeating units. In general, these polysaccharides can be either linear by containing, for example, only 1,4-β-glycoside bonds (e.g., cellulose) or branched by containing a mixture of 1,4-β-glycoside bonds as well as other bonds such as 1,6-α-glycoside bonds (e.g., glycogen). Polysaccharides may also contain sugars which are missing an oxygen from a given position (deoxy sugars), sugars with amine units (amino sugars), and sugars with sulfate/sulfonate moieties (sulfate/sulfonate sugars). The presence of the amine and sulfate/sulfonate groups allows for other chemical modifications. Table 1 briefly highlights a few examples of different types of polysaccharides.

A hydrogel is a network of polymer chains which absorb water but do not dissolve due to either physical or chemical cross-links. As polysaccharides are already polymeric in nature and possess a plurality of nucleophilic moieties (hydroxyls and sometimes amines) along their backbone, they are easily converted into hydrogels. This, along with their typically low price and natural biodegradability, makes them attractive starting materials for forming various hydrogels. Linear homoglycans are typically used for mechanical stiffness and strength in hydrogel applications (Chang et al. 2009, 2010; Sannino et al. 2009). Branched and linear heteroglycans, such as locust bean gum, xanthan gum, and others, tend to form supermolecular complexes based on their respective chain entanglement which naturally leads to strong gel formation even without modifications beyond a simple heating–cooling cycle (Fernandes 1991; Lundin and Hermansson 1995; Mannion et al. 1992; Urayama et al. 2008). Notably, aminoglycans possess an amine unit which acts as a stronger nucleophile than hydroxyl units, allowing for a

Table 1 Types of polysaccharides and examples

Linear homoglycans	Branched homoglycans	Linear heteroglycans		Branched heteroglycans
Amylose/ cellulose	Amylopectin	Alginate	Chitin	Galactomannans
Chrysolaminarin	Dextran	Gellan	Chitosan	Guar gum
Curdlan	Schizophyllan	Laminarin	Carrageenan	Locust bean gum
	Scleroglucan	Pullulan	Chondroitin sulfate	Xanthan gum
		Welan		
		Xylan		

From references Pigman et al. (1950), McNaught and Wilkinson (1997), Matthews et al. (1999), McMurry (2000); BeMiller (2007), Varki et al. (2009), Heinze et al. (2012)

greater flexibility in chemical modifications. Additionally, the amine unit is weakly basic, making the polymer pH sensitive (Qu et al. 2000; Risbud et al. 2000; Wang et al. 2004). Figure 1 shows examples of polysaccharide structures.

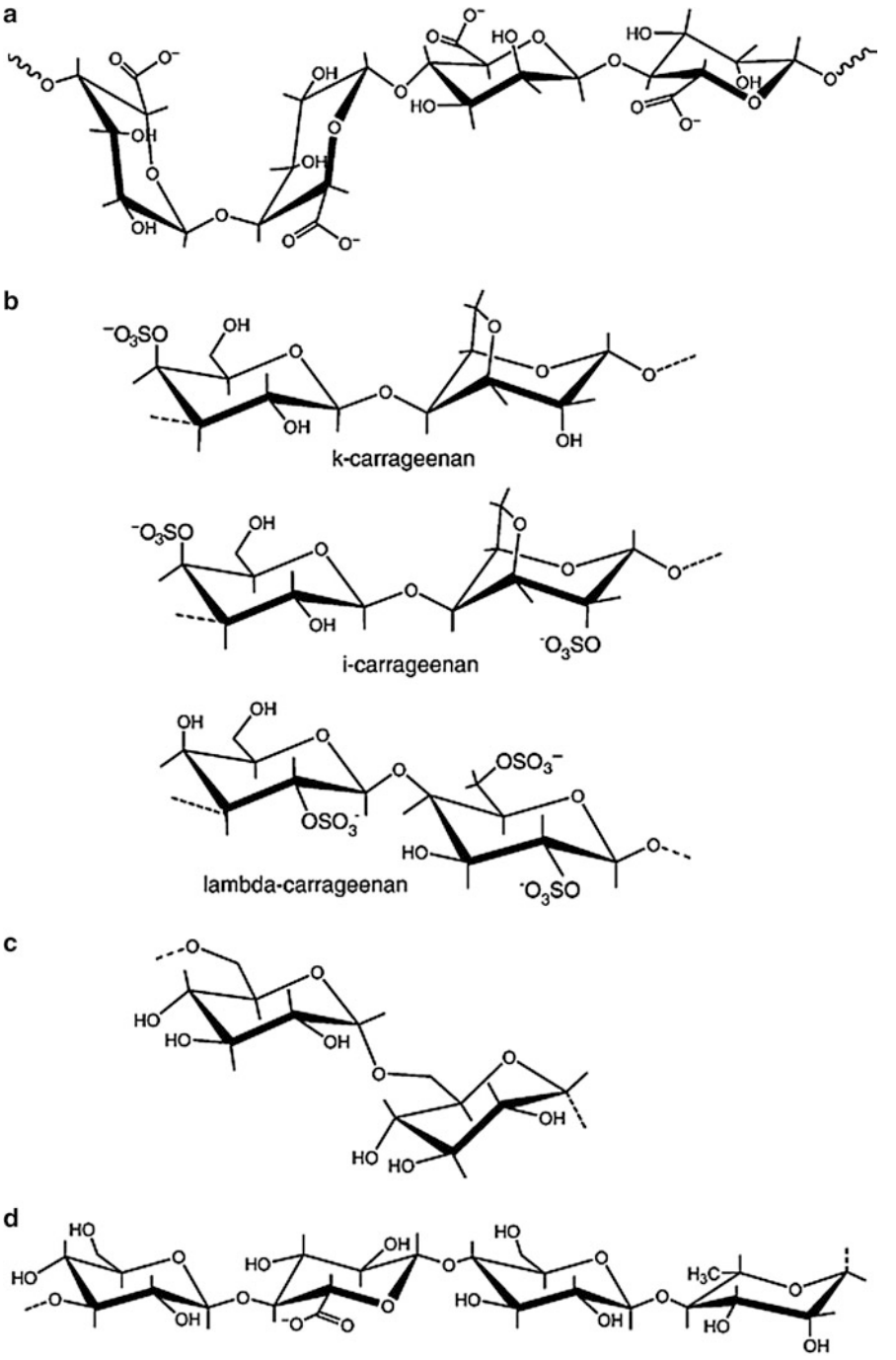
2 Chemical Modifications

With a plurality of nucleophilic groups ranging from alcohols to amines as well as highly reactive acid moieties, polysaccharides represent an ideal platform for a wide array of chemical modifications. However, there are some drawbacks which must be considered for chemical modification of polysaccharides. First, polysaccharides inherently have poor solubility in organic solvents, and thus, they must be processed, at least initially, in highly polar solvents or water. Second, the presence of a multitude of nucleophilic moieties makes it rather difficult to control the extent of modification, i.e., degree of substitution. Third, polysaccharides are susceptible to hydrolysis, although fairly resilient.

2.1 Ether Conjugation

Most chemical modifications of polysaccharides generally rely on the plurality of nucleophiles present along the chain in the form of hydroxyl or amine units. Several hydrogel-forming modifications, particularly of water-insoluble cellulose, have been used to generate water-soluble/gel-like cellulose derivatives for many years. Most of these reaction require cellulose to be activated by conversion to alkali cellulose (cellulose which has been treated with sodium hydroxide) prior to subsequent conjugation (Fig. 2). Once formed into alkali cellulose, the material can be transferred into a variety of derivatives including carboxymethylcellulose (formed by reacting alkali cellulose with sodium monochloroacetate), hydroxyethyl cellulose (formed by reacting alkali cellulose with ethylene oxide), hydroxypropyl cellulose (formed by reacting alkali cellulose with propylene oxide), and methyl-cellulose (formed by reacting alkali cellulose with methyl chloride). Additionally, combinations of modifying agents, e.g., propylene oxide and methyl chloride, can be used to generate polysaccharides with multiple modifications, e.g., hydroxypropyl methylcellulose. These ether derivatives of cellulose are popular for use as thickeners and gums, as they form physical hydrogels (BeMiller 2007).

Carboxymethylcellulose (CMC, Fig. 3a), also known as sodium carboxymethylcellulose to reflect its deprotonated state, notably has high water solubility and is utilized traditionally in foods and other household applications as a thickener and emulsifier. Due to the presence of the carboxymethyl moiety, this material has a pKa of roughly 4.0 (Abu-Ghoush et al. 2009) and can be converted between protonated and deprotonated forms by changing solution pH. Naturally it is fully water soluble and usually does not form a hydrogel. CMC, however, has the capacity to form hydrogels by ionic interaction with multivalent cations such as iron (III) and calcium (Yakup Anca 2000; Davidson et al. 2013). Additionally CMC



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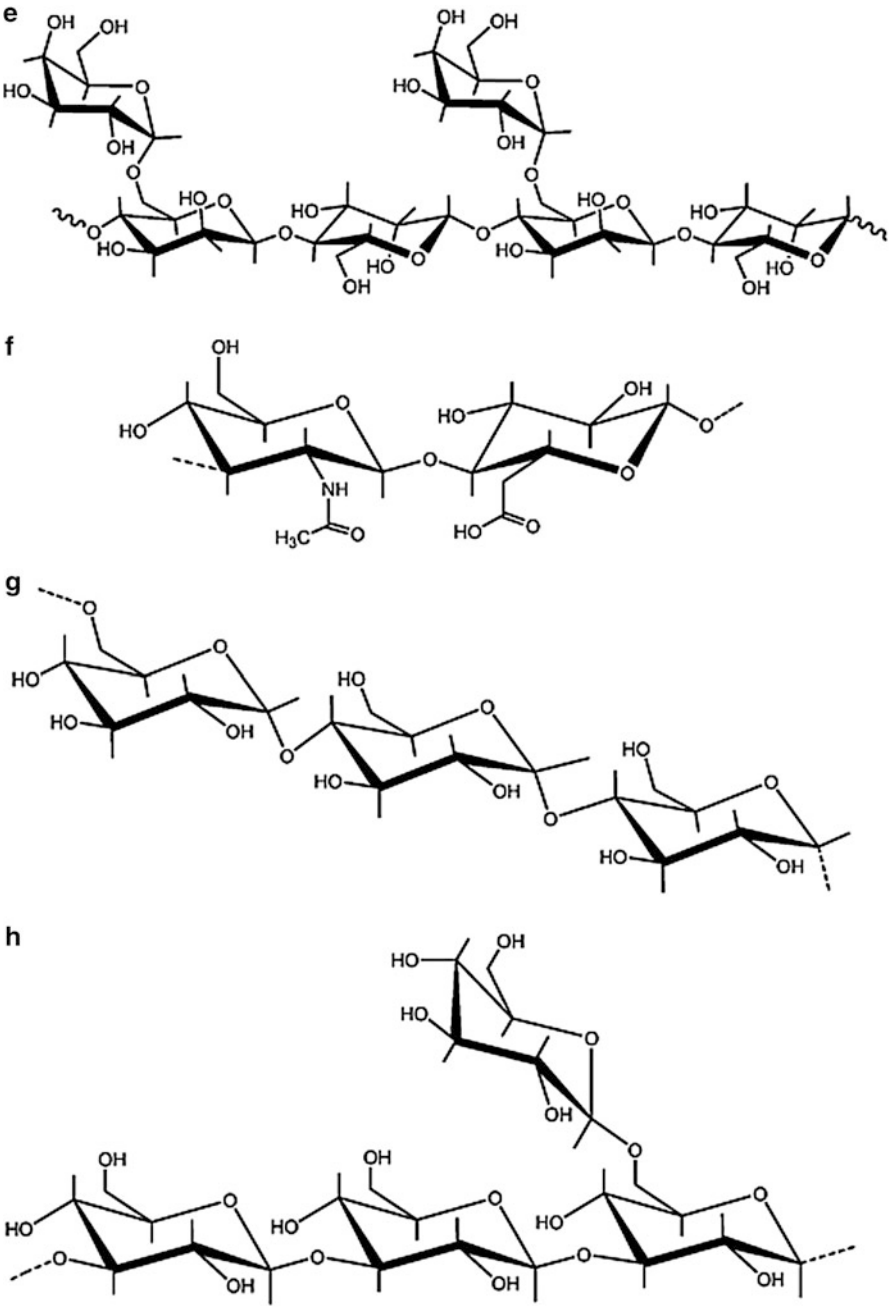

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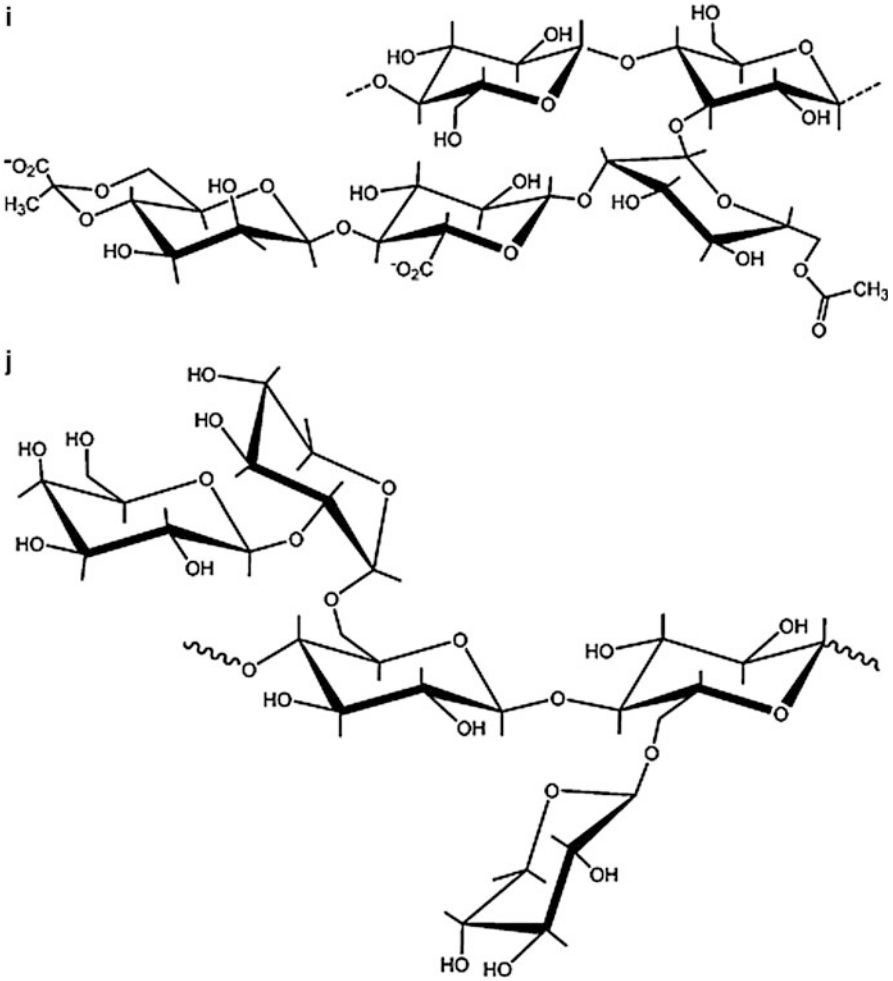

Fig. 1 Chemical structures of alginate (**a**), carrageenan (**b**), dextran (**c**), gellan (**d**), guar gum (**e**), hyaluronic acid (**f**), pullulan (**g**), scleroglucan (**h**), xanthan (**i**), and xyloglucan (**j**) (Reproduced with permission from Coviello et al. (2007))

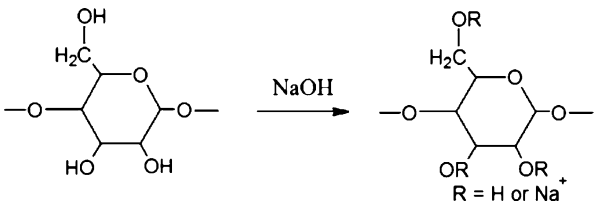

Fig. 2 Conversion of cellulose to alkali cellulose

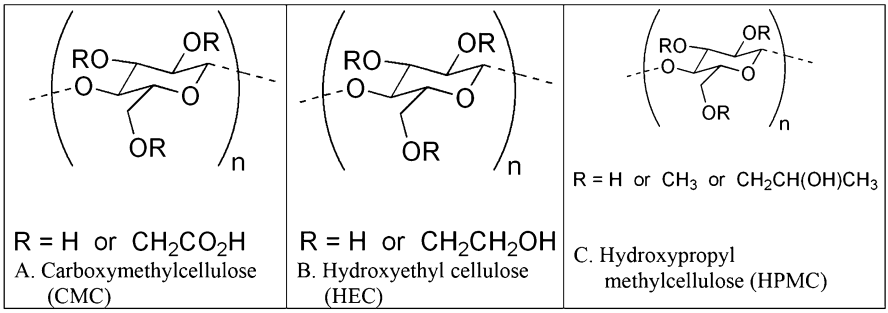

Fig. 3 Examples of chemically modified cellulose. (**a**) Carboxymethylcellulose (CMC). (**b**) Hydroxyethyl cellulose (*HEC*). (**c**) Hydroxypropyl methylcellulose (*HPMC*)

hydrogels can be achieved by a multitude of further chemical modifications including reactions with dialdehydes (Rokhade et al. 2006), divinyl sulfone (Sannino et al. 2004), and diepoxies (Kono et al. 2013), as well as with interaction with radiation (Wach et al. 2001) and other methods. CMC hydrogels tend to display pH sensitivity due to the presence of the carboxymethyl units. In deprotonated state, i.e., at high pH, CMC hydrogels swell more rapidly due to both higher hydrophilicity of the anionic COO^- and the lack of hydrogen bonding which occurs in the protonated state (Barbucci et al. 2000). Figure 3 also shows the repeating units of hydroxyethyl cellulose (HEC) and hydroxypropyl methylcellulose (HPMC). Methylcellulose, despite the hydrophobic modification, has improved water solubility over cellulose, because the modification prevents the polysaccharide chains from forming crystalline domains that prevent solubility of native cellulose.

Methylcellulose possesses a thermal sensitivity and exhibits a lower critical solubility temperature (LCST) between 40 °C and 50 °C (Ruel-Garipy and Leroux 2004). LCSTs of HPC and HPMC are known to be ~42 °C (Winnik et al. 1992) and 69 °C (Joshi 2011), respectively. HEC does not display a thermal sensitivity (Kan et al. 2004). An additional ether-type addition of cellulose is ethyl(hydroxyethyl)cellulose (EHEC). It forms into a gel upon cooling; however, when mixed with surfactants such as sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide (CTAB), the solution becomes a reverse thermogel capable of transitioning around body temperature (Ruel-Garipy and Leroux 2004). Ether-modified celluloses are highly stable and can be utilized for further chemical modifications (Lee et al. 2005).

3 Chemically Cross-Linked Polysaccharide Hydrogels

Polysaccharide hydrogels can be generated by chemically cross-linking the polysaccharide using permanent covalent bonds. This can be done in a one-step process by direct addition of a cross-linking agent or can be done in a two-step process in which the polysaccharide is modified to contain activated groups (such as vinylic groups)

and then subsequently cross-linked in a second step. The benefits of the first method are speed and ease of a "one-pot"-type reaction, though the second type offers more flexibility as the activated polysaccharide can be subsequently combined with other monomers or components to create complex multicomponent hydrogels with varying properties.

3.1 Direct Cross-Linking Methods

There are several methods and conditions under which polysaccharides can be cross-linked directly to form into chemically conjugated hydrogels. One method which utilizes no chemicals is the application of high-energy ionizing radiation. This method has been utilized previously to cross-link water-soluble polysaccharide derivatives, such as carboxymethyl starch, carboxymethylcellulose, carboxymethyl chitin, and carboxymethyl chitosan, by exposing paste-like suspensions of each to electron beams or gamma irradiation. In this condition, water hydrolysis products create free radicals that interact with the polysaccharide chains which can induce chemical reactions between the chains leading to cross-linking (Yoshii et al. 2003). Recently, gamma radiation was applied to solutions of carboxylated locust bean gum to create superabsorbent hydrogels. It should be noted that the cross-linking reaction by exposure of polysaccharide to radiation competes against degradation of the polysaccharide which places some limits on this method in terms of exposure and cross-linking degree (Hayrabolulu et al. 2013).

Chemicals which may be applied to directly cross-linking polysaccharide primarily include multifunctional moieties which are reactive towards nucleophiles. One example of this is cross-linking by dialdehydes. Figure 4 shows a schematic overview of an aldehyde–nucleophile reaction commonly used for cross-linking. Typical aldehydes and nucleophiles react in a rapid and spontaneous manner at room temperature allowing for simple one-step reactions. Typically, this has been done with low molecular weight dialdehydes. For example, chitosan is reacted with a variety of phthalaldehydes that cross-link preferentially with the amines (Hirano and Takeuji 1983). Another example is the reaction of glutaraldehyde with alginate to form hydrogels to control delivery of pesticides for agricultural uses. Alginate does not contain amine units but the aldehyde reaction can also occur with other nucleophiles such as alcohol units (Kulkarni et al. 2000).

In addition to low molecular weight dialdehydes, large multifunctional aldehydes can be utilized to achieve this reaction. Recently, an interesting variation of this chemistry was used to create an in situ forming aldehyde-linked hydrogel. In this

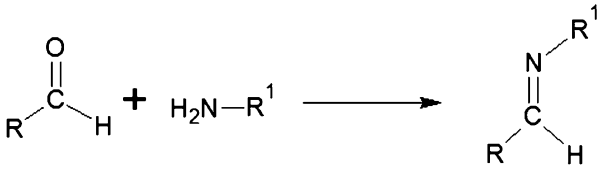

Fig. 4 Example of aldehyde reaction with a nucleophile

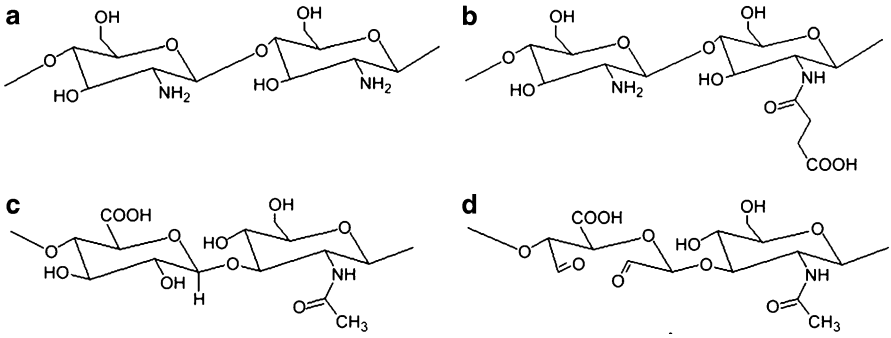

Fig. 5 Chemical structures of chitosan (**a**), *N*-succinyl-chitosan (**b**), hyaluronic acid (**c**), and aldehyde hyaluronic acid (**d**) (Reproduced with permission from Tan et al. (2009))

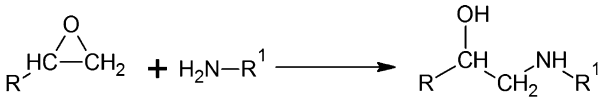

Fig. 6 Reaction between amine and epoxide

technique, chitosan was reacted with succinic anhydride to add carboxylic acid moieties along the backbone. This allowed the chitosan to remain soluble at higher pH. Separately, hyaluronic acid was reacted with sodium periodate in the dark to generate aldehyde units along the backbone of the hyaluronic acid (Fig. 5). When solutions of these polymers were combined, they reacted spontaneously to form a strong cross-linked hydrogel. This reaction, also known as a Schiff base reaction between and amine and an aldehyde, proceeded rapidly in 1–4 min to form a hydrogel system which is biodegradable and biocompatible enough to support growth of bovine chondrocytes on the surface of the hydrogel (Tan et al. 2009). Similarly, aldehyde-converted dextran was reacted with chitosan to form a chemically cross-linked hydrogel. Bovine serum albumin (BSA) was released in a controlled manner from the matrix showing promise for potential use as a drug delivery material as well as for tissue engineering and cell culture (Cheng et al. 2013).

Another method of cross-linking polysaccharides to form hydrogels is to apply epoxide moieties to the polysaccharide. In the presence of nucleophiles, epoxide rings undergo ring opening to conjugate to the nucleophile. Due to ring strain, epoxides are highly susceptible to nucleophilic attack, and thus, this reaction proceeds quite easily even at room temperature with no catalysts. An example schematic of this reaction is shown in Fig. 6. Examples of this cross-linking reaction include reactions involving amine-bearing polysaccharides, such as chitosan, with ethylene glycol diglycidyl ether or epichlorohydrin (Wan Ngah et al. 2002). In this case, the amine serves as the nucleophile. Additionally, alcohol units can be used as the nucleophile in reactions, for example, between poly(ethylene glycol) diglycidyl ether and hyaluronic acid (Collins and Birkinshaw 2008). In general, the amine group is a much more suitable nucleophile for this and other reactions. The hydroxyl reaction may occur but is very slow unless the pH is quite high (Lawal et al. 2011).

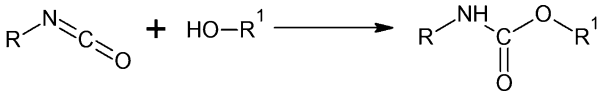

Fig. 7 Generalized isocyanate reaction to form urethane linkage

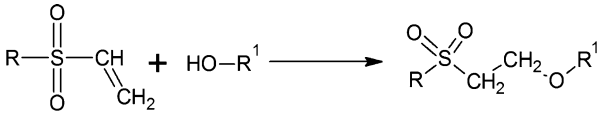

Fig. 8 Generalized vinyl sulfone–alcohol reaction

Polysaccharides can be cross-linked as well by utilizing difunctional isocyanates to form polyurethanes. A generalized schematic of this reaction is shown in Fig. 7. Similar to previous items, the urethane moiety (R-N=C=O) is susceptible to reacting with nucleophiles. It has a higher reactivity towards alcohols, allowing for easier reactions along this route. There are, however, some drawbacks for reacting polysaccharides directly with isocyanates, as this reaction typically does require increased temperatures, specific solvents, and catalysts. This reaction is also highly susceptible to the presence of water which leads to degradation of the isocyanates into carbon dioxide gas. Due to polysaccharides' crystallinity, poor solubility, tendency to contain water, susceptibility to temperature, and acid/alkaline, native polysaccharides are poorly suited for conversion into PEU hydrogels. Typical modifications include depolymerization or alkylation, although this often leads to plastics instead of thermogels (Donnelly et al. 1991). Despite this, there are examples of hydrogels generated utilizing this chemistry typically when mixed with other alcohol/amine-bearing ingredients. For example, urethane reactions have been utilized to cross-link mixtures of chitosan, poly(ethylene glycol) (PEG), and alcohol-terminated polydimethylsiloxane (Rodkate et al. 2010). Additionally, hyaluronic acid has been cross-linked utilizing star-shaped polyethers with isocyanate groups on the distal arms to form biodegradable hydrogels for drug delivery applications (Dhanasingh and Groll 2012).

Another method of cross-linking is by utilizing divinyl sulfone. A generalized schematic of this reaction is shown in Fig. 8. Divinyl sulfone operates as a Michael's type reaction acceptor and is reactive preferentially towards nucleophiles such as amines and thiols. Vinyl sulfone can also react with alcohols at high pH. This reaction has been done with hyaluronic acid to form hydrogels (Collins and Birkinshaw 2008). This vinyl-sulfone-linked hyaluronic acid has been investigated for drug delivery as well as for use as biocompatible coatings and tissue scaffolds. In addition to direct divinyl sulfone, a variety of sulfone-terminated PEGs have been used for this as well. The presence of the hydrophilic PEG spacer leads to an overall lower cross-linking density which allows for greater swelling and faster drug release (Hahn et al. 2004). HPC has also been reacted with divinyl sulfone at high pH (pH~12) to form a temperature-responsive hydrogel which shrinks upon warming. This system was investigated for its potential to serve as an actuator or "artificial muscle" (Hinkley et al. 2004). In addition to divinyl sulfone and PEG/vinyl sulfone, vinyl sulfone macromers have also been produced. To create this, mercaptoalkanoic

acid was modified with divinyl sulfone to yield vinyl sulfone alkanoic acid which was subsequently conjugated to dextran using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) as catalyst. This formed dextran/vinyl sulfone with varying degrees of substitution was reacted with thiol-terminated PEGs linear and star shaped. This in situ reaction formed dextran–PEG hydrogels rapidly which were biodegradable over the course of 3–21 days based on the degree of substitution (Hiemstra et al. 2007). These examples highlight some of the capabilities of this versatile reaction mechanism.

3.2 Activated Cross-Linking Methods

The above sections highlight a variety of methods for generating polysaccharide hydrogels directly in a "single-step" reaction. This chemical method is robust and simple. There is often a requirement for the hydrogel to include other monomers or be reacted in a certain manner. Thus, it becomes desirable to first convert the polysaccharide into an activated form. Typically, this takes the form of attaching vinyl groups onto the polysaccharide which can subsequently participate in radical chain reaction. Several of the linking chemistries are similar to those described above but will be highlighted briefly with an emphasis on their functionality.

3.2.1 Epoxide–Acrylate

One common method is to react polysaccharide with epoxide–acrylates to generate a vinylically reactive macromer. The initial reaction typically involves an epoxy reaction between glycidyl methacrylate and glycidyl acrylate with a polysaccharide. The epoxide ring opens against one of the polysaccharide nucleophiles (alcohol or amine) binding the vinyl group to the nucleophile. Since this reaction occurs easily at room temperature with no need for a catalyst, the vinyl group remains unreacted, allowing for the subsequent material to be purified and mixed with other reagents easily. Examples of this include the grafting of glycidyl methacrylate onto cashew gum (Guilherme and Reis 2005), galactomannan (Reis et al. 2003), dextran (Hennink et al. 1996; De Smedt et al. 1995), and hyaluronic acid (Leach et al. 2004). This allows these macromers to be subsequently reacted into semisynthetic hydrogels. Recently, this technique has been applied to synthesize biocompatible cryogels for cell growth and seeding. The components used were low molecular weight hyaluronic acid–methacrylate generated by reacting glycidyl methacrylate with autoclaved hyaluronic acid (having reduced chain length) as well as full molecular weight hyaluronic acid–methacrylate. Dextran–methacrylate was synthesized by dissolving dextran in dimethyl sulfoxide (DMSO) and reacting with glycidyl methacrylate utilizing 4-(dimethylamino) pyridine as a catalyst. Solutions of the thus formed polysaccharide–acrylates were then dissolved in water and degassed by sparging/ultrasound. They were then frozen and irradiated directly with electron beam irradiation to achieve cross-linking. The resultant cryogels were found to be highly biocompatible and readily allowed for ingrowth of 3T3 cells (Reichelt et al. 2014).

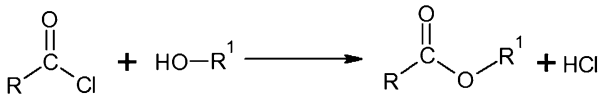

Fig. 9 Generalized acyl chloride–nucleophile reaction. If "R" is acrylate or methacrylate, the resultant molecule is capable of participating in radical chain polymerization

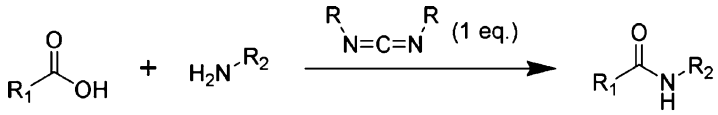

Fig. 10 Example of generic carbodiimide-mediated reaction between an acid and an amine

3.2.2 Vinyl-Oyl Chloride

Vinyl groups can also be attached to polysaccharide using vinyl-oyl chlorides, such as acryloyl chloride and methacryloyl chloride, both of which are readily commercially available. The acyl chloride moiety is highly reactive towards any nucleophile including alcohol and water, and thus, care must be taken to ensure that the reaction is performed under anhydrous conditions. The resultant formation of HCl limits the reaction from proceeding, and so typically this reaction is performed in the presence of an HCl scavenger such as triethylamine (Fig. 9) (Tran et al. 2011). This reaction has been previously applied to acrylate starch which had been previously modified to contain sulfate groups to provide hydrophilicity. This reaction was performed at 60 °C in DMF utilizing pyridine as an acid scavenger. The resultant modified starch was subsequently combined with a comonomer, acrylic acid, and initiated via ammonium persulfate/*N*,*N*,*N*′,*N*′-tetramethylethylenediamine redox pair utilizing a blowing agent sodium bicarbonate to simultaneously increase the rate of reaction by raising pH and generate carbon dioxide gas bubbles. The formed superporous hydrogel (SPH) was capable of absorbing up to 200 times its weight in water (Kuang et al. 2011).

3.2.3 Carbodiimide Conjugation

Conjugation of polysaccharides can also be accomplished via carbodiimide-mediated chemistry. A carbodiimide is any reagent with the N=C=N moiety typically close to the middle of the reagent. Popular carbodiimides include *N*,*N*′-dicyclohexylcarbodiimide (DCC) (preferably in hydrophobic solvents) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (preferable in aqueous or polar solvents). The reaction initiates with the acid end cap of the material reacting with the carbodiimide to form an unstable *O*-acylisourea intermediate. The intermediate proceeds to react with nucleophiles (preferentially with amines over hydroxyls) forming an amide bond with a side product being an insoluble organo-urea which is typically removed by filtration (Fig. 10) (Monagle 1962). This chemistry has been applied to forming polysaccharide gels by reacting hyaluronic acid with *N*-(3-aminopropyl)methacrylamide hydrochloride utilizing EDC as a carbodiimide reagent. The resulting hyaluronic acid derivative (Fig. 11) had chemical conjugates

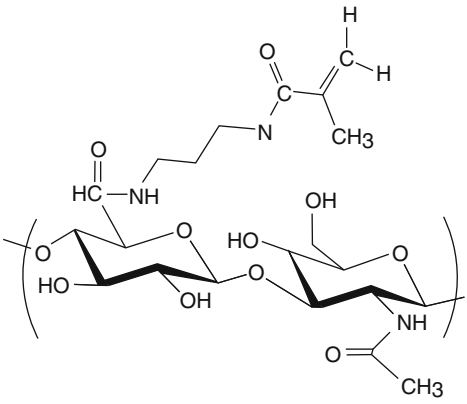

Fig. 11 Hyaluronic acid conjugated to methacrylate units by carbodiimide-mediated attachment (Reproduced with permission from Kim and Park (2002))

between the carboxylic acid units of the hyaluronic acid and the aminopropylmethacrylamide, allowing for subsequent cross-linking with poloxamer diacrylate to form a thermally sensitive, chemically cross-linked hydrogel which served well as a scaffold for controlled release of human growth hormone (Kim and Park 2002).

3.3 Enzymatic Methods

One of the more unusual chemistries recently applied to adding vinyl reactive groups is the utilization of biological enzymes to catalyze the conjugation of vinyl acrylate onto dextran in order to activate it for subsequent use in radical chain-initiated reaction. Solutions of proleather and lipase were utilized to catalyze the reaction between dextran and vinyl acrylate. The subsequent dextran acrylate was then cross-linked to form hydrogels which showed good biocompatibility and biodegradability (Ferreira et al. 2002, 2004).

4 Physically Cross-Linked Polysaccharide Hydrogels

Physically cross-linked polysaccharide hydrogels are formed through chain entanglement, ionic interactions, hydrogen bonding, and hydrophobic interactions.

4.1 Chain Entanglement

Polymer chains of polysaccharides can intertwine to form physical cross-links. The "gum" series of polysaccharides form chain-entangled hydrogels upon simple heating and cooling of their solutions. Xanthan gum is a heteropolysaccharide composed of a 1-4-linked β-D-glucose backbone. This backbone has a trisaccharide side chain composed of two mannoses and one glucuronic acid at every other residue. Locust bean gum is a 1-4-linked β-D-mannan backbone with 1-6-linked

α-D-galactose side groups. When respective solutions of these two gums are mixed and heated, the chains gain motility, allowing for them to intertwine. Upon cooling a gel is formed which is highly resilient to redissolution except for at extremely high temperatures (Higiro et al. 2006). These kinds of gels are typically used in the food industry as viscosity modifiers for a wide variety of products as well as for forming edible films and other substances (Aydinli and Tutas 2000). Although chain entanglement is not typically a desired method of physical cross-linking in semisynthetic polysaccharides, it is often a contributing factor along with other modes of physical cross-linking.

4.2 Ionic Interaction

Several anionic polysaccharides react to form hydrogels directly by interaction with multivalent cation metals, such as calcium (Olivas and Barbosa-Canovas 2008; Betigeri and Neau 2002), iron (Kroll et al. 1996), and others. Cationic polyglucosamines have the capacity to react with polyanions, such as sodium tripolyphosphate(Betigeri and Neau 2002), to form gels. Most notable examples of this kind of cross-linking include cross-linking interaction between sodium alginate and calcium chloride as well as the cross-linking interaction between chitosan and sodium tripolyphosphate. These ionic interactions have the capacity to form hydrogels very quickly, so fast that discrete beads of alginate/calcium or chitosan/tripolyphosphate can be formed by simply dripping the polysaccharide solution into the polyvalent ion solution.

4.2.1 Alginic Acid–Calcium

Alginic acid (Fig. 1a) is a linear copolymer comprised of blocks of (1-4)-linked β-D-mannuronate (M) and the C-5 epimer α-L-guluronate (G) residues, respectively. Alginic acid is extracted from the cell walls of brown algae and used for a wide variety of medical, food, and industrial purposes as a water-absorbent gum/thickener. When processed at high pH utilizing sodium hydroxide or potassium hydroxide, it is converted to sodium alginate or potassium alginate. In this form, it is freely water soluble at low concentrations (Remminghorst and Rehm 2009). When a solution of alginate interacts with calcium, the sodium or potassium exchanges with calcium. Divalent calcium ions interact with two carboxyl groups of alginate to form ionic cross-links and a gel (Fig. 2). This versatile chemistry can be applied for a wide variety of applications including drug delivery (McLennan et al. 2000) and medical devices/tissue scaffolds (Christensena 2011; Livnat et al. 2005).

4.2.2 Chitosan/Tripolyphosphate

An analogous situation occurs with a cationic polysaccharide (chitosan) which interacts with tripolyphosphate to form a hydrogel (Vimal et al. 2013). Chitin is found naturally as the structural component in the exoskeleton of crab, shrimp, and insects. This material is readily available in bulk at an extremely low price from commercial fishing industry. Shell pieces are chopped/ground and then soaked in

hydrochloric acid and deproteinized typically by hot alkali treatment with refluxing sodium hydroxide. This, along with oxidization procedures using oxidizing agents (e.g., KMnO4 + oxalic acid + H2SO) to remove pigmentation, allows for chitin to be isolated from shells. Subsequently, chitin can be converted to chitosan by deacetylation of the chain using alkali treatment (Abdou et al. 2008). Chitin can also be extracted from fungal sources as well (Rane and Hoover 1993; Zikakis 1984). Chitosan is inherently water insoluble at neutral and high pH due to the deprotonated form of the amine units along the chain. When exposed to low pH (typically pH < 5, easily achieved with even 1 % acetic acid), however, the chitosan becomes protonated and fully soluble in water. When this chitosan solution is exposed to a multivalent anion, such as sodium tripolyphosphate which is dibasic, a chemical cross-link is formed between the chains, creating a polysaccharide hydrogel held by ionic influences (Mi et al. 1999). This hydrogel can occur with other materials to create mixed hydrogels as well as has the capacity to have anionic materials, such as alginate, absorb onto the hydrogel due to ionic attraction. These capabilities make this type of hydrogel desirable for microparticle drug delivery systems (Ko et al. 2002). The mechanical strength, in the absence of reinforcement, of chitosan/tripolyphosphate is not as high as that of other ionic polysaccharide hydrogels such as calcium ion-cross-linked alginate. For this reason, it is not typically used for devices or other larger structures (Shu and Zhu 2000).

4.2.3 Other Ionic Polysaccharides

Although chitosan and alginate are the most popular materials for generating ionic polysaccharide hydrogels, any polysaccharides which have a plurality of charges along its backbone can be used for this purpose. Carrageenan (Fig. 1) is a family of polysaccharides which have anionic sulfonate (SO^{3-}) groups present along their backbone. These anionic sulfonate groups have also been used as sites for ionic cross-linking with divalent and trivalent counterions as well as by interaction with other natural polymers such as gelatin. Gellan gum forms thermoreversible gels in the presence of divalent counterions. Scleroglucan, when derivatized to introduce carboxylic acid units, can be cross-linked by calcium ions to form hydrogels (Coviello et al. 2007). Ionically cross-linked polysaccharide hydrogels are inherently sensitive to both pH and ionic strength of the media. Since it is the balance between ions that generates these gels, changes in pH can shift the protonation degree of the ions. When applied in vivo or in a sink system over time, the low molecular weight cross-linking ion (e.g., calcium and tripolyphosphate) leaches out of the system, and eventually the hydrogel redissolves.

4.2.4 Polyelectrolyte Complex

A polyelectrolyte complex forms when two polymers having opposite charges interact. This is a popular method of forming an ionic gel with polysaccharides as they typically contain or can be modified to contain opposing charges. These ionic interactions are stronger than other interactions, such as van der Waals or hydrogen bonding. Polysaccharide polyelectrolyte complexes are formed by interacting a polycation (e.g., chitosan) with an anionic polysaccharide. Anionic polysaccharides

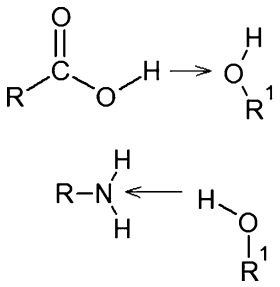

Fig. 12 Examples of hydrogen bonding of carboxylic acid and amine with alcohol units. *Arrows* indicate hydrogen bonds

include alginic acid, hyaluronic acid, chondroitin sulfate, carboxymethylcellulose, dextran sulfate, pectin, and xanthan. Polysaccharide polyelectrolyte complexes can also be formed with proteins, such as gelatin, fibroin, albumin, keratin, or collagen, or anionic synthetic polymers such as polyacrylic acids (Bhattarai et al. 2010).

4.3 Hydrogen Bonding

Hydrogen bonding is a strong electromagnetic attraction between hydrogen and an electronegative atom such as oxygen or nitrogen. As shown in Fig. 12, hydrogen bonding in polysaccharides is common due to the presence of several participatory groups such as alcohols, carboxylic acids, and amines (Blackburn 2004). Hydrogen bonding is an environmentally sensitive process. For hydrogen bonding to occur, the hydrogen-bearing groups must be appropriately protonated for carboxylic acid or deprotonated for amine (Fig. 12) and at an appropriate temperature with a low salinity for a strong hydrogen bond to form. Agarose only forms hydrogen bonds at relatively low temperatures, and chitosan only forms these bonds at pH higher than 5 (Francis Suh and Matthew 2000). Semisynthetic polysaccharides, such as carboxymethylcellulose, also undergo hydrogen bonding in a pH-sensitive manner (Barbucci et al. 2000). Hydrogen bonding is sensitive towards other components in the solution, such as urea, which disrupt this form of physical bond and reduce its capacity to gel a material (Moon et al. 2012).

4.4 Hydrophobic Physical Cross-Linking

One effective way of forming a hydrogel is to attach hydrophobic units to the chain for hydrophobic association. The hydrophobic association is primarily due to the lack of attraction between water and the hydrophobic moieties rather than any attraction among hydrophobic moieties (Chandler 2005). The presence of an excess of hydrophobic moieties will simply yield a water-insoluble polymer (Miyamoto et al. 1995). One good example of this is cellulose acetate with high levels of acetate conjugation. They are water-insoluble plastics used for a variety of films, photographic and consumer purposes (Kamide 2005). The thermal sensitivity is strongly tied to the relative content of hydrophobic and hydrophilic portions of the polymer,

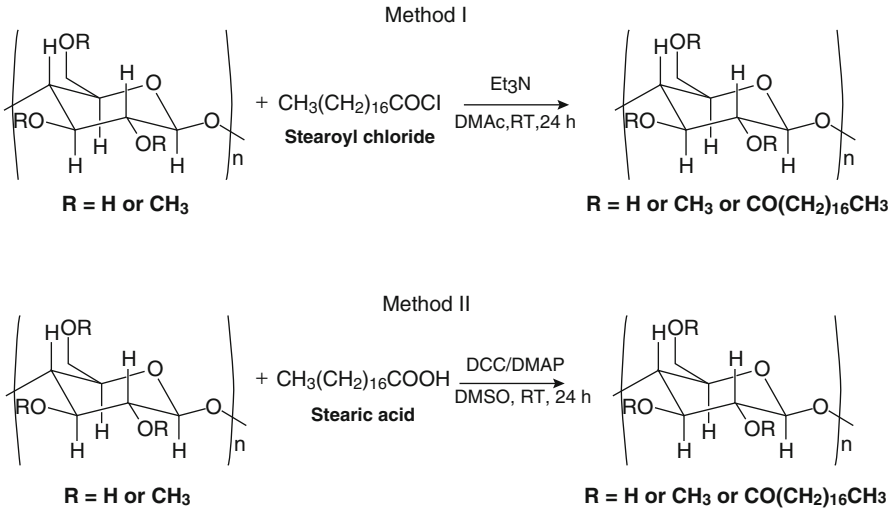

Fig. 13 Synthetic methods for generating stearate-modified methylcellulose (Reproduced with permission from Lee et al. (2005))

and thus, it can be controlled by adjusting the hydrophobic content of a polysaccharide. Methylcellulose, which has thermogelation properties but with a relatively high transition temperature, can be modified to contain additional stearate (C18 fatty acid) groups along the chain. Stearoyl chloride was reacted with methylcellulose in the presence of triethylamine (which serves as an HCl scavenger). In another method, stearic acid was reacted with methylcellulose utilizing carbodiimide-mediated chemistry and dimethylaminoethylpyridine (DMAP) as a catalyst. Both of these conjugation techniques were successful in generating hydrophobically modified cellulose (Lee et al. 2005) (Fig. 13). Similarly cellulose has been reacted with hydrophobic modifiers, such as 1,2-epoxydecane and butyl isocyanate, to form thermogelling derivatives based on hydrophobic associations (Miyamoto et al. 1995). Additionally reacetylated chitosan can serve as a thermogel based on hydrophobic modifications as well (Li et al. 2013).

5 Stimuli-Responsive Polysaccharide Hydrogels

An increasingly popular field of research is into "smart" or "environmentally sensitive" hydrogels. This is a popular field both for polysaccharide hydrogels and hydrogels in general. The phrase "smart" typically refers to hydrogels which undergo a change in their physical bonds or attraction towards water due to a change in the environmental factor, such as pH, ionic strength, temperature, light, and presence of specific molecules, such as glucose or enzymes. In some instances, the phrase is applied to hydrogels which undergo chemical changes such as those which are sensitive towards enzymatic hydrolysis of a specific enzyme which may be utilized for drug delivery.

Stimuli-responsive polysaccharide hydrogels display a phase transition behavior. There are two (or three for un-cross-linked polymers) different phases these responsive hydrogels can exist in as defined by the Flory–Huggins theory (Bromberg and Ron 1998). The polymer–solvent interaction creates osmotic pressure ($\Delta\pi_{mix}$) attracting the polymer chains to the solvent, thus driving the polymer to either expand or, if there are no other bonds holding it together, to ultimately dissolve. Additionally, there are polymer–polymer interactions related to the strength of the polymer chain attraction for each other as an elastic force ($\Delta\pi_{elast}$). These forces in a given polymer system are always in balance, and the following equation applies:

$$\Delta\pi = \Delta\pi_{mix} + \Delta\pi_{elast} = 0$$

In a general hydrogel system, the opposing forces balance and thus define the swelling of the hydrogel. However, in a responsive system, the relative balance of these forces is affected by a specific stimulus. As this occurs, the system transitions through three stages (Harsh and Gehrke 1991):

1. Precipitate stage ($\Delta\pi_{mix} \ll \Delta\pi_{elast}$): There is maximum polymer–polymer interaction with minimal polymer–solvent interaction, and thus, the polymer is precipitated from the solution and behaves in a hydrophobic manner resisting water infiltration.
2. Gel stage ($\Delta\pi_{mix} \sim \Delta\pi_{elast}$): The opposing interaction forces are close to balance with one another. The polymer chains retain enough interaction to hold the hydrogel together as one piece but still be highly hydrated with water. Depending on the system, this transition may be very sharp or broad.
3. Solution/fully swollen stage ($\Delta\pi_{mix} \gg \Delta\pi_{elast}$): The polymer–solution interaction is maximized, and the polymer either completely dissolves (in the situation where the physical bonds were holding it together) or in the case of a chemically cross-linked hydrogel expands to reach their maximum swelling.

This general theory applies to the interaction of physical bonds with the environment, but with several exceptions and nuances. For example, altering the relative attractive forces is a reversible change, whereas breaking of chemical bonds by enzymes or formation of chemical bonds by light is an irreversible change. Additionally, the method in which these interactions change depends drastically on the system being altered. Several types will be detailed and highlighted with examples below (Qiu and Park 2001).

5.1 Enzymatic/Special Molecule Sensitivity

In general, polysaccharide hydrogels display enzymatic sensitivity in that they are degraded by the enzyme which is a nonreversible change to the polymer backbone. A good example of this is the degradation of dextran-based hydrogels by dextranases leading to increased release of the drug payload. This method was used as a means to develop a hydrogel for controlled protein delivery based on enzymatic

degradation rate (Hennink et al. 1997). One valuable aspect of this application is towards colon-specific drug delivery where dextranases are present in higher concentrations than elsewhere in the body (Hovgaard 1995). Additionally "dual-sensitive" hydrogels have been made by forming interpenetrating polymer networks (IPNs) consisting of oligopeptide-terminated PEG and dextran. This requires the presence of both papain and dextranase to degrade the hydrogel and release the drug under very specific conditions.

There are a wide range of hydrogels which display sensitivity towards a variety of biomolecules including glucose, peptides/proteins, nucleic acids, and other materials (Miyata et al. 2002). Glucose sensitivity can be achieved by incorporation of concanavalin A (Con-A), which is a carbohydrate-binding protein with a strong affinity for glucose. A dextran–PEG-based cross-linked hydrogel incorporating Con-A was made to provide glucose sensitivity. As part of forming this, hydrogel dextran was modified using glycol methacrylate to form dextran–methacrylate, and Con-A was modified by Michael's addition with ethylene glycol acrylate–methacrylate. These were reacted with PEG-dimethacrylate to form a chemically cross-linked hydrogel. In the absence of glucose, Con-A is attracted to the dextran chain and binds to this chain. When glucose is present, however, it competes for this binding and as such increases the swelling of the hydrogel, thus making the hydrogel as a whole sensitive towards glucose. The goal of this type of research is to generate an intelligent pump which releases certain quantities of insulin based on the glucose content in the blood (Yin et al. 2010).

5.2 pH Sensitivity

Some hydrogels exhibit pH sensitivity primarily due to the presence of ionizable groups. Several natural polysaccharides, such as alginic acid and chitosan, already have ionizable groups available and display pH sensitivity. Weak acid, such as –COOH, present in hydrogels ionizes at high pH and thus swells to a greater degree or dissolves. On the other hand, hydrogels containing weak bases, such as amines, become ionized at low pH for higher swelling or dissolution. In all cases, the sensitivity towards pH is related to the materials' acidity constant which defines the equilibrium state of the ionized fraction at a given pH. The equilibrium constants for weak acids and weak bases can be written as shown in Table 2. K_a and K_b are the acidity constant and basicity constant of the weak acid and weak base, respectively. A weak acid at low pH (where $[H^+]$ is high) is primarily in the protonated (non-ionized) form which is not water soluble (or not swellable). At high pH, it is primarily in the deprotonated (ionized) form which is water soluble (or swellable).

Table 2 Equilibrium constants for weak acids and weak bases

Weak acids	Weak bases
$HA \leftrightarrow H^+ + A^-$	$B + H_2O \leftrightarrow BH^+ + OH^-$
$K_a = \frac{[H^+][A^-]}{[HA]}$	$K_b = \frac{[BH^+][OH^-]}{[B]}$

A weak base dissolves (or swells) at low pH, but become water insoluble (or not swellable) at high pH. Due to the wide range of pH, it is common to convert the K_a and K_b values into pK_a and pK_b, the sum of which for a conjugate acid/base pair equals 14 in an aqueous system. One simple and practical implication of pH sensitivity with natural polysaccharides is that chitosan is normally insoluble in water at neutral pH. It dissolves quite easily, however, in dilute acids (e.g., 0.1 M acetic acid) due to ionization of the amine units along its backbone (Rinaudo et al. 1999).

Polyelectrolyte complex beads formed by mixing chitosan and alginate have been utilized to control the release of bitter gourd extract in a pH-dependent manner (Lin et al. 2014). Sometimes the pH-sensitive portion of the hydrogel is not from the polysaccharide. Radical chain polymerization of acrylic acid was performed in the presence of tragacanth gum. During this reaction, the initiator abstracts a hydrogen atom from tragacanth gum chain, and poly(acrylic acid) initiates from this point leading to a grafted polymer. Due to the presence of the plurality of –COOH units along the poly(acrylic acid) chain, the system displayed a reduced swelling and drug release at low pH (Singh and Sharma 2014).

5.3 Thermal Sensitivity

A gel can react towards changes in solution temperature in two primary ways. The first is referred to as the "normal" thermogel response in which heating of the solution improves overall polymer solubility. The increase in temperature reduces chain entanglement and hydrogen bonding, leading to either dissolution (for a physically cross-linked gel) or increased swelling for a chemically cross-linked gel. This type of response is common for gels made from gelatin or mixtures of xanthan and locust bean gum (Acharya et al. 2010; Carafa et al. 2011). Typically, however, when one refers to a thermogel or thermally sensitive hydrogel, this is not the response they are indicating. Responsive thermogels are generally considered to be "inverse" thermogels. In this situation, heating of the solution leads to a change in the water–polymer entropy or physical bonding that leads, counterintuitively, to gelation or shrinking of the hydrogel at increased temperature. As temperature increases, hydrogen bonding decreases while hydrophobic interaction increases.

Generally, the process of thermogelation is considered to be driven by entropy relative to the water–polymer attraction system. When water molecules are associated with the polymer chain, they are highly organized leading to a decreased entropy relative to the entropy of free water. The attractive force of water–polymer binding energy is an enthalpic term (ΔH_f, heat of fusion). The energy of the system as a whole can be generally described by the Gibbs free energy equation as follows:

$$G = H - TS$$

where G, H, S, and T are Gibbs free energy, enthalpy, entropy, and temperature, respectively. At low temperatures, the entropy drive is reduced, and the heat of fusion (ΔH_f) dominates so that the water molecules prefer to be associated with the polymer

chain and the physically cross-linked hydrogel dissolves freely while a chemically cross-linked hydrogel swells to its maximal extent. As temperature increases, the total energy of the system, as described by Gibbs free energy of the system, favors the entropic term rather than the enthalpic term, and the water molecules prefer to be in unorganized form in free solution rather than bound to the polymer chain (Oesterhelt et al. 1999; Harris 1992; Wang et al. 1997). The condition at which these two forces are in balance is referred to as the lower critical solubility temperature (LCST), and it is at that point that the material gels to a solid. The nucleophilic groups along the polysaccharide chain bond strongly to water. Thus, the requirement to generate a thermogel is simply to modify the polysaccharide to introduce hydrophobic moieties (Li et al. 2010; Kuang et al. 2006; Zhai et al. 2012).

Several examples have already been discussed, including methylcellulose, HPMC, etc. Chitosan, dextran, pullulan, and carboxymethyl curdlan have been hydrophobically modified to form thermogels (Lee et al. 2005; Hennink and van Nostrum 2012; Jeong et al. 2002). Polysaccharide hydrogels can also be generated relying on an additional, typically synthetic polymer for providing the thermogelation property. Examples of this include graft polymers of alginate with poloxamer (a known synthetic thermogel containing blocks of polypropylene oxide and polyethylene oxide) (Chen et al. 2011) and hyaluronic acid with poly(*N*-isopropylacrylamide) (PNIPAM) (Mortisen et al. 2010). In both these situations, the polysaccharide provides general hydrophilicity as well as other desired properties (biodegradability, biocompatibility), while the thermogel (poloxamer or PNIPAM) provides the thermogelling property.

Thermal gelation LCST is sensitive towards other components present in the solution. For instance, some materials (NaCl, KF, $(NH_4)_2SO_4$, and poly(ethylene glycol)) lower the LCST due to their water structure formation properties (Lee et al. 2005). Conversely, the addition of hydrotropic agents, materials which reduce water structure formation such as salicylate, diethylnicotinamide, urea, etc., will raise the LCST. This property can be controlled by the degree of hydrophobic modification. For example, methylcellulose has thermal sensitivity with LCST of $>45\ ^\circ C$. This makes the methylcellulose useful for some applications, such as food preparation as a batter viscosity modifier (Sanz 2005), but physiologically not applicable as a tissue scaffold or drug delivery gel. To lower the LCST of methylcellulose, additional hydrophobic groups may be added. Stearate (C18) groups were introduced to methylcellulose of 14,000–40,000 Da and a degree of methyl substitution around 1.6–1.9. Stearates were conjugated onto a methylcellulose chain at 0.3 or 1.6 mol%. The original methylcellulose could not thermogel at 33–37 °C, regardless of NaCl concentration, but that the stearate-modified methylcellulose could gel easily at these conditions (Lee et al. 2005).

Chitosan can also be converted into a thermogel by carefully controlling the degree of deacetylation. A highly controlled thermogelling glycol chitin was generated by reacetylating the chitin chain to contain the desired quantity of acetylation units (Li et al. 2013). This was done by reacting glycol chitosan with acetic anhydride at room temperature for varying reaction times to achieve varying degrees of reacetylation (Fig. 14). Glycol-modified chitosan has enhanced water

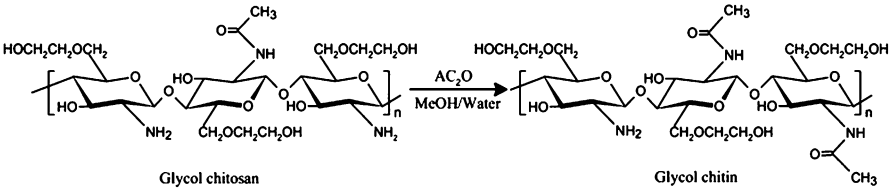

Fig. 14 Reacetylation reaction of glycol chitosan with acetic anhydride (Image reproduced with permission from Li et al. (2013))

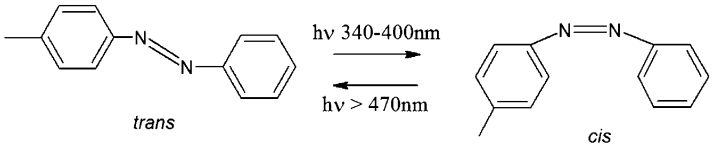

Fig. 15 The cis/trans conversion of [4-(phenylazo)phenyl]carbamate upon exposure to light Yashima et al. (1995)

solubility over conventional chitosan due to glycol unit which allows the polymer to dissolve at high pH. Further modification to add the acetyl units achieves a material which carefully balances the hydrophobic interactions leading to gel formation and the hydrophilic attractions which lead to solution formation. The formed hydrogel was found to be rapidly gelling allowing for gelation upon injection into a warm body. With higher degrees of acetylation, the polysaccharide was more susceptible to degradation in the presence of lysozymes. The material displayed good biocompatibility and allowed for controlled release of doxorubicin, a chemotherapeutic agent, over 13 days. Overall this research showed that there is good promise for modified chitosan to be utilized as thermogels in the fields of drug delivery and tissue engineering. A great deal of these properties (biodegradability, biocompatibility) can be attributed to the polysaccharide itself which lends the use of these materials to medical applications.

5.4 Light Sensitive

Light-sensitive hydrogels refer to the hydrogels with reversible sensitivity towards light without light-induced chemical cross-linking. A typical strategy to form a light-sensitive hydrogel is to utilize a thermally sensitive polymer and then incorporate a material which generates heat from light such as melanin (Ninh et al. 2014) or graphene oxide (Lu et al. 2014). Photosensitive moieties, such as [4-(phenylazo) phenyl]carbamate which switched between cis and trans upon exposure to either UV light or light >470 nm, can be grafted to cellulose/amylose (Fig. 15) (Yashima et al. 1995). This light-sensitive moiety has been utilized to modify HPMC which, in the presence of cyclodextrin, forms a photosensitive hydrogel. Another method

of inducing light sensitivity is to conjugate a light-inducible zwitterion, such as spiro benzopyran or spiro napthoxazines, which undergoes reversible ring opening upon light impingement. This method has been utilized to convert methylcellulose, dextran, and other polysaccharides into a form that had varying water/organic solubility based upon light exposure. More information can be found in a review article (Wondraczek et al. 2011).

6 Conclusion

The possibilities for generating hydrogels out of polysaccharide precursors are boundless. The inherent biocompatibility/biodegradability as well as the presence of a plurality of nucleophilic groups along the polysaccharide backbone provides many benefits. Polysaccharides can be modified, allowing for both chemical and physical hydrogels. Several natural as well as modified polysaccharides display sensitivity to environmental conditions such as temperature, pH, salinity, and light, allowing for the generation of environmentally responsive or "smart" polysaccharide hydrogels. The many capabilities of polysaccharide hydrogels are useful for applications to industrial, cosmetic, food, and healthcare fields.

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Part IV

Bioactivity

Extraction, Structure, and Bioactivities of the Polysaccharides from *Fructus*

51

Yanfang Wu, Xinsheng Wang, and Enguo Fan

Contents

Abstract

Phytochemical and pharmacological investigations identified that polysaccharides are major active compounds in *Fructus Corni*, which is a well-known traditional Chinese medicine, which shows various beneficial effects, such as immune regulation, antioxidation, antitumor, and antiaging functions. This chapter summarizes the techniques of polysaccharide extraction and purification from *Fructus Corni*, as well as their structural determination and related bioactivities. The prospects of future investigations and polysaccharide use are also discussed.

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Keywords

Fructus Corni • Polysaccharides • Extraction • Structure • Pharmacological activities

Abbreviations

AChE	Acetylcholinesterase
ALAT	Alanine aminotransferase
ALT	Alanine transaminase
ANOVA	Analysis of variance
Bcl-2	B-cell lymphoma 2
BHT	2,6-Di-tert-butyl-4-methylphenol
CD4	Cyclin-dependent kinase 4
$CD4^+$	Cluster of differentiation 4
$CD8^+$	Cluster of differentiation 8
DEAE-C	Diethylaminoethyl-cellulose
EAE	Enzymatic assisted extraction
EC50	Concentration for 50 % of maximal effect
GC	Gas chromatography
HDF	Human dermal fibroblast
HPGPC	High-performance gel permeation chromatography
HPLC	High performance liquid chromatography
HWE	Hot water extraction
IL-2	Interleukin 2
IL-4	Interleukin 4
IR	Infrared spectroscopy
MAE	Microwave-assisted extraction
MAP	Microwave-assisted process
MDA	Malondialdehyde
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUAE	Microwave and ultrasound assisted extraction
NBT	Nitroblue tetrazolium
NGF	Nerve growth factor
NMR	Nuclear magnetic resonance
RIA	Radioimmunoassay
RSM	Response surface methodology
RT-PCR	Reverse transcription polymerase chain reaction
SGPT	Serum glutamic-pyruvic transaminase
SOD	Superoxidase dismutase
TCA	Trifluoroacetic acid
TFA	Trifluoroacetic acid
UAE	Ultrasonic assisted extraction

1 Introduction

Cornus officinalis Sieb. Et Zucc. is a plant species known also as Japanese cornel, Japanese cornelian cherry, or cornelian cherries, which is one of the traditional and valuable Chinese medicinal plants and is scientifically classified into the *Cornus* genus, Cornaceae family, Cornales order, Magnoliopsida class and Magnoliophyta phylum. *Cornus officinalis* is mainly distributed in China, Korea, and Japan at latitudes between 26° and 37°40′ parallel north and altitudes from 600 to 1,400 m (optimal at 800–1,000 m) above sea level. The optimal cultivation climate is a yearly average temperature of 14.9 °C, lowest average temperature −14 °C, frost-free season ~230 days, and rainfall >822.3 mm (Chen et al. 2012). *Cornus officinalis* are deciduous trees or shrubs usually 4–10 m tall; their bark is light brown, with a feature of flaky stripping; their twigs are green, with a slender cylindrical shape that is glabrous covered with sparsely pubescent winter buds, while mature branches become blackish brown; winter buds are located terminal or axillary, displaying an ovate to lanceolate shape and covered with yellowish brown pubescent. Blade elliptic or long elliptic leaves grow in an opposite direction and are usually 5- to 12-cm long, 2.5- to 4.5-cm wide, green and glabrous at the upper face, and light green and covered with lean white paste sparsely pubescent at the lower face; leaves usually have 6–7 pairs of arcuate recurved lateral veins and are covered with densely pale brown floccose between veins; the petiole is thin and cylindrical, with a length of 0.6–1.2 cm and covered with slightly posted rusty pilose. *Fructus Corni* flowers in March–April, which precedes the leaves opening, and the flowers are clustered in umbels that are grown on apical branchlets or axils; flowers are small and bisexual; each flower has 4 crack calyxes yellowish green, 4 crack petals yellow, and 4 stamens; the fleshy disk is annular; below is the ovary with 2 rooms. *Fructus Corni* fruits in September–October, with oblong drupe that is 1.2- to 1.7-cm in length and 5- to 7-mm in diameter, with a red to purplish red color; nuclear bone, narrowly elliptic, ~12 mm, with a few irregular ribs (Fig. 1).

The dried fruit (*Fructus Corni*, Shan Zhu Yu in Chinese) of *Cornus officinalis* has been used for at least 2,000 years in Chinese herbal medicine and is officially listed in the Chinese Pharmacopoeia (2010). It has been used to invigorate the liver and kidney, to maintain kidney essence, to reduce urination, and to reduce perspiration and hemorrhage (China Pharmacopoeia Committee 2010). It has also been used in hygienic food and for cosmetic purposes (Cao et al. 2012). A number of functional compounds, including iridoid glycoside, aglycone, tannins, polysaccharides, organic acids, and esters, have been isolated and identified from *Fructus Corni* (Liu et al. 2011). Among them, polysaccharide is recognized as one of the major active compounds responsible for a wide range of healthy properties and thus has attracted ever-increasing attention (Tomshich et al. 1997; Ammar et al. 2010). Modern pharmacological investigations have indicated that polysaccharides of *Fructus Corni* have various bioactivities, including immunomodulatory (Du et al. 2008a; Miao et al. 2002), antioxidant (Li et al. 2003a, 2010; Zhang et al. 2008), antitumor (Zou et al. 2012), anticancer (Wang et al. 2012a, b), antiaging (Fu et al. 2007;

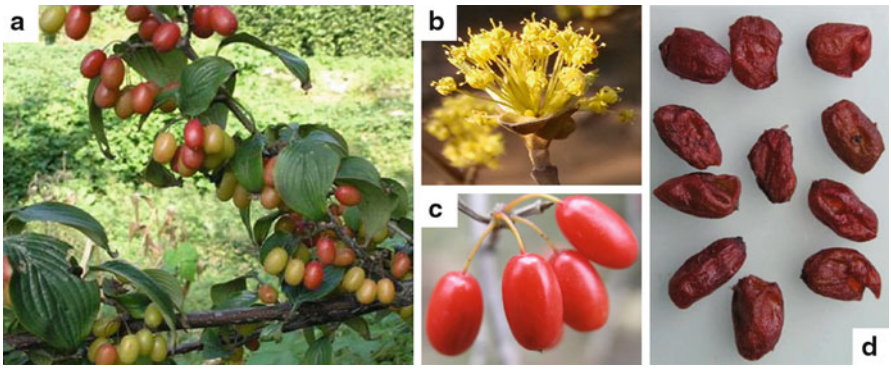

Fig. 1 (**a**) Tree, (**b**) flowers (**c**) fruits, (**d**) dried fruits of *Cornus officinalis*.

Wang et al. 2008a; Ou et al. 2010; Zhou et al. 2011), and sexual function effects (Shao et al. 2010). Because immunological modulation is believed to be involved in the prevention of various diseases such as cancer or pathogenic infections, the modulation function of polysaccharides on the immune system is particular interesting. Moreover, the physical–chemical properties of polysaccharides, such as the molecular weight, primary structure, solution conformation, and polymer charges, might play an important role in determining their affinity on various receptors and thus affect their biological activities and ultimately their biological effects on various diseases. Therefore, it is critically important to optimize their extraction, isolation, and purification from *Fructus Corni* in order to determine their structures for a better understanding of the underlying molecular mechanisms of their beneficial effects.

2 Extraction and Purification Processes

The application and further investigation of polysaccharides rely on an efficient extraction and isolation from *Fructus Corni*, a process that is theoretically affected by various factors, such as extraction temperature, pressure, pH, and ionic strength of the solvent. Commonly, aqueous solvents, such as water or basic aqueous solutions, in combination with various extraction "helpers" are used for polysaccharide extraction. Moreover, because polysaccharides of *Fructus Corni* exist as a structural compound of the cell wall, the nature of the cell wall structure constitutes a basic determinant for the selection of a suitable extraction method. Based on the structure and water solubility of *Fructus Corni* polysaccharides, the basic rule is to break the cell wall from outer layer to the inner layer with mild-to-strong conditions (for example, by varying solvent pH and temperature). In principle, polysaccharide extraction can be performed using water at room temperature, which is followed by a centrifugation process to remove nonsolubilized material that can be further extracted using hot water or other aqueous solutions. Acidic solutions can be employed for those water insoluble polysaccharides.

2.1 Hot-Water Extraction

Hot-water extraction (HWE) is the most commonly used technology for polysaccharide extraction in industrial applications. To improve the extraction efficiency of HWE, various reports have been reported in order to optimize the potential parameters, such as the extracting temperature and time, the ratio of solid-to-solvent and the number of extraction times all affecting the extraction of polysaccharides (Guo et al. 2010; Ye and Jiang 2011; Zhang et al. 2014a; Bin 2010). Zhang et al. (2007a; Zhang and Zhang 2007) performed HWE by selecting the extraction temperature, extraction time, and ratio of solvent to material as extraction parameters to optimize the extraction process using a uniform experimental design, which is a kind of space-filling design used for computer or industrial experiments when the underlying model is unclear. A uniform scattering of its design points on the experimental domain is desired by the uniform experimental design (Khattree et al. 2003). To determine the content of polysaccharides, Zhang et al. used a widely applied stable and simple method called phenol–vitriolic colorimetry (Dubois et al. 1956; Han et al. 2014; Wang et al. 2012c; Zhao et al. 2014), which employs the phenol–sulfuric-acid reaction, i.e., phenol undergoes sulfonation in situ, and the phenol–sulfonic acid generates a decreased color intensity for many hexoses and pentoses (Masuko et al. 2005). The final determined optimum extraction conditions were as following: temperature 100 °C, extraction time 180 min, and ratio of solvent to material 36 (mL/g). Extraction yield and polysaccharide content were 199.36 mg/g and 16.04 %, respectively.

By use of orthogonal arrays (or Taguchi methods) of L_9 $(3)^4$, which are frequently employed in industrial experiments to investigate the effect of several control factors, Li et al. (2003b) investigated also the factors, including extraction temperature and time, ratio of solvent to material, and material size, which all affect the extraction process. Usually, for an orthogonal array, columns for the independent variables are "orthogonal" to one another; thus, this method has the advantage of being easy to perform for large-scale analysis but with less experimental effort, and conclusions are valid over the entire region spanned by the control factors and their settings. In the work of Li et al. (2003b), the single-factor experiments were assayed and then the levels of parameter were selected. Based on polysaccharide content, range analysis was performed. The authors concluded that the optimal processing conditions for HWE were temperature 80 °C, extraction time 120 min, ratio of solvent to material 16 mL/g, and size of material particle 300–450 μm. According to the results of orthogonal arrays, another parameter – number of extractions–was further investigated; it was concluded that 4 extractions is the best.

In order to evaluate interactive effects among various parameters, response surface methodology (RSM) is used to optimize the process parameters. RSM is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data. It can be well applied when a response or a set of responses of interest are influenced by several variables (Bezerra et al. 2008). Using this method, Du et al. (2008b) optimized the extraction process by investigating the effects of extraction temperature, extraction time, and ratio of solvent to material on the extraction yield. After analyzing the data with MATLAB 7.0

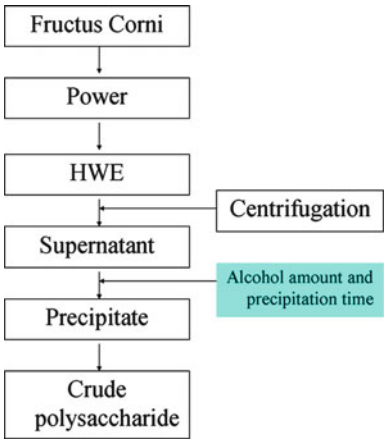

Fig. 2 Diagram of the process of hot-water extraction (*HWE*) of polysaccharides from *Fructus Corni*

software, the optimum extraction conditions were temperature 84 °C, extraction time 142 min, and water volume 276 mL for 20 g of raw material. The value of polysaccharide extraction yield from *Fructus Corni* was 89.79 μg/mL, which is in good agreement with the predicted value of 89.34 μg/mL by the RSM model. Based on these results (Du et al. 2008b), another study, reported by Wang (2008), further investigated the amount of alcohol that should be used for polysaccharide precipitation and the length of precipitation during the extraction of polysaccharides from *Fructus Corni*. The diagram of the extraction process is presented in Fig. 2. In this work, five levels of different factors were performed using RSM, and the experimental data variances were statistically analyzed using MATLAB 7.0 designed for the analysis of variance (ANOVA). The results indicated that the quadratic polynomial model is significant ($P < 0.05$), thus suggesting that the experimental data fit well with the model. By analyzing regression equation and inverse matrix, it was determined that 652 mL of alcohol should be used for 100 mL supernatant and the precipitation should take place for 59.7 h. Under such extraction conditions, the content of crude polysaccharide is determined to be 35.22 g/100 mL.

Although HWE is simple and safe, the disadvantage of this method is a rather low extraction yield and a long extraction time at high temperature, which could accelerate degradation of polysaccharides and thus constitutes a further contribution to low extraction efficiency.

2.2 Ultrasonic-Assisted Extraction

Ultrasonic-assisted extraction (UAE) is an emerging technique that is believed to have various advantages over traditional extraction methods and is regarded as one of the simplest extraction techniques. The mechanism of UAE involves two types of physical phenomena: diffusion through cell walls (acoustic cavitation effect) and washing out the cell contents once cell walls are broken. Ultrasound wave can facilitate swelling and hydration of cell walls, leading to an enlargement of the

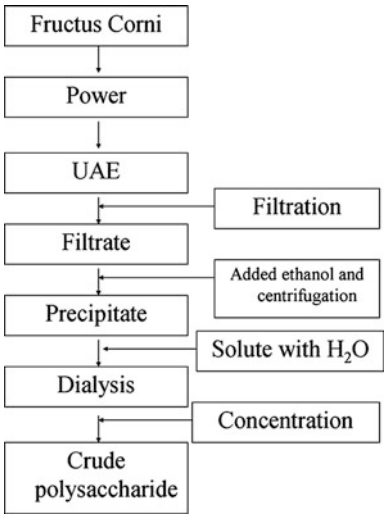

Fig. 3 Process of ultrasound-assisted extraction (*UAE*) of polysaccharides from *Fructus Corni*

pores formed on cell walls. This consequence will further contribute to cell disruption and diffusion of content from the plant-cell walls and ultimately washing out of the target compounds (Vinatoru 2001; Hromadkova et al. 1999; Yang et al. 2008a). Accumulated evidence suggests that the process parameters, including ultrasound frequency, ultrasound power, ratio of solvent to material, extraction time and temperature, number of repeated extractions, as well as the size of the material, must all be optimized to improve polysaccharide extraction efficiency.

UAE has been applied widely in traditional Chinese medicine to extract both single and combined medical herbs (Tian et al. 2013; Fan et al. 2012; Teng and Choi 2014). Its application on polysaccharide extraction has been described in a number of reports (Wen et al. 2011; Tahmouzi 2014; Chen et al. 2014). Figure 3 summarizes an extraction process described by Lei and Li (2013) who used UAE to extract polysaccharides from *Fructus Corni*. The process variables, such as ultrasonication time, extraction temperature, and ratio of solvent to material were used to investigate the extraction of polysaccharides in single-factor experiments, which provided the basis for a further orthogonal design based on three levels of different parameters. The data were finally analyzed by ANOVA, and the results showed that the optimal process parameters were 40 min of ultrasonic pretreatment, extraction temperature 80 °C, and ratio of solvent to material 30, which led to an average polysaccharide extraction yield of 5.29 %. Compared with conventional HWE, UAE uses less energy and has a higher extraction efficiency. In a study reported by Zhang and Kong (2007), a different ultrasonic device was used, but the whole extraction process was quite similar to that of Lie et al. (Fig. 3). Factors including extraction time, extraction temperature, and ratio of solvent to material were investigated using orthogonal design at three levels. The final optimized extraction condition was 60 min of ultrasonic pretreatment, extraction temperature 80 °C, and ratio of solvent to material 20, which is slightly different from the results of Lei and Li (2013). Although the

reason for this discrepancy between the two reports for optimized conditions is not known, it might be reasonable to assume that the differences in ultrasonic devices might generate a different acoustic cavitation effect, thus resulting in a different extraction yield. Therefore, the ultrasound power and frequency should be further investigated, which was not addressed in either study.

2.3 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE), also called microwave-assisted process (MAP), or simply microwave extraction, is a newly evolved technique that combines microwave and traditional solvent extraction. Applied microwaves heat the extraction solvents and plant tissues during the process because the penetrated microwave energy into raw materials and extraction solvent generates a volumetrically internal heat source due to molecular friction. Thus, the mass transfer of target compounds is accelerated as a consequence of an increased extraction kinetic. The advantages of MAE include a shorter extraction time, less solvent, lower cost, and a higher extraction rate compared with traditional extraction methods (Rodriguez-Jasso et al. 2011; Eskilsson and Björklund 2000; Srogi 2006). Generally, a selective and quick extraction of natural chemical ingredients can be achieved by MAE, which thus can be regarded as a cost-effective and environmentally friendly extraction method (Zhang et al. 2014b; Taghvaei et al. 2014; Barrera Vázquez et al. 2014). In our previous work, extraction of polysaccharides from *Fructus Corni* using UAE was investigated using a single-factor design and orthogonal test. Various factors, such as microwave power, length of microwave treatment, ratio of solvent to material, and extraction time were optimized to improve the extraction yield of polysaccharides. Based on the results of single-factor experiments, orthogonal test design were carried out using $L_9(3^4)$. The optimal extraction parameters were concluded to be the following: microwave treatment for 10 min with a power of 560 W after a preextraction for 70 min, with the ratio of material to water 1:25 (g/mL). The average yield was 10.53 %, which is higher than using traditional extraction methods (Wu et al. 2011). In another study, RSM was used to investigate the interactions of various factors affecting polysaccharide extraction from *Fructus Corni* using MAE. After employing the single-factor designs to obtain different levels, extraction conditions were optimized using RSM, and the final optimized extraction conditions were as following: soak time 120 min, microwave power 456 W, solvent-to-material ratio 33 (mL/g), and microwave extraction time 3.2 min. Using these optimal extraction parameters, the extraction yield of polysaccharides was 11.12 % (Hu et al. 2011).

2.4 Enzymatic-Assisted Extraction Technique

Another alternative environmentally friendly extraction method is enzymatic-assisted extraction (EAE), which has the advantage that enzymes serve as ideal catalysts because they hold inherent abilities to specifically and region-selectively catalyze

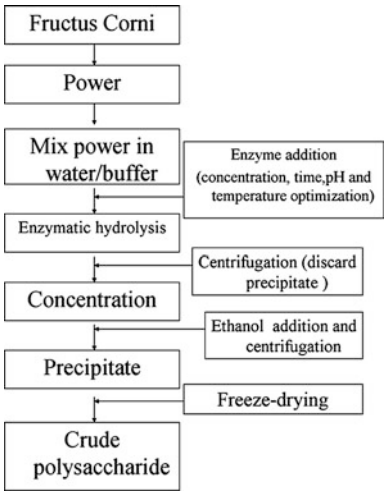

Fig. 4 Process of enzyme-assisted extraction (*EAE*) of polysaccharides from *Fructus Corni*

the degradation or disruption of cell walls and membranes, thus accelerating the mass transfer of target compounds into extraction solvent and as a consequence enabling a better release and more efficient extraction of bioactive compounds (Bahramian et al. 2011). Therefore, this method is regarded as an eco-friendly, greener, and cleaner chemical tool for food industry and pharmaceutical companies to extract new compounds. In comparison with conventional extraction methods, EAE exhibits properties of faster extraction, higher recovery, reduced solvent usage, and lower energy consumption. Enzymes such as pectinases, cellulases, and hemicellulases have been widely used for pretreatment of plant material prior to conventional methods for extraction (Puti et al. 2012), which can also significantly reduce the amount of solvent requirement. Application of EAE has been proved to be effective for natural ingredients, such as phenolic compounds, oils, and pigments when aiming for high extraction yields (Wu et al. 2005; Le and Le 2012; Jiao et al. 2014; Sowbhagya and Chitra 2010). Accumulated reports exist also for the application of EAE for polysaccharide extraction (Bahramian et al. 2011; Jiang 2014; Wang 2014; Qian 2014; Zhu et al. 2014). To obtain a more effective enzyme-assisted extraction, it must be optimized first by using a single enzyme or a combination of various enzymes, as well as optimal operation parameters such as temperature, pre- and actual extraction time, enzyme concentration, and optimal pH depending on the sources of plant materials selected. Figure 4 provides a summary of the application of EAE for crude polysaccharide extraction from *Fructus Corn*. Cheng et al. (2010) used a combination of various enzymes, including cellulase, pectic enzymes, and neutral proteases. The extraction process was optimized for the pretreatment of *Fructus Corni*, and the amount of used cellulase, pectic enzyme, and neutral protease was studied based on orthogonal test. The results showed that the amount of enzyme is 2.0 % cellulase, 2.0 % pectic enzyme, and 1.5 % neutral protease. Furthermore, the length of time for pretreatment, solvent pH, and temperature were all optimized using RSM. Data were analyzed using Design-Expert 7.0 software. ANOVA indicated that the quadratic polynomial model

is significant ($p < 0.01$), thus clearly indicating that the model fit well with the response. Moreover, the coefficient of multiple determination (R^2) is 0.9555, suggesting that a very high correlation was obtained. The determined optimum conditions for enzyme hydrolysis were temperature 50 °C, treatment for 69 min, and pH 3.8, with an extraction yield of 5.54 %. In another study by Chang (2011), the effect of pectin enzymes, variables such as the ratio of solvent to material, extraction time, extraction temperature, and amount of enzyme were investigated using the orthogonal test. Based on a single experiment, three levels of different parameters were selected, and the results showed that the extraction temperature was the most significant factor and extraction time is less significant compared with the others. This study determined the optimal extraction conditions were ratio of solvent to material 5 (mL/g), extraction time 6 h, extraction temperature 80 °C, and amount of pectin enzyme 0.55 g/L. Obviously, there is a discrepancy compared with the results of Cheng et al. (2010), thus further addressing the importance of thorough optimization of EAE depending on the selected enzymes for polysaccharide extraction from *Fructus Corn*.

2.5 Ultrasonic Microwave-Assisted Extraction

Considering the obvious advantages of both UAE and MAE techniques, particularly their abilities to reduce the extraction time and the consumption of solvent (Sporring et al. 2005), it is highly attractive to consider the combination of both methods to take advantage of UAE-enhanced mass transfer in inter- and intrafiber pores and MAE-intensified heat transfer. Thus, the combination of UAE and MAE is assumed to produce a high yield within a short reaction time. To this end, microwave- and ultrasound-assisted extraction (MUAE) has been developed to accelerate the extracting process and to improve extraction efficiency of natural ingredients (Cheng et al. 2011; Zhang and Liu 2008; Gergely et al. 2000; Chen et al. 2010). Optimization of MUAE-based polysaccharide extraction from *Fructus Corni* was reported by Shi (2014), who performed pretreatment of *Fructus Corni* powder using MAE prior to UAE. Variables including ultrasound time, temperature, ratio of solvent to material, and concentration of ethanol were evaluated using orthogonal array design. It was found that ultrasound time is the most significant factor for polysaccharide extraction, and the ratio of solvent to material is the least significant based on ANOVA analysis. The final determined optimal extraction conditions were extraction time 80 min, extraction temperature 60 °C, ratio of solvent to material 14 (mL/g), and concentration of ethanol 65 %, with a yield of 16.83 %.

3 Purification of *Fructus Corni* Polysaccharides

Extracted polysaccharides from plant materials using the above-mentioned methods are usually not pure enough for a direct characterization; therefore, commonly, further purification steps must be performed to remove undesired substances, such as proteins, phenolic compounds, amino acids, or monosaccharides.

In general, phenolic compounds can be eliminated by performing a methanolic extraction step (Palacios et al. 2011), which could even increase the effectiveness of extraction (Park et al. 2009). While remove of proteins can be achieved by performing a protease treatment followed by a trifluoroacetic acid (TCA, 10–20 %, w/v) precipitation or a direct TCA precipitation, both finally followed by centrifugation to remove aggregated proteins.

To purify polysaccharides, as the final step, various techniques, including ethanol precipitation (a ratio of 2:1, v/v) or combined with concentrated sodium chloride solutions to favor precipitation, fractionation by ammonium sulfate precipitation based on the molecular size of polysaccharides (Ragaee et al. 2008; Li et al. 2006), acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration, and affinity chromatography (Jin et al. 2012), can be applied. In general, gel chromatography, such as size-exclusion chromatography, separates polysaccharides based on their molecular weights, while ion-exchange filtration separates neutral from acidic polysaccharides by changing elution buffers. To date, polysaccharides isolated from *Fructus Corni* are mainly neutral and acidic. Yang et al. (2008b) separated an acidic polysaccharide, which has a molecular weight of 8.7×10^4 Da, using ion-exchange chromatography packed with positively charged diethylaminoethyl-cellulose (DEAE-C) resin. In another study reported by Yang et al. (2008b), a neutral polysaccharide composed of glucose, galactose, xylose, and fucose with a molecular weight of 2.47×10^4 Da was identified using DEAE-C and a Sephadex G-200 column.

4 Physiochemical and Structural Features

The physiochemical and structural features of a polysaccharide are defined by its molecular weight, chain composition, configuration and conformational isomers, sequence order of monosaccharide residues, presence and position of branches, presence of glycosidic linkages, as well as number and location of appended noncarbohydrate functional groups (Johansson et al. 2006; Zhang et al. 2007b). Current available techniques to characterize the structural details of polysaccharides include both chemical and analytical methods. Chemical methods are usually used as the first step and followed by analytical strategies for a detailed determination. For example, to determine the chain composition of monosaccharides, acid hydrolysis using such compounds as trifluoroacetic acid (TFA), H_2SO_4, or HCl, is first applied then followed by monosaccharide profile determination using high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with mass spectrometry (MS) (Agrawal 1992). For complex products, an *O*-methylation of polysaccharides is preferred to prepare samples for MS analysis because the *O*-methylated polysaccharides have the advantage of increased stability of ions and sensitivity during MS detection. While nuclear magnetic resonance (NMR) spectroscopy has a poor sensitivity in structural determination, it is a helpful tool for determining the configuration of polysaccharides (Laws et al. 2008; Wang et al. 2008b). Other biophysical techniques, like infrared spectroscopy

(Calonje et al. 1996; Kulicke et al. 1997), multiangle laser light scattering (Jelsma and Kreger 1975), or X-ray crystallography (Lee et al. 2010) can also be used for the structural determination of polysaccharides, but all have disadvantages of a high cost and limited availability. At this point, it is interesting to mention that chemical dyes that specifically interact with certain conformations can be applied under certain cases. For example, calcofluor, a chemofluorescent stain, can nonspecifically bind to β-linked polysaccharides, producing an intense green fluorescent sample, and therefore could enable distinction of the β-configuration of polysaccharides from that of α-configuration (Ko and Lin 2004). Other examples include detection using aniline blue in solution for single-helix conformation (Ogawa et al. 1994) and Congo red in alkaline solutions for glucans with high degrees of β-(1 → 6)-glycosidic bonds, which is usually arranged into a triple helix and can thus lead to a complex formation between the polysaccharide and the dye molecule through hydrogen-bond formation and/or hydrophobic interactions (Yang et al. 1999).

The molecular weight and composition of monosaccharides from polysaccharides isolated from *Fructus Corni* seem to vary, as evidenced by various reports (summarized in Table 1), which might be due to different purification processes or the sources of raw materials. Yang et al. (1999) isolated a water-soluble polysaccharide (Co-4), which has a molecular weight of 24,700 Da as determined using high-performance gel-permeation chromatography (HPGPC). The composition of this polysaccharide was determined using thin-layer chromatography, paper chromatography, GC–MS, and ^{13}C-NMR techniques, and results showed that it is composed of glucose, galactose, xylose, and fucose. Another polysaccharide, SZYP-2, separated by Chen et al. (2002) using HWE, macroporous resin decoloration, ethanol precipitation, and BUTYL-Sephadex G-200-column chromatography, is composed of rhamnose, arabinose, galactose, and glucose in a molar ratio of 1:3.3:3:3.8. Furthermore, another two polysaccharides, PFCC-I and PFCA-Ⅲ, were isolated from *Fructus Corni* by Li et al. (2003a, b), which they derived from physiochemical characterization and antioxidation activity investigation. The molecular weight of PFCC-I and PFCA-Ⅲ was determined to be 75,700 and 17,400 Da, respectively. The composition of monosaccharide includes xylose and glucose in a molar ratio of 18.8:81.2 for PFCC-I, while for PFCA-Ⅲ, it is composed of rhamnose, arabinose, and glucose in a molar ratio of 13.74:50.54:35.72. FCP5-A is an acidic polysaccharide that was isolated from *Fructus Corni* using water extraction and column chromatography by Yang et al. (2008b). Monosaccharide analysis using gas chromatography showed that FCP5-A has a molecular weight of 87,000 Da and is composed of rhamnose, arabinose, galactose, and galacturonic acid in a molar ratio of 1:5.7:0.6:1.2. While a CSZP polysaccharide with a molecular weight of 81,000 Da was separated by Wei and Cui (2006) using HWE, DEAE-32 ion-exchange chromatography and Sephacryl n300 HR gel filtration chromatography, its structure and the composition of monosaccharide still needs determination.

In contrast to the determination of molecular weight and composition of monosaccharide for those isolated polysaccharides from *Fructus Corni*, greater

Table 1 Polysaccharides isolated from *Fructus Corni*

No.	Compound name	Molecular weight (Da)	Composition of monosaccharide	Structures	Reference
1	Co-4	24,700	Glucose, galactose, xylose, and fucose	(1 → 4)β-D-glucan	(Yang et al. 1999)
2	SZYP-2		Rhamnose, arabinose, galactose, and glucose in the molar ratio of 13.3:3:3.8	Ara(1 → 4)Rhm (1 → 4)Ara(1 → 2,4)Rhm(1 → 3) Glc(1 → 3)Gal (1 → 4)Gal(1 → 6)Glc(1 → 2,6) Glc(1 → 4,6)Glc	(Chen et al. 2002)
3	PFCC-I	75,700	Xylose and glucose in the molar ratio of 18.8:81.2		(Li et al. 2003a)
4	PFCA-III	17,400	Rhamnose, arabinose, and glucose in a molar ratio of 13.74:50.54:35.72		(Li et al. 2003b)
5	FCP5-A	87,000	Rhamnose, arabinose, galactose, and galacturonic acid in molar ratio of 1:5.7:0.6:1.2	Rha(1 → 4)Gal with side chain attached to O-4 of Rha	(Yang et al. 2008b)
6	CSZP	81,000			(Wei and Cui 2006)

efforts are required to determine structure and molecular conformation of polysaccharides. Structural analysis of a water-soluble polysaccharide (Co-4) showed that Co-4 contains a backbone composed of 1,4-linked β-D-glucan residues, with branches attached to O-6 in some residues. The branches contain α-D-Xyl (1→,α-D-Xyl(1 → 2)) α-D-Xyl(1→),β-D-Glc(1→,β-D-Gal(1 → 2)) α-D-Xyl(1→,Fuc (1 → 2) β-D-Gal(1 → 2) α-D-Xyl(1 →)) (Yang et al. 1999) (Fig. 5). The structural feature of SZYP-2 was assayed by capillary gas chromatography. The glycosidic linkages of SZYP-2 is Ara(1 → 4)Rhm(1 → 4)Ara(1 → 2,4)Rhm(1 → 3) Glc(1 → 3)Gal(1 → 4)Gal(1 → 6)Glc(1 → 2,6)Glc(1 → 4,6)Glc (Fig. 6) (Chen et al. 2002). The structural feature of an acidic polysaccharide (FCP5-A) from *Fructus Corni* was also investigated using various strategies, including infrared spectroscopy (IR), GC, partial hydrolysis with acid, uronic acid reduction, methylation, GC-MS, and ^{13}C-NMR analysis. The results indicate that a backbone is composed of 1,2-linked Rha and 1,4-linked Gal, with branches attached to O-4 of rhamnosyl residues. The branches contain highly branched arabinan and short linear 1,3-linked galactan (Fig. 7) (Yang et al. 2008b).

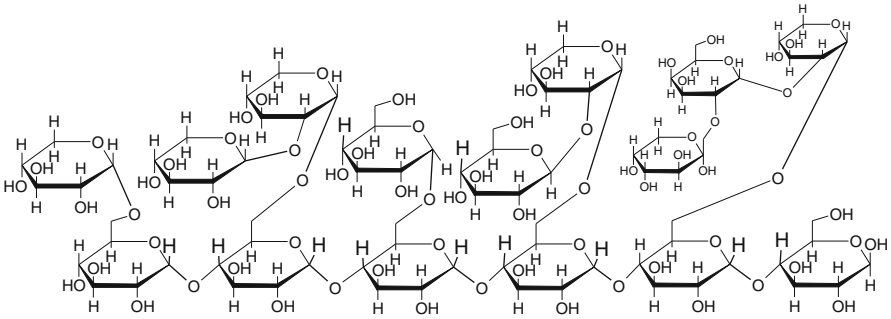

Fig. 5 Molecular structure of water-soluble polysaccharide (Co-4)

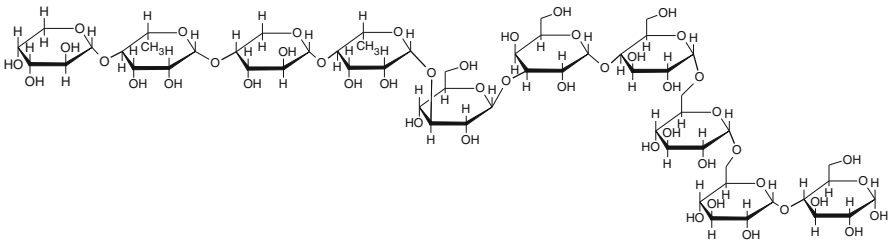

Fig. 6 Molecular structure of SZYP-2

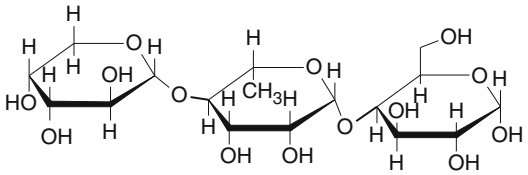

Fig. 7 Molecular structure of FCP5-A

5 Bioactivity of Polysaccharides from *Fructus Corni*

5.1 Immunomodulatory Activity

Polysaccharides are one of the main classes of bioactive substances identified in fungi, algae, and higher plants. They display broad immunomodulatory activity properties (Kouakou et al. 2013; Yao et al. 2009). *In vitro* cell culture analysis revealed that polysaccharides could improve the biological activity of interleukin 2 (IL-2), a protein that regulates the activities of white blood cells (leukocytes, often lymphocytes) responsible for immunity; in other words, IL-2 constitutes a part of the body's natural response in discriminating "own" and "not own" factors under microbial infection conditions. This result thus provides clear evidence of the specific immunomodulatory

activity of polysaccharides from *Fructus Corni* on immune cells (Jiang et al. 2010). In another study (Du et al. 2008a), it was demonstrated that polysaccharides from *Cornus officinalis* both in crude and processed form showed a beneficial effect on an immune-suppressed rat model induced by cyclophosphamide. The polysaccharides showed an enhanced effect on nonspecific immunity, specific humoral immunity, and specific cellular immunity when applied in immune-depressed mice; the processed form possesses a dramatically increased function. Improved effects of processed polysaccharides are in agreement with another study performed by Yao et al. (2009), who used a low-immunity mice model. After 9 days of polysaccharide treatment, the blood sample was taken from the eye pit to analyze the concentration of serum agglutinin, which was used as an indicator to see whether *Fructus Corni* polysaccharides can improve humoral immunity. The data demonstrated that compared with unprocessed polysaccharides, the processed form significantly improves the concentration of serum agglutinin and thus can benefit the body-specific immune function.

5.2 Antioxidant Activity

Free radicals induced by oxidative stress are believed to be one of the leading causes of aging and various diseases, including cardiovascular diseases, cancer, multiple sclerosis, Parkinson's disease, autoimmune disease, and senile dementia (De Lima et al. 2004; Lu et al. 2010; Zeng et al. 2014). Accumulated evidence suggests that natural polysaccharides function as a promising antioxidant (Zhang et al. 2008; Sun et al. 2014; Zhao et al. 2013; Li et al. 2003c). Isolated polysaccharides from *Cornus Officinalis* Seib. et Zucc were further fractionated using ethanol precipitation at various concentrations to investigate the antioxidant activities of different polysaccharides (Zhang et al. 2008). The antioxidation capacity of fractionated polysaccharides was measured using methods based on Fenton reaction, a commonly used nitroblue tetrazolium (NBT) photo-reduction method. It was found that the polysaccharides precipitated by 50% ethanol had the strongest free radical scavenging activity at a polysaccharide concentration of 1.2 mg/mL, with a scavenging rate for hydroxyl free radical and superoxide anion of 89.9 % and 87.1 %, respectively. Polysaccharides prepared with 90 % ethanol precipitation can inhibit lipid oxidation, even showing a comparable antioxidant activity against autoxidation of lard stored in an oven with that of 2,6-Di-tert-butyl-4-methyl phenol [$C_{15}H_{24}O$, (BHT), also known as antioxidant 264], a widely used natural antioxidant in food chemistry.

In vitro evaluation of purified polysaccharides PFCC-I and PFCA-Ⅲ from *Fructus Corni* as a potential antioxidant (Li et al. 2003a, b; Ou and Liu 2007) found that PFCA-III not only showed a strong protection activity on axunge and gingili against oxidation, but also showed scavenging actions towards Fenton-reaction-generated hydroxyl free radicals as well as superoxide anions generated by a pyrogallol autoxidation system. The EC50 of PFCA-III on hydroxyl free radicals was 430 mg/L, and the scavenging rate of PFCA-III on superoxide anion was 21.5 % at a concentration of 100 mg/L. The EC50 of PFCC-I on hydroxyl free radical and superoxide anion was 80 and 34.9 μg/mL, respectively.

5.3 Antitumor Activity

Because of the immunological enhancing property of polysaccharides, they have also been widely used in tumor therapies, as confirmed by biochemical/medical evidence (Bejima et al. 2000). This is because polysaccharides can activate various immune responses in the host without observed harmful body effects. Using an S180 rat model, the antitumor effects and underlying immunologic mechanism of polysaccharides from *Fructus Corni* were found to noticeably restrain tumor growth in S180 rats ($P < 0.01$) (Zou et al. 2012). Further mechanism analysis revealed that the expression of the cluster of differentiation 4 ($CD4^+$) T-helper white blood cells was increased while the expression of $CD8^+$ T cells in peripheral blood was decreased, indicating an improved immunity. In addition, an increase in IL-2, a protein that regulates the activities of white blood cells and serves as part of the body's natural response in discriminating between foreign ("nonself") and host ("self") organisms. A decrease in IL-4 was also recorded. All the above observed effects are in a dose- and concentration-dependent manner.

Effects of polysaccharides from *Fructus Corni* on apoptosis were also investigated (Wang et al. 2012a) using lung carcinoma A549 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a colorimetric assay to assess cell viability. The results showed that polysaccharides of *Fructus Corni* displayed a strong inhibiting effect against cell multiplication. The expression pattern of apoptosis-related genes in lung carcinoma A549 cells was also analyzed using agarose-gel electrophoresis (AGE), which showed a ladder-shaped DNA strap with ~200 base-pairs of difference among various ladders. Further immunohistochemistry using streptavidin–peroxidase demonstrated that B-cell lymphoma 2 (Bcl-2, an important antiapoptotic protein involved in cell-death regulation) expression was down-regulated, while the expression of apoptosis regulator Bax (also known as Bcl-2-like protein 4, a protein that promotes apoptosis) was up-regulated, thus suggesting polysaccharides exert an antitumor effect. Increase of Bax expression in HeLa cells was also observed when the cells were treated with various concentrations of polysaccharides for 24 h (Wang et al. 2012b), which was correlated with a morphological change in the shape of HeLa cells, as observed using an upside-down microscope. Together, these results suggest that polysaccharides of *Fructus Corni* can indeed serve as an antitumor compound, as they promote Bax expression and thus induce tumor-cell apoptosis.

5.4 Antiaging Effect

Although aging is an inherently complex process, accumulation of oxidative stresses is believed to be an important contributor involved in the aging processes (Zhang et al. 2003; Jin et al. 2009). Antiaging effects of *Fructus Corni* have been recorded and demonstrated using both *in vitro* and *in vivo* methods. Ou et al. (2010) investigated the effects of polysaccharides of *Fructus Corni* using human dermal fibroblast (HDF) cell system and found that the cell viability of HDF can be improved by polysaccharides.

In another study, Wang et al. investigated HDF cell morphology after supplementation with polysaccharides of *Fructus Corni* from the 40th generation of HDF cell culture. They determined the cell activity by MTT assay and the expression pattern of key genes involved in aging, including cyclin D1 (a protein required for G1/S transition of cell cycle, whose aberrant behavior such as mutation or overexpression leads to altered cell-cycle progression and thus contributes to tumorigenesis) and cyclin-dependent kinase 4 (CD4, also known as cell-division protein kinase 4, which is a catalytic subunit of the protein kinase complex important for cell-cycle G1-phase progression, and mutations of this protein lead to tumorigenesis) using reverse transcription polymerase chain reaction (RT-PCR). They found that polysaccharide treatment increased cell activity and the content of CDK4 messenger RNA (mRNA) but decreased the content of cyclin D1 mRNA. These data suggest that a possible mechanism of antiaging effect of polysaccharides of *Fructus Corni* is to regulate the expression of cyclin D1 and CDK4 in HDF-senescence-model cells.

Using aging in a rat model induced by D-galactose (Zhou et al. 2011; Qin et al. 2011), it was found that polysaccharide treatment enhanced the antioxidation activity of superoxide dismutase (SOD), inhibited lipid peroxidation, and decreased the activity of acetylcholinesterase (also known as AChE, or acetylhydrolase, a hydrolase that hydrolyzes the neurotransmitter acetylcholine involved in a range of central nervous system diseases), and up-regulated the mRNA level of nerve growth factor (NGF, a small protein important for growth, maintenance, and survival of certain target nerve cells). In an aging mouse model of kidney deficiency (Fu et al. 2007), polysaccharides of *Fructus Corni* were observed to prolong the swimming time of mice with weight loading and the ability of antihypoxia, to promote SOD activity and to decrease the content of malondialdehyde (MDA, a naturally occurring reactive species and indicative marker for oxidative stress). All these results suggest that polysaccharides of *Fructus Corni* can be used as antiaging reagents to benefit body health.

5.5 Sexual Function Effect

Shao et al. (2010) demonstrated that polysaccharides of *Fructus Corni* increase the sexual function of hemicastrated rats, possibly by adjusting the hypothalamus–pituitary–gonadal axis. In that study, polysaccharides at various doses were given to Sprague–Dawley (SD) rats with right testis extirpated. Both mating and erective tests were assessed. The level of serum sex hormone testosterone was increased, as determined with radioimmunoassay (RIA). It was found that the incubation period of penis erection and the mounting period were shortened in the group treated with *Fructus Corni* polysaccharides and the percentage of mounting rats increased. Significant increase in the organ coefficient of foreskin gland and seminal vesicle-prostate gland as well as sperm count and vigor were observed ($P < 0.01$). All results suggest that polysaccharides have a positive effect on sexual function. In another study (Jiang et al. 2013), *Fructus Corni* polysaccharides were shown to benefit damaged testis in a dose-dependent manner, as investigated on rats in which

testis-tissue damage was induced by a warm bath. This conclusion was based on the investigation of morphological changes in testis organ, sperm amount and motility in epididymis, SOD activity, MDA content, and germ-cell apoptosis after 14 days of intragastric treatment of SD rats with *Fructus Corni* polysaccharides.

5.6 Hepatoprotective Activity

Yao et al. (2009) reported that the administration of *Fructus Corni* polysaccharides at a dose of 10 g/kg body weight prevents liver injury induced by CCl_4. The contents of the alanine transaminase [ALT, also called serum glutamic–pyruvic transaminase (SGPT) or alanine aminotransferase (ALAT), a transaminase enzyme clinically measured as a specific indicator of liver inflammation for diagnostic evaluation of hepatocellular injury to determine liver health] in serum of injured mice and MDA in injured liver tissue decreased, while an increased SOD activity in injured liver tissue was observed. It was therefore hypothesized that *Fructus Corni* polysaccharides have a nourishing effect on liver and kidney. A similar hepatoprotective effect of processed *Corni Fructus* extracts on D-galactose-induced liver injury in mice was also observed Jiang et al. (2013).

6 Conclusion

Despite accumulated investigations dealing with the extraction of polysaccharides from *Fructus Corni*, and mounting evidence suggests that polysaccharides have important pharmacological activities, the underlying molecular mechanisms of how the chemical structures of polysaccharides correlate with their respective bioactivities are still ill-defined, particularly because of the lack of detailed chemical structures and their chain conformations. Therefore, future extensive investigations focussing on such mechanisms are urgently required for application for pharmaceutical purposes. Moreover, although traditionally *Fructus Corni* has been used as a common method to nourish liver and kidney, and although it is believed that polysaccharides function as one of the bioactive compounds involved in that function, the specific utilization of *Fructus Corni* polysaccharides remain to be determined. Considering the wide application of polysaccharides from other sources being used in the food industry, it is reasonable to assume that *Fructus Corni* polysaccharides and their derived specific products hold high potential for broad applications in food or for pharmacological purposes pending extensive scientific investigations.

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Bioactivity of Chitosan Derivatives

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Contents

Abstract

Chitosan, a natural-based polymer obtained by alkaline deacetylation of chitin, is composed of glucosamine and *N*-acetylglucosamine monomers. Chitosan and its derivatives have a great potential for a wide range of applications in biomedical, pharmaceutical, food, cosmetics, and environmental fields due to its biodegradability, biocompatibility, nontoxicity, and versatile chemical and physical properties. For a breakthrough in utilization, especially in the biomedical field, chemical modification of chitosan will be a key point that can introduce desired properties and enlarge the field of the potential applications of chitosan. Different approaches have been reported to prepare chitosan derivatives and chitosan oligomers with low, medium, and high molecular weights. In this chapter, the properties such as biodegradation, antimicrobial activity, anti-inflammatory and tissue regeneration activity, wound-healing properties, antioxidant activity, and antitumor activity of chitosan and its derivatives are discussed in detail.

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1 Introduction

Chitin, which is the most abundant biopolymer in nature after cellulose, is a polysaccharide that is widely spread among marine and terrestrial invertebrates and lower forms of a plant kingdom (Jayakumar et al. 2011). Chitosan is a poly (aminosaccharide), normally obtained by alkaline deacetylation of chitin as shown in Fig. 1. This naturally occurring polymer has a repeating structural unit of 2-acetamido-2-deoxy-β-D-glucose (Prabaharan et al. 2008). Chitosan is semicrystalline and the degree of crystallinity is a function of the degree of deacetylation. Chitosan allows specific chemical modifications since it has primary amine groups at the C-2 position and primary alcoholic groups at the C-6 position of its monomeric units. These reactive sites enable the grafting of a large variety of properly functionalized molecules (Prabaharan and Mano 2005b; Jayakumar et al. 2005). Chitosan is available in a variety of useful forms, and its unique chemical and biological properties such as biodegradability, bioactivity, and biocompatibility make it very attractive biomaterials. It is extensively used in many types of applications such as treatment of wastewater, chromatographic support, enzyme immobilization, wound-healing dressing, dental application, adhesion bandages for surgery, and drug-delivery system (Prabaharan et al. 2007; Prabaharan and Mano 2005a, 2007). The unique properties of chitosan, such as biodegradability, non- toxicity, antimicrobial activity, and polycationic nature, make it suitable for a number of applications.

Bioactivity of chitosan and its derivatives is associated with several phenomena, including biodegradation, stimulation of natural resistance, and direct action against pathogens. Biocompatibility has been defined as an ability of the biopolymers to perform with an appropriate host response in the specific application (Struszczyk 2002). The presence of these phenomena in the formation of chitosan can be linked to the type of chitosan, pathogens, or organisms attacked. Action of chitosan and its derivatives depends on the type of existing hazards, like infection of phytopathogenic bacteria and fungi, viruses or plant growth stimulation, wound healing, and the structure of chitosanous agents (Struszczyk 2002). Bioactivity and biocompatibility of chitosan and its derivatives is a function of several phenomena related mainly to their structural parameters such as average molecular weight, degree of deacetylation, distribution of both *N*-acetylglucosamine and glucosamine in polymer chain, crystallinity, porosity, charge character, and type of existing hazards (Struszczyk 2002). This chapter focuses on the biological activities of chitosan and its derivatives, which are correlated with their structures and physicochemical properties. This overview provides insights into these activities and for developing chitosan and its derivatives with special biological activity.

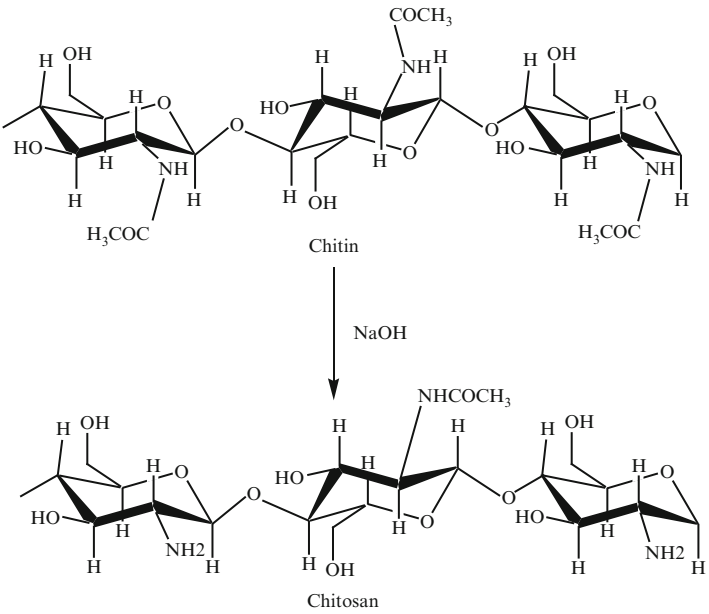

Fig. 1 Reaction scheme for the preparation of chitosan from chitin

2 Biodegradation of Chitosan

Chitosan and its derivatives are degraded by various lysozymes, which are present in plants and animals. A residual number of acetyl groups and the free hydroxyl group at C3 of the sugar units are required in order to observe reasonable hydrolysis rates (Tokura et al. 1984). Chitin deacetylase, detected in many fungi and insect species, catalyzes the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan (Tsigos et al. 2000). Chitinases are another type of enzymes that are present in plant seeds, tubers, and flower organs, and they are associated with stimulation of self-defense in response to an exogenous attack by plant pathogens and/or contact with chitooligosaccharides (Santos et al. 2007). They have been detected in extracellular, cytosolic, and microsomal fractions from those organisms.

The presence of chitinases in human serum plays a defensive role against chitin-containing pathogens (Overdijk et al. 1996). Papain, an enzyme that is used in the food industry for the tenderization of meat by controlled hydrolysis of muscle protein, may be applied as a hydrolyzing agent of chitosan. This enzyme degrades long chain chitosan at room temperature, producing low molecular oligosaccharides, which show a number of attractive biological actions and can be used as elicitors of plant defense mechanisms against pathogens, accelerators of the root nodule formation, antitumor agents, material for synthesis of biologically active compounds, anti-inflammatory drug, etc. (Kobayashi et al. 1997).

The biodegradability of thiolated chitosans in comparison to unmodified chitosan was reported by Laffleur et al. (2013). In this study, two chitosan–thioglycolic acid conjugates and one chitosan–mercaptonicotinic acid conjugate were obtained. The obtained chitosan–thioglycolic acid conjugate displayed 267.7 μmol and 116.3 μmol of immobilized thiol groups. With 325.4 μmol immobilized thiol groups, chitosan–mercaptonicotinic acid conjugate displayed the most content of thiol groups. The enzymatic degradability of these thiomers was investigated by viscosity measurements with a plate–plate viscometer. In rheological studies subsequently the modification proved that chitosan–thioglycolic acid conjugates with a higher coupling rate of thiol groups were not only degraded to a lesser extent by 20.9–26.4 % but also more slowly. Chitosan–mercaptonicotinic acid was degraded by 31.4–50.1 % depending on the investigated enzyme and even faster than unmodified chitosan. According to these results the biodegradability can be influenced by various modifications of the polymer which showed in particular that the rate of biodegradation is increased when mercaptonicotinic acid is the ligand, whereas the degradation is hampered when thioglycolic acid is used as ligand for chitosan.

Enzyme-degradable dextran–chitosan hydrogels with aligned pores were prepared via unidirectional freezing followed by cryogelation method (Wu et al. 2013). In this study, microtubular pores from 20 to 100 μm were obtained in the swollen state of the hydrogels. It was found that chitosan can be hydrolyzed by chitosanase solution into short oligosaccharide fragments that leads to collapse and degradation of the gel network. In this study, the degradation rate was found to be optimized by adjusting the hydrogel composition and chitosanase concentration. Recently, Costa Pinto et al. (2014) studied the degradation of poly(butylene succinate) and chitosan using lipase and lysozyme, respectively. In this study, the subcutaneous implantation of the scaffolds was performed to assess tissue response. The type of inflammatory cells present in the scaffold was determined histologically and immunohistochemistry. The results showed that the water uptake of the scaffolds was found to be increased in the presence of lipase or lysozyme. The lysozyme combined with lipase had a notable effect on the in vitro degradation of the scaffolds. The in vivo implantation study showed a normal inflammatory response in the presence of neutrophils and macrophages, lymphocytes, and giant cells.

3 Antimicrobial Activity

Chitosan and its derivatives are found to have antimicrobial properties. They have several advantages over commercial disinfectants such as a high antibacterial activity, broad spectrum of activity, and a low toxicity over animal and plant cells (Liu et al. 2001). The antimicrobial action of the chitosan and its derivatives depends on the following factors: (i) target organism, (ii) intrinsic factors of the chitosan (positive charge density, molecular weight, hydrophobic and hydrophilic characteristics, chelating capacity), (iii) physical state factors, and (iv) environmental factors (ionic forces, pH, temperature, time) (Badawy and Rabea 2011).

3.1 Action Against Plant Pathogens

Since chitosan and its derivatives have antimicrobial and plant defense elicit function, they are considered as useful pesticides in the control of plant diseases. Chitosan's inhibition was observed on different development stages such as mycelia growth, sporulation, spore viability and germination, and the production of fungal virulence factors (Badawy and Rabea 2011). The experimental data indicate that chitosan or its oligosaccharides may inhibit the transcription or accumulation of RNA. Chitosan induces different mechanical defensive reactions in plant cells, such as plugging intercellular spaces with amorphous electron opaque substances, accumulation of chitinases, synthesis of proteinase inhibitors, lignification, induction of callous synthesis, and the formation of cell wall appositions, which may be implicated in restricting fungal invasion (Ghaouth et al. 1992). It is also known that the positively charged amine groups in chitosan form complexes with the cell DNA, which limit the growth of fungus (Li et al. 2011). Chitosan can also induce a set of plant genes known as disease resistance response and pathogenesis-related genes. Some of these genes encode enzymes for secondary pathways of phytoalexins and hydrolytic enzymes such as RNAase, chitinase, and B-glucanase, thionins or plant defensins, and peroxidases that are directly antimicrobial or capable of generating phenolic polymers such as lignin (Hadwiger 1999).

The fungicidal activity of chitosan samples was tested against plant pathogenic bacteria of *Agrobacterium tumefaciens*, *Corynebacterium fascians*, *Erwinia amylovora*, *E. carotovora*, *Pseudomonas solanacearum*, and *Sarcina lutea* (Badawy et al. 2006). The results indicated that chitosans with higher molecular weight were more potent in bactericidal activity than low molecular weight chitosan. The oligochitosans prepared by enzymatic depolymerization were found to be more effective than the original chitosan in inhibiting mycelial growth of nine phytopathogens: *F. graminearum*, *Phytophthora capsici*, *Verticillium dahliae*, *A. solani*, *B. cinerea*, *C. orbiculare*, *Exserohilum turcicum*, *F. oxysporum*, and *Pyricularia oryzae* (Xu et al. 2007). Hirano and Nagao (1989) reported the effect of high and low molecular weight chitosan on different fungal species, and they found that the best fungicidal activity on mycelia occurred in media supplemented with low molecular weight chitosan. Meng et al. (2010) reported that both of chitosan (350 kDa) and oligochitosan (6 kDa) strongly inhibited spore germination and mycelial growth of two phytopathogenic fungi *A. kikuchiana* Tanaka and *Physalospora piricola* Nose.

Chittenden and Singh (2009) tested the control of two sapstain fungi *Leptographium procerum* and *Sphaeropsis sapinea* by a combination of chitosan or chitosan oligomer and an albino strain of *Trichoderma harzianum*. There was no mycelial growth of the fungi regardless of chitosan concentrations used when *L. procerum* or *S. sapinea* was simultaneously inoculated with *T. harzianum*. The dose response of chitosan or chitosan oligomer was found to be apparent when *T. harzianum* was not inoculated with test fungi. There was a greater growth reduction at higher concentrations (0.075–0.1 %) of chitosan, and overall chitosan oligomer was more effective than chitosan aqueous solution. The results of this study showed that chitosan controlled the germination of spores and the combination of chitosan and *T. harzianum* inhibited spore germination.

Chitosan shows an antiviral activity against plant viruses. It was found that chitosan inhibited the productive infection caused by the bacteriophage. The efficiency of inhibition of bacteriophage was found to be depending on the final concentration in the medium (Kochkina et al. 1995). Major factors of suppressing phage infections by chitosan are phage particle inactivation and inhibition of bacteriophage reproduction at the cellular level. Obviously, chitosan may be used for induction of phagoresistance in industrial microorganism cultures to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture.

Anusuya and Sathiyabama (2013) investigated the defense proteins present in the exudates from water-imbibed and chitosan-imbibed seeds of chickpea. The results showed that chickpea seeds immersed in chitosan released a higher amount of proteins in the exudates when compared to the seeds immersed in water. The exudate obtained from chickpea seeds immersed in chitosan solution exhibited a new isoform of chitinase, chitosanase, and protease inhibitors. These exudates have shown an in vitro inhibitory effect on the growth of the fungus, *Fusarium oxysporum f.sp. ciceri*. These results suggest that seed exudates protect seeds during their germination from soil pathogens.

Antimicrobial properties of chitosan coatings and chitosan enriched with bioactive compounds and essential oils were determined by in vitro and in vivo assays on minimally processed broccoli (Alvarez et al. 2013). The efficiency of chitosan with bioactive compounds and essential oils in improving the safety of broccoli was tested against the native microflora. Also, its effects on the survival of *Escherichia coli* and *Listeria monocytogenes* inoculated in broccoli were evaluated. In vitro assays performed in tea tree, rosemary, pollen, and propolis demonstrated significant inhibitory effects on *E. coli* and *L. monocytogenes* counts, while pomegranate and resveratrol presented reduced activity. In vivo application of these bioactive compounds on broccoli exerted a bacteriostatic effect on mesophilic and psychrotrophic populations except for rosemary. The application of chitosan or enrichment with bioactive compounds and essential oils resulted in a significant reduction in mesophilic and psychrotrophic counts. Between 5 and 7 days, significant reductions of mesophilic and psychrotrophic counts were observed in samples treated with chitosan with bioactive compounds. The enrichment with bioactive compounds improved the antimicrobial action of chitosan. The application of these coatings did not introduce deleterious effects on the sensory attributes of broccoli. The results showed that chitosan coatings enriched with bioactive compounds and essential oils were a good alternative for controlling not only the microorganisms present in broccoli but also the survival of *E. coli* and *L. monocytogenes*.

Different types of chemically modified chitosan derivatives were reported as improved antimicrobial active compounds. For example, Muzzarelli et al. (2001) prepared chemically modified chitosans and tested their antifungal activities against *Saprolegnia parasitica*. Results showed that *S. parasitica* did not grow normally on the first day for methylpyrrolidinone chitosan and *N*-phosphonomethyl chitosan, and on the second day for *N*-carboxymethyl chitosan, a tightly packed precipitate was present at the bottom of the test tubes instead of the fluffy fungal material as in

the control. In contrast, *N*-dicarboxymethyl chitosan was found to favor fungal growth, while dimethylaminopropyl chitosan did not significantly differ from the control.

N,*N*,*N*-Dimethylalkyl chitosans as quaternary and water-soluble chitosan compounds were recently prepared to test their antimicrobial activities against the plant pathogenic bacteria *A. tumefaciens* and *E. carotovora* and fungi *B. cinerea*, *F. oxysporum*, and *P. debaryanum* (Badawy 2010). Quaternary chitosans enhanced the antibacterial activity, and *N*,*N*,*N*-dimethylpentyl chitosan was found to be active with MIC 750 and 1,225 mg/L against *A. tumefaciens* and *E. carotovora*, respectively. Both of *N*,*N*,*N*-dimethylpentyl chitosan and *N*,*N*,*N*-dimethyloctyl chitosan were active in fungal mycelial growth inhibition of *B. cinerea*, *F. oxysporum*, and *P. debaryanum*.

Charged derivatives of chitosan, *N*-sulfofurfuryl chitosan and *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride, were prepared by Channasanon et al. (2007). The bioactivity of these films on the treated PET substrate was tested against selected proteins having a distinctive size and charge. It has been demonstrated that the proteins adsorbed onto the assembled film in a multilayer fashion, implying that the diffusion of the proteins within the multilayer structure has occurred. Since both *N*-[(2-hydroxyl-3-trimethylammonium)propyl]chitosan chloride and *N*-sulfofurfuryl chitosan are soluble over a broader pH range and possess different bioactivity from chitosan, these two charged derivatives can be potential candidates for biomedical applications.

3.2 Action Against Animal Pathogens

Several mechanisms of antimicrobial action of chitosan have been suggested in the literature. One of the mechanisms is the interaction between the positive charge of the chitosan and the negative charge of the microbial cell wall for the agglutination of the microbial cells and inhibition of growth (Senel et al. 2000; Avadi et al. 2004). It was also reported that when interacting with the bacterial cell, the chitosan promotes displacement of Ca^{2+} of the anionic sites of the membrane resulting in cell damages. Other studies proposed that the antimicrobial action is closely related to the physical–chemical properties of the polymer and the features of the cell wall of the microorganism (Costa Silva et al. 2006). It was reported that chitosan with low molecular weight penetrates in the cell and is linked to the microorganism DNA inhibiting the transcription and consequently the translation, whereas the high molecular weight chitosan acts as a chelant agent, binding to the cell membrane (Pedro et al. 2009).

Chung et al. (2004) have observed that the chitosan presents a better bactericidal and bacteriostatic action for Gram-negative bacteria than Gram-positive due to the composition of phospholipids and carboxylic acids of the bacterial cellular wall. These results proposed that the effects of the chitosan are distinct in Gram-positive and Gram-negative bacteria. In the Gram-positive bacteria, the chitosan of high molecular mass may form films around the cell that inhibit the absorption of

nutrient. In the Gram-negative bacteria, chitosan of low molecular mass penetrates more easily that may cause riots in the metabolism of these microorganisms. It was observed that the antimicrobial activity of chitosan is directly related with the absorption of the polysaccharide to the bacterium that will cause alterations in the cellular wall structure, consequently, in the permeability of the cellular membrane (Ikinci et al. 2002; Goy et al. 2009).

Chitosan microparticles have been shown to reduce *E. coli* shedding in a cattle model. However, the underlying mechanism of chitosan microparticles on reducing the shedding of this pathogen remains unclear. To understand the mode of action, Jeon et al. (2014) studied molecular mechanisms of antimicrobial activity of chitosan microparticles using in vitro and in vivo methods. They report that chitosan microparticles are an effective bactericidal agent with capability to disrupt cell membranes. Binding assays and genetic studies showed that outer membrane protein OmpA of *E. coli* is critical for chitosan microparticles binding, and this binding activity is coupled with a bactericidal effect of chitosan microparticles. This activity was also demonstrated in an animal model using cows with uterine diseases. Chitosan microparticles treatment effectively reduced shedding of intra-uterine pathogenic *E. coli* (IUPEC) in the uterus compared to antibiotic treatment. It was found that chitosan microparticle treatment did not induce bacteriophage or Shiga toxins in *E. coli* that suggests that chitosan microparticles can be a potential candidate to treat infections caused by this pathogen.

The antibacterial activities of *N*-alkylated disaccharide chitosan derivatives against *E. coli* and *S. aureus* were investigated (Yang et al. 2005). The results showed that the antibacterial activity of chitosan derivatives was affected by the degree of disaccharide substitution (DS) and the kind of disaccharide present in the molecule. Regardless of the kind of disaccharide linked to the chitosan molecule, a DS of 30–40 %, in general, exhibited the most pronounced antibacterial activity against both test organisms. *E. coli* and *S. aureus* were the most susceptible to cellobiose–chitosan derivatives and maltose–chitosan derivatives, both with DS values of 30–40 %, among the various examined chitosan derivatives. Although the disaccharide chitosan derivatives showed less antibacterial activity than native chitosan at pH 6.0, they exhibited higher activity at pH 7.0. The antibacterial activity of the chitosan derivatives against *E. coli* increased as the pH increased above 5.0 and reached a maximum around pH 7.0–7.5. The effect of pH on the antibacterial activity of chitosan derivatives against *S. aureus* was not as significant as that observed with *E. coli*. Population reductions of *E. coli* or *S. aureus* in nutrient broth increased markedly when the concentration of chitosan derivatives was increased from 0 to 500 mg/kg, while no marked increase in population reduction was found with further increases, even up to 2,000 mg/kg. From these studies, it is clear that although there are many reports discussing chitosan's antimicrobial activity in different conditions with conflicting results, they all confirmed that chitosan and its oligosaccharides have strong antimicrobial effects and are safe for human use.

Recently, Jena et al. (2012) synthesized chitosan-stabilized silver nanoparticles and analyzed their activities. In this study, silver nanoparticles were synthesized

using chitosan as both a stabilizing and a reducing agent. Chitosan–silver nanoparticles exhibited potent antibacterial activity against different human pathogens and also impeded bacterial biofilm formation. SEM analysis indicated that chitosan–silver nanoparticles kill bacteria by disrupting the cell membrane. Chitosan–silver nanoparticles showed no significant cytotoxic or DNA damage effect on macrophages. Propidium iodide staining test pointed out that active endocytosis of chitosan–silver nanoparticles resulted in reduced intracellular bacterial survival in macrophages. This study indicates that chitosan-based silver nanoparticles kill bacteria without harming the host cells at a specific dose.

4 Anti-inflammatory and Tissue Regeneration Activity

The anti-inflammatory activity of chitosan is due to the acid hydrolysis of chitosan to glucosamine hydrochloride or its sulfate, phosphate, and other salt preparation by salt conversion. These monosaccharides are structural units of the proteoglycans contained in connective tissue and cartilage. These tissues can be repaired and regenerated by absorbing these monosaccharides directly when they are damaged or inflamed (Olivier et al. 2004). Therefore, these monosaccharides are an effective treatment for preventing and curing rheumatoid arthritis as well as bone hyperplasia and are used as antiarthritic drugs in clinical practice. Moreover, unlike some common antiarthritic steroidal anti-inflammatory drugs or analgesic and anti-inflammatory drugs, these monosaccharides have no side effects and can be taken for a long period. The clinic experiments indicated that taking glucosamine for 2 weeks can eliminate arthritic pain and improve movement in patients suffering from severe arthritis.

Chung et al. (2012) studied the anti-inflammatory effects of low molecular weight chitosan oligosaccharides prepared from high molecular weight chitosan by enzymatic digestion against allergic reaction and allergic asthma in vivo and in vitro. The low molecular weight chitosan (<1 kDa), consisting of glucosamine $(GlcN)_n$, $n = 3–5$, was found to be capable of inhibiting both antigen-stimulated degranulation and cytokine generation in rat basophilic leukemia RBL-2H3 cells. In this study, the protective effect of low molecular weight chitosan against ovalbumin-induced lung inflammation in asthma model mice was also examined. Oral administration of low molecular weight chitosan (16 mg/kg body weight/day) resulted in a significant reduction in both mRNA and protein levels of interleukin-4, interleukin-5, interleukin-13, tumor necrosis factor-α in the lung tissue, and bronchoalveolar lavage fluid compared to those in the ovalbumin-sensitized/challenged asthma control group. These results showed that the oral administration of low molecular weight chitosan is effective in alleviating the allergic inflammation in vivo and thus can be a good material for the development of a potent therapeutic agent against mast cell-mediated allergic inflammatory responses and airway inflammation in allergic inflammatory diseases.

Chitosan–tobermorite composite membrane was prepared by solvent casting a mixture of chitosan and tobermorite from acetic acid solution (Hurt et al. 2014).

The bioactivity and biocompatibility of the composite were evaluated with respect to its potential for use as a biodegradable guided tissue regeneration membrane. The in vitro bioactivity of the composite membrane was confirmed by the formation of crystalline bone-like substituted hydroxyapatite on the surface of the embedded tobermorite particles in simulated body fluid. The tobermorite began to degrade on contact with simulated body fluid, and its alkaline dissolution products buffered the acidic breakdown products of the chitosan polymer. MG63 osteosarcoma cell viability was enhanced by up to 30 % in the presence of the chitosan–tobermorite membrane. These results indicated that the chitosan–tobermorite composite may be a suitable material for tissue regeneration applications.

5 Wound-Healing Properties

Chitosan can facilitate wound healing by stimulating granulation tissue formation or reepithelialization. It can influence the formation of postsurgical adhesions and the repair of surgical incisions and anastomosis since it improves reepithelialization and wound healing (Dai et al. 2011). The role of chitosan in wound healing was found to be associated with their immune stimulating property, which involves higher production of macrophages that release cytokines necessary for the healing process (Okamoto et al. 2003). The wound-healing property of chitosan oligomers is due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor and subsequent collagen production in order to facilitate the formation of connective tissues (Howling et al. 2002).

The nanofibrous composite chitosan/collagen membrane for wound-healing application was reported by Chen et al. (2008). The composite membrane was found to promote wound healing and induce cell migration and proliferation. The results showed that the composite chitosan/collagen membrane was found to be better in wound healing than commercial wound-healing materials. A wound dressing material based on chitosan/collagen with high liquid absorbing, biocompatibility, and antibacterial properties was designed by Wang et al. (2008). In their study, different weight ratios of collagen to chitosan, which were pre-grafted with acrylic acid or *N*-isopropyl acrylamide (NIPAAm), were used to immobilize on the polypropylene nonwoven fabric to construct a durable wound dressing membrane with high water absorbing, easy removal, and antibacterial activity. The results showed that NIPAAm-grafted and collagen-/chitosan-immobilized polypropylene nonwoven fabric showed a better healing effect than acrylic acid-grafted and collagen-/chitosan-immobilized polypropylene nonwoven fabric. The excellent remodeling effect in histological examination with respect to the construction of vein, epidermis, and dermis at 21 days after skin injury was observed when the wound was treated with this composite fabric.

A bilayer composite film consists of an upper layer of a soybean protein nonwoven fabric and a lower layer of a genipin-cross-linked chitosan film was developed as a wound dressing material by Liu et al. (2008). In this study, the degree of cross-linking and the in vitro degradation rate of the genipin-cross-linked

chitosan films was found to be controlled by varying the concentration of genipin. The contact angle analysis showed that the genipin-cross-linked chitosan film is not highly hydrophilic, and therefore, the genipin-cross-linked chitosan layer is not entangled with the soybean protein nonwoven fabric, which forms an easily stripped interface layer between them. It was observed that this wound dressing material provides adequate moisture, thereby minimizing the risk of wound dehydration, and exhibits good mechanical properties.

6 Antioxidant Activity

Chitosan and its derivatives have antioxidant properties. They act as antioxidants by scavenging oxygen radicals, and it is dependent on their molecular weights as well as their degree of deacetylation (Park et al. 2003). Low molecular weight chitosan oligomers are preferred than that of higher molecular weights for the activity. Highly deacetylated (90 %) chitosan oligomers also act as scavengers of hydroxyl, superoxide, alkyl, as well as highly stable DPPH radicals tested in vitro. It is reported that chitosan and their derivatives act as hydrogen donors to prevent the oxidative sequence (Je et al. 2004a, b).

Liu (2008) reported the antioxidant activity of chitosan in vitro and in vivo. The results showed that 0.02 % chitosan had antioxidant effects in lard and crude rapeseed oil, but the activity was less than ascorbic acid. When the concentration was increased, chitosan and ascorbic acid had similar activities; chitosan could significantly reduce serum FFA and MDA concentrations and elevate SOD, CAT, and GSH-PX activities, the latter being the major antioxidant enzymes in the body, indicating that chitosan regulated the antioxidant enzyme activities and reduced lipid peroxidation.

The cellular antioxidant effects of chitosan oligosaccharides produced by acidic hydrolysis of crab chitin were identified (Ngo et al. 2008). This study showed that chitosan oligosaccharides have free radical scavenging effects in a cellular system. It was observed that chitosan oligosaccharides can inhibit myeloperoxidase activity and decrease free radical oxidation of DNA and membrane proteins. Furthermore, they stimulate an increase in intracellular GSH levels. Based on the results, the authors concluded that chitosan oligosaccharides have free radical scavenging effects, acting in both indirect and direct ways to inhibit and prevent biological molecular damage by free radicals in living cells. Hence, chitosan and chitosan oligosaccharides can be used as a scavenger to control radical-induced damage to cellular systems and promise further applications in the future. Li et al. (2014) investigated the in vitro antioxidant activity of chitosan scaffold material by the chemiluminescence signal generated from the hydroxyl radical (•OH) scavenging assay. The results showed that the free radical scavenging ability of chitosan scaffold probably depends on the chitosan concentration and its molecular weight.

In recent years, more attention has been given on the preparation of chemically modified chitosan derivatives for the improved antioxidant activity. For example, modified chitosans with 3,4-dihydroxy benzoyl groups and 3,4,5-trihydroxy

benzoyl groups were synthesized and their antioxidant activity before and after sonication was investigated by the radical scavenging activity method using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Taghizadeh and Bahadori 2014). The results showed that the DPPH scavenging free radical capacity of chitosans with 3,4-dihydroxy benzoyl and chitosans with 3,4,5-trihydroxy benzoyl groups increased up to 89 % and 74 % respectively, when the concentration reached 6 μg/ml. The ultrasonic treatment of chitosans with 3,4-dihydroxy benzoyl and chitosans with 3,4,5-trihydroxy benzoyl groups after 30 min decreased the DPPH free radical scavenging activity but ultrasonic treatment of chitosan increased the DPPH free radical scavenging activity. Lee et al. (2014) reported the antioxidant and antimicrobial activities of chitosan–caffeic acid, chitosan–ferulic acid, and chitosan–sinapic acid conjugates with different grafting ratios. The antioxidant activities of the conjugates were found to be increased compared to the unmodified chitosan, by 1.79–5.05-fold (DPPH scavenging assay), 2.44–4.12-fold (hydrogen peroxide scavenging assay), and 1.34–3.35-fold ($ABTS^+$ radical scavenging assay). The conjugates showed excellent lipid peroxidation inhibition abilities in a linoleic acid emulsion system. The conjugates exhibited antimicrobial activity against various clinical isolates, standard methicillin-resistant strains, and foodborne pathogens.

7 Antitumor Activity

The anticancer activities of charged chitooligosaccharides were reported using three cancer-cell lines namely HeLa, Hep3B, and SW480 (Huang et al. 2006). Cell-viability studies showed that highly charged chitooligosaccharides could significantly reduce cancer-cell viability, regardless of their charge. Fluorescence microscopic observations and DNA-fragmentation studies confirmed that the anticancer activity of these charged chitooligosaccharides were due to necrosis. It was reported that chitooligosaccharides inhibited tumor growth through an increase in immune effects (Suzuki et al. 1986). Moreover, the chitooligosaccharides (DP4-7) showed strong inhibition of ascites cancer in BALB/c mice, whereas (GlcNAC)6 and (GlcN)6 showed strong inhibiting effects on S-180 and MM156 solid tumor growth in syngeneic mice.

The inhibitory effect of chitosan salts and chitosan gel with high molecular weight on cancer growth was observed (Guminska et al. 1996). Chitosan gel with a high degree of deacetylation showed a greater inhibition than chitosan salts with the same molecular weight and lower degree of deacetylation. These results indicate that chitosan blocks metabolic cascades connected with the transmission of signals from the cell membrane into its interior and leads to a specific inhibition of the tumor cell metabolism (Ignacak et al. 1998). Chitosan oligomers with degree of polymerization >6 with medium degree of deacetylation were found to be effective in blocking absorption of cholesterol and lipids in the intestinal tract. This observation might be due to the protonation of chitosan and thereby forming gels in the intestinal tract that entrap lipids and cholesterol (Razdan et al. 1997).

Kato et al. (2005) reported the mitomycin C (MMC) and chitosan derivative conjugates as antitumor materials. The MMC–*N*-succinyl-chitosan conjugates showed good antitumor activities against various tumor models due to their predominant distribution into the tumor tissue and sustained-release characteristics. Salah et al. (2013) determined anticancer activities of chitin, chitosan, and low molecular weight chitin using a human tumor cell line (THP-1). In this study, a molecular weight–activity relationship and an electrostatic interaction–activity relationship were determined. The cytotoxic effects of chitin and its derivatives were determined using a normal human fetal lung fibroblastic cell line (MRC-5). The results showed that when low molecular weight chitin is bound by YKL-40, an antiapoptotic effect of YKL-40 glycoprotein was found to be inhibited. Therefore, THP-1 cancer-cell death and MRC-5 were found to be proliferated.

Recently, Kim et al. (2013) reported the mechanism of the anticancer activity of water-soluble chitosan on the human leukemia cells (U937, K562, HL60, and THP-1). This study showed that water-soluble chitosan inhibited cell proliferation and induced apoptosis in leukemic cells via the mitochondria-dependent pathway. It was observed that this effect was accompanied by a marked increase in caspase activation and the cleavage of poly(ADP-ribose) polymerase. Water-soluble chitosan was found to decrease Bcl-2 expression in all leukemic cells and reversed the ectopic expression of Bcl-2 in U937 cells. Moreover, it suppressed the phosphorylation of Akt, thereby inhibiting cell proliferation. These observations suggest that water-soluble chitosan can inhibit proliferation and induce apoptosis in leukemia cells through the suppression of Bcl-2- and the Akt-dependent signaling pathways. From the above studies, it is clear that chitosan and its derivatives are potential candidates for tumor therapy.

8 Conclusion

Chitosan is a non-toxic, biocompatible and biodegradable biopolymer. Due to these properties, it is being widely used across a broad spectrum of industries like pharmaceutical, biotechnology, food, textile, cosmetic, etc. The applicability of the chitosan is directly related with its physicochemical properties such as degree of deacetylation, molecular weight, thermal stability, and degree of crystallinity. From this review, it is clear that chitosan and its derivatives have excellent antibacterial activity against plant and animal pathogens. Therefore, they can be used in a number of ways to reduce plant disease levels and prevent the development and spread of pathogens and hence the improved crop yield and quality. Based on the improved antibacterial activity, cell attachment ability, and oxygen permeability, it is clear that chitosan and its derivatives can be a promising candidate for wound dressing. To improve the wound-healing properties, chitosan-based membranes have been developed with different types of polymers such as collagen, acrylic acid, polypropylene, and *N*-isopropyl acrylamide. Due to their composite in nature, these membranes were found to have desired properties for wound-healing applications. While several studies have reported the antioxidant and antitumor activity

of chitosan and its derivatives, no clear information is available describing the detailed molecular mechanisms. Therefore, future research should be directed toward understanding the molecular mechanisms of these materials, which may help in the better understanding of the unknown biochemical functions of chitosan and its derivatives as well as to widen their potential applications.

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Gellan

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Contents

Abstract

Gellan gum is an anionic extracellular bacterial polysaccharide identified in 1978 by CP Kelco (San Diego, USA). It is available in two forms in normal commercial production, native gellan gum and deacylated gellan gum. For deacylated gellan gum, acyl groups present in the native polymer are removed by alkaline hydrolysis resulting deacylated polymer which is known generically as "gellan gum." Gellan gum can form gels at low concentrations when hot solutions are cooled in the presence of gel promoting cations which provides it as a texture modifier to control the viscoelasticity in foods, as delivery vehicle in pharmaceuticals, and as cell encapsulation material in tissue engineering etc. Gellan gum has been extensively used as a culture medium in microbiology, as a rheology modifier in foods and cosmetics industries since the early stage of its

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discovery. Recently, gellan gum as a natural polymer and its hydrogel show a wide range of application perspective in drug delivery and tissue engineering. Gellan gum is nontoxic, biocompatible, biodegradable and the resulting hydrogel is transparent and stable. However, gellan gum-based hydrogels have intrinsic defects such as lack of toughness and tissue tolerance as tissue engineering materials that restrict their use in biomedicine field. In order to solve these problems, quite a large number of studies on chemical modification of gellan gum have been carried out. In this chapter, the gelation behaviors, mechanism as well as various modification methods of gellan gum are summarized. Applications of gellan gum and modified gellan gum in food and biomedicine are highlighted.

Keywords
Hydrogel • Gelation Mechanism • Food • Chemical Modification • Biomedicine

1 Introduction

Gellan gum is a microbial polysaccharide derived from *Sphingomonas elodea* (ATCC31461), previously known as *Pseudomonas elodea*. It was recognized to have commercial potential (Sanderson 1990) in 1978, during an extensive screening program of soil and water bacteria by Kelco (San Diego, USA), the company that was also the first to produce xanthan as an industrial polysaccharide. The first applications of commercial gellan (Gelrite) were in growth media for microbial cultures, as a replacement for agar (Harris 1985; Lin and Cassida 1984). Gellan substrates have the advantages of clarity, purity, and the ability to withstand prolonged incubation at high temperature. Gellan was also identified (Morris et al. 2012; Shimomura and Kamada 1986) as a promising substitute for agar in plant tissue culture, because of the absence of impurities found in agar, the lower polysaccharide concentrations needed, and the greater clarity of the gels, allowing clearer observation of the development of roots and tissue (Arregui et al. 2003). Gellan gum was first used as a culture medium in horticulture because it formed a transparent gel (Ichi et al. 1986; Koda et al. 1988; Turner and Singha 1990).

Gellan gum has been approved for food use first in Japan in 1988, and then the approval propagated to USA in 1992, and then to the EU and many other countries. In 1989 a collaborative research group for gelation of gellan was organized in conjunction with the research group of polymer gels affiliated to the Society of Polymer Science of Japan. The common gellan was used to study its properties with various techniques. The results of the collaborative studies were published in special issues of *Food Hydrocolloids* (7, 361–456 in 1993), *Carbohydrate Polymers* (20, 75–207 in 1996), and *Progress in Colloid and Polymer Science* (114, 1–131 in 1999). It has been used as a texture modifier to control the viscoelasticity in foods and as a culture medium, as delivery vehicle in pharmaceuticals, and also in tissue engineering.

Recently, hydrogels from gellan gum show a wide range of application perspectives in drug delivery and tissue engineering. Gellan gum is nontoxic, biocompatible, and biodegradable and the resulting hydrogel is transparent and stable. The mechanical properties of gellan gum hydrogel are similar to those of normal human tissues under certain conditions. These advantages provide gellan gum versatile characteristics as a source of biomaterials. However, gellan gum-based hydrogels have intrinsic disadvantages such as lack of toughness and tissue tolerance as tissue engineering materials. These defects restrict their use in the biomedical field. In order to solve these problems, quite a lot of work on chemical and physical modification of gellan gum has been carried out. Modified gellan gum reveals a much more promising perspective in the development of biomedical materials.

2 Toxicity

In 1991, toxicological evaluation of gellan gum was done at the 37th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in Geneva WHO food additives series 28 (1991). The comments of the committee were as follows (Bajaj et al. 2007).

Gellan gum was shown to be poorly absorbed and did not cause any deaths in rats, which received a single large dose (5 g per kg of body mass) in the diet or by gavage. Short-term (90-day) exposure of rats to gellan gum at levels up to 60 g/kg in the diet did not cause any adverse effects. In prepubertal monkeys, toxicity of gellan gum was studied for 28 days at the highest dose level of 3 g per kg of body mass per day. Signs of any overt toxicity were not observed during this study. In reproduction and teratogenicity studies in rats in which gellan gum was given up to 50 g/kg in the diet, there was no evidence of interference with the reproductive process, and no embryotoxic or developmental effects were observed. Study in dogs, which were treated at dose levels up to 60 g/kg in the diet for 1 year, showed that there were no adverse effects that could be attributed to chronic exposure to gellan gum. In long-term carcinogenicity studies, gellan gum did not induce any adverse effects in mice or rats at the highest dose levels of 30 and 50 g/kg in the diet, respectively. Twenty-three-day dietary studies on humans revealed no significant effect on plasma biochemistry, hematological indices, urinalysis parameters, blood glucose, plasma insulin concentrations, or breath hydrogen concentration. No significant changes were seen in concentrations of HDL cholesterol, triglyceride, or phospholipid, but a decrease in serum cholesterol of ~12 % was noted. For the majority of volunteers participating in the human dietary study, an increase in fecal bulking was observed. Toxicological studies show that gellan gum is relatively nontoxic. An acute oral toxicity test on rat found that the LD50 of gellan gum is higher than 5,000 mg/kg, while a similar inhalation toxicity test caused no death in a group of ten animals, and an eye irritation test indicated gellan gum to be safe for eye contact. Thus, it was concluded that gellan gum produced no adverse effects and that increases in fecal bulking and decreases in serum cholesterol were desirable dietary attributes.

Gellan gum is also reported to shorten gastrointestinal transit time (Giavasis et al. 2000). As gellan gum is a relatively novel biopolymer, it is unlikely that the gut microflora have enzymes that can degrade the polysaccharide. However, there are reports of microbially produced gellan gum lyases (Giavasis et al. 2000; Kennedy and Sutherland 1994), and it will be interesting to see whether in the future gut microorganisms adapt to degrade this biopolymer.

3 Structure/Properties/Modification of Gellan Gum

3.1 Conformation

Gellan gum consists of a tetrasaccharide unit β-D-glucose, β-D-glucuronic acid, β-D-glucose, and α-L-rhamnose. X-ray diffraction studies of polycrystalline and well-oriented samples of the lithium, potassium, and calcium salts of gellan gum have shown that gellan adopts a double-helical structure with two left-handed threefold chains, each of which is translated by half a pitch (5.63 nm) with respect to the other (Chandrasekaran et al. 1988, 1992).

It is believed that gellan molecules take double-helical conformations in solutions at lower temperatures, and above a certain critical concentration, they form aggregates which act as junction zones resulting in a three-dimensional network.

The conformation of gellan gum in solutions has been studied by light scattering, optical rotation, and circular dichroism (Crescenzi et al. 1987; Milas et al. 1990). Since the potassium-type gellan molecules have a strong tendency to form aggregates, it was necessary to convert gellan gum to tetramethyl ammonium type or sodium type. It is necessary to mention here that almost all the commercially available samples are potassium type and contains different amounts of various cations because cations generally increase the gelling ability except when an excessive amount is added.

The collaborative researches reached to some conclusions; from optical rotation, light scattering, osmotic pressure, and intrinsic viscosity measurements, two disordered chains of deacylated sodium-type gellan at higher temperatures make a double helix at lower temperatures in dilute solution, and from rheological measurements gellan forms a gel under conditions favorable for the aggregation of double helices. The aggregation of double helices follows the coil-to-helix transition on further cooling above a certain gellan and cation concentrations.

Takahashi et al. (2004), using a sodium-type gellan gum, determined the molar mass by light scattering at 25 °C and 40 °C, and the molar mass ratio at these temperatures was shown to be 1.99~2.07. Furthermore, they got the persistence length 9.4 nm at 40 °C and 98 nm at 25 °C. These findings confirm that the gellan gum takes a double-helical conformation at lower temperatures and the helix–coil transition occurs at a temperature between 25 °C and 40 °C.

The helix–coil transition temperature was shown to be determined by the total concentration of cations as shown in Fig. 1 (Morris et al. 2012). The inverse of the midpoint temperature of the transition is a linear function of the logarithm of cation activity (Rochas and Rinaudo 1980). In solutions where the concentration of added

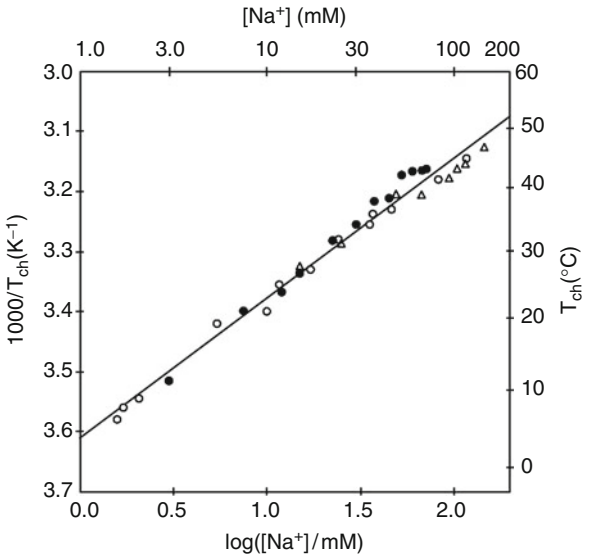

Fig. 1 Dependence of T_{ch} on total concentration of Na^+ (counterions to the polymer plus added NaCl, where present) observed for different independent measurements (Morris et al. 2012)

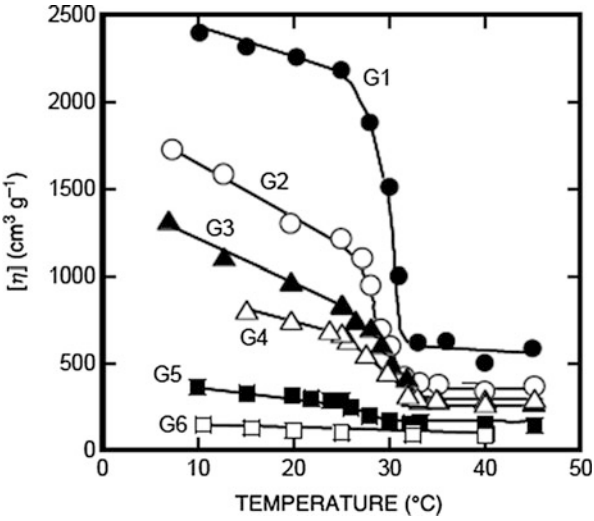

Fig. 2 Temperature dependence of intrinsic viscosity for Na–gellan aqueous solutions with 25 mM NaCl. Molar mass G1 > G2 > G3 > G4 > G5 > G6 (Ogawa et al. 2006)

salt is at least comparable to the polymer concentration and the activity coefficient is high or moderate, cation activity can be replaced by the total cation concentration without any serious loss of linearity, although the resulting plots have somewhat steeper slope than those obtained using the cation activity.

Ogawa et al. (2006) prepared six fractions of deacylated gellan samples with different molar masses which are converted to sodium type. The temperature dependence of the intrinsic viscosity for these six fractions is shown in Fig. 2.

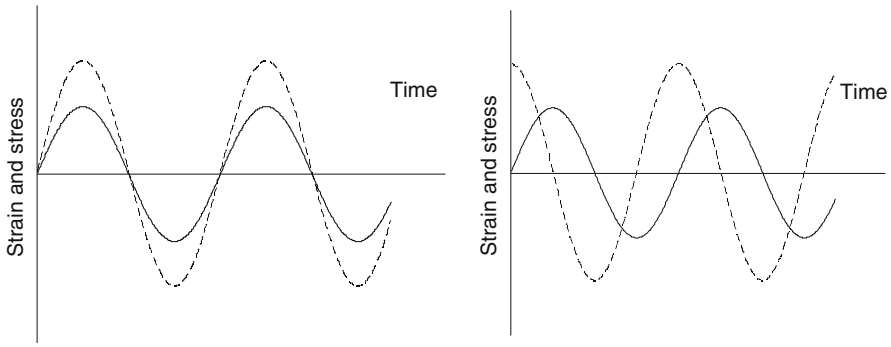

Fig. 3 Sinusoidal oscillation of strain (*solid line*) and stress (*dashed line*) for (**a**) a purely elastic solid and (**b**) a purely viscous fluid

3.2 Gelling Behavior

3.2.1 Rheology of Gellan Solutions and Gels

Dynamic oscillatory measurements are useful for gelation analysis since it is possible to shorten the measuring time so that samples remain unchanged with time during measurement. Figure 3 shows a sinusoidal oscillation of strain applied to (a) purely elastic solid and (b) purely viscous fluid and the resultant stress. If the material is perfectly elastic, the resultant stress wave is exactly in phase with the strain wave. On the other hand, if the material is purely viscous fluid, the resultant stress wave is exactly $\pi/2$ out of phase with the imposed deformation. The stress wave of the viscoelastic material has a phase difference δ ($0<\delta<\pi/2$).

In dynamic viscoelastic analysis, the storage and loss shear moduli, G' and G″, are obtained. The storage and loss Young's moduli E' and E'' are obtained by longitudinal oscillation. G' and E' are proportional to the elastic energy stored in viscoelastic material in a period of oscillation; G' and E' characterize solid-like response of the sample. G'' and E″ are proportional to the dissipated energy as heat in a period of oscillation; G'' and E″ characterize liquid-like response of the sample.

The variation of G' and G'' with frequency is known as the "mechanical spectrum" of the material. Typical mechanical spectra for polysaccharide solutions and gels are shown in Fig. 4.

A power law $G' \sim \omega^2$ and $G'' \sim \omega$ is found in dilute solutions of polysaccharide at low frequencies (Fig. 4d). Another rheological behavior where G' and G'' cross at a certain frequency, below which G'' is higher than G', is found in semi-dilute solution (Fig. 4c). In elastic polysaccharide gels, G' is higher than G'' at all frequencies examined and both moduli show a plateau even at low frequencies (Fig. 4a). At low frequencies, where there is sufficient time for entanglements to come apart within the period of oscillation, semi-dilute solutions respond predominantly by flow, and their mechanical spectra (Fig. 4c) are similar to those of dilute solutions (Fig. 4d). However, at higher frequencies, where there is less time for

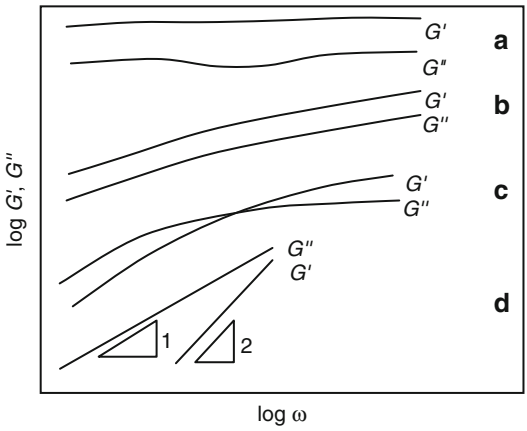

Fig. 4 Mechanical spectra for polysaccharide solutions and gels

disentanglement, the predominant response to the oscillatory strain becomes elastic distortion of the entangled network, and the mechanical spectra become similar to those observed for gels (Fig. 4a).

Some polysaccharide solutions show a gel-like behavior in mechanical spectra, but if subjected to a steady shear flow, they will apparently flow rather than fracture. In this case the behavior is called a "weak gel" type, where G' is higher than G'' at all frequencies examined and both moduli show only a slight frequency dependence (Fig. 4b). Some gellan solutions, sodium-type gellan solutions at lower temperature, for example, show "weak gel"-type behavior. Systems of this type show predominantly elastic (gel-like) response to small perturbations but cannot support their own weight and can be stirred and poured like normal solutions (Picout and Ross-Murphy 2003). "Weak gels" should not be confused with conventional gels that are "weak" only in the sense of having low moduli. Conventional gels, which are often described as "true gels," respond to high stress by fracturing, whereas "weak gels" flow. Mechanical spectra of "weak gels" normally differ from those of true gels in having greater frequency dependence of G' and G'' and smaller separation between the two moduli, but the main distinction is the difference in response to unidirectional stress or large strain. To avoid such confusion, other descriptions such as "structured liquids" have been used recently.

Although the term "weak gel" seems to have been used in many papers for a long time, the term "structured liquid" seems to be more logical. Polysaccharides that form true gels on cooling under quiescent conditions can also give dispersions of microscopic gel particles with "weak gel" properties if they are subjected to shear on cooling through the temperature range of the sol–gel transition (Norton et al. 1999). By applying this approach to gellan, Sworn et al. obtained "weak gel" networks capable of suspending small particles by mixing solutions of commercial gellan (Kelcogel) and NaCl at 80 °C and cooling (at 0.5 °C/min) to 25 °C under shear (at a constant shear rate of 100 s^{-1}) (Sworn et al. 1995). This is called "fluid gels" and can be also formed by dispersing gellan solution in oil phase at higher temperatures and then by lowering the temperature.

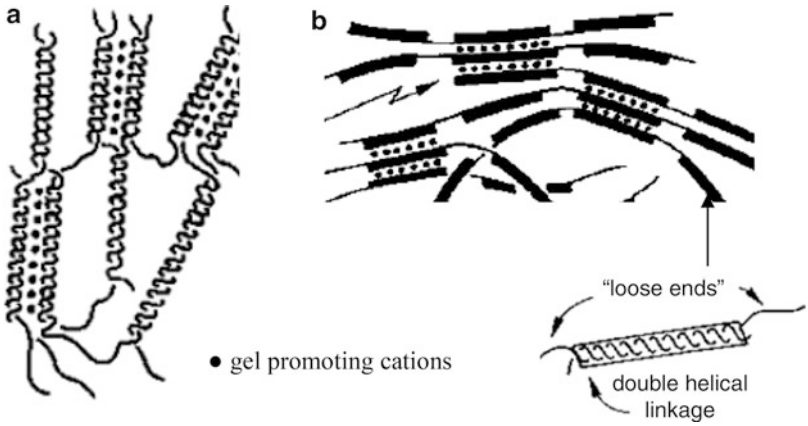

Fig. 5 Schematic model of gellan gel network proposed by (**a**) Robinson et al. (1991) and (**b**) Gunning and Morris (1990)

3.2.2 Gel Networks

Gel network and the origin of the elasticity of gellan gels have been investigated extensively. However, it has not been established well. Two schematic models proposed for the internal structure of gellan gels are shown in Fig. 5. The gel elasticity is often discussed based on the rubber elasticity. In rubber, disordered polymer chains are cross-linking, which leads to three-dimensional network formation. The main response to applied stress is stretching of the flexible regions of disordered polymer chains, and the elastic resistance to deformation comes predominantly from the consequent reduction in conformational entropy. The decrease of the mesh size (i.e., increase of number of cross-links) in the network leads to the increase of the elastic modulus. Some gellan helices are considered to play a role of junction zone which connects disordered polymer chains and make three-dimensional networks (Fig. 5a). Aggregates of helices are thought to form junction zones, out of which flexible gellan chains are released and connected with other junction zones, as proposed by "reel-chain" model (Nishinari et al. 1985). An alternative model has been proposed; the enthalpic elasticity rather than the entropic elasticity (rubber elasticity) dominates gellan gel elasticity (Morris 1995). In this model gellan helices form long "filaments" and networks are formed by lateral association of fibrillar strands (Fig. 5b). The elasticity of such networks would come predominantly from the increase in enthalpy on distortion of the aggregated filaments or the entire fibrillar network (Morris et al. 1999a).

It was shown that the helix–coil transition occurred in the self-supporting gellan gels (Nitta et al. 2001). They used cylindrically molded gels of a potassium-type gellan, and an endothermic DSC peak and a sigmoidal increase in circular dichroism at 202 nm were found on heating (Fig. 6). The gels kept the cylindrical shape up to highest temperature 60 °C at which the observation of longitudinal vibration could be made. Both storage and loss Young's moduli showed a sigmoidal decrease showing weakening of the network structure at this temperature range. The opposite

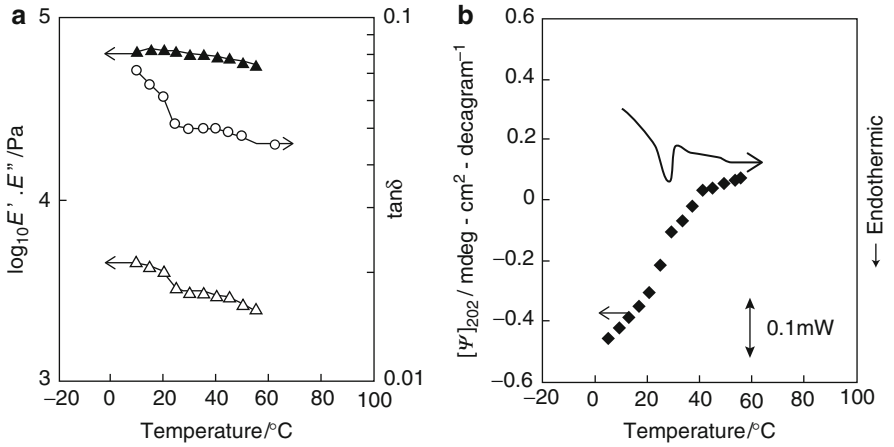

Fig. 6 (**a**) Temperature dependence of the storage modulus E' (▲),the loss modulus E'' (Δ), and mechanical loss tanδ (○) on heating and (**b**) the heating DSC curve (*solid curve*) and temperature dependence of the specific ellipticity at 202 nm (◆) on heating for 1.6 %(w/w) gellan gum gels. E' and E'' measurements were done after 15 min equilibration at each temperature. Heating rates of DSC and CD measurements: 0.5 ° C/min (Nitta et al. 2001)

tendency was observed on cooling. It is evident, therefore, that disordered chain sequences formed during heating of the gel have sufficient freedom of movement within the surviving cross-linked network to revert to the double-helix state on cooling.

Observations of chain release from gellan gels which were immersed in aqueous solutions (Hossain and Nishinari 2009; Tanaka and Nishinari 2007) suggested the existence of gellan chains that do not participate the network.

3.2.3 Effect of Ions

Kani et al. (2005) examined the effects of monovalent cations and anions on the conformation of gellan chains in aqueous solutions by optical rotation and fluorescence anisotropy measurement. They confirmed that the electrostatic shielding ability of cation species between intramolecular segments and intermolecular chains increased with increasing ionic radius. The coil–helix transition of gellan chains was affected by the cation concentration as shown in Fig. 1 rather than by the type of cation species; however, the latter was the predominant factor with respect to the aggregation of gellan chains. They found that the anion species F^- lowered the coil–helix transition temperature and suppressed the aggregation of gellan chains, while the anion species Cl^-, Br^-, and I^- showed no influence on the coil–helix transition temperature or the aggregation behavior.

The aggregation of gellan helices is inhibited by electrostatic repulsion between the helices. Monovalent cations are thought to screen electrostatic repulsion between the gellan helices and to promote double-helix formation and aggregation of the helices. The effectiveness is observed in order of $Cs^+ > K^+ > Na^+ > Li^+$ (Miyoshi and Nishinari 1999a). Aggregates lead to thermal hysteresis between the

temperatures at which helices form on cooling and aggregates melt on heating. The helix-forming temperature is usually from 30 °C to 40 °C, and the temperature increases with increasing cation concentration, while the aggregate melting temperature changes widely from 40 to over 90 °C depending on cation concentrations.

At low concentrations of monovalent cations, solutions of ordered gellan give mechanical spectra similar to those of solutions of disordered coils (Fig. 4c or d). On further increase in cation concentration, there is a region of "weak gel" response (Fig. 4b) before reaching the threshold concentration for formation of true gels. This progression is attributed to progressive suppression of electrostatic repulsion between the gellan double helices. For the true gel formation, at least 50 mM Na^+ or 20 mM K^+ is required (Morris et al. 2012).

Excessive addition of cations has been shown to decrease the elastic modulus and the fracture stress (Moritaka et al. 1991; Milas and Rinaudo 1996; Morris et al. 1999b). Morris et al. (1995) attributed this phenomenon to the formation of inhomogeneous structure in the presence of excessive amount of cations.

Divalent cations seem to bind directly with gellan molecules to form aggregates of gellan helices with the effectiveness of $Ca^{2+} > Mg^{2+}$. Firm gels are obtained above ~2 mM Ca^{2+}. Maximum gel strength is attained (Tang et al. 1996) when the concentration of divalent cations reaches ~100 % of stoichiometric equivalence to the carboxyl groups of the polymer. The gels induced by divalent cations have greater thermal stability than those induced by monovalent cations. A proposed interpretation is that divalent metal ions promote aggregation by site binding between pairs of carboxylate groups on neighboring helices, rather than by suppressing electrostatic repulsion by binding to individual helices.

3.2.4 Effect of pH

Gelling agents that form a gel at low pH are important to the production of, e.g., dessert jellies containing fruit juices. Gelling agents such as agarose and carrageenan show reduced gelling capacity at low pH, whereas gellan gum can form strengthened gels (Moritaka et al. 1995; Yamamoto and Cunha 2007). Moritaka et al. (1995) showed that the breaking stress of all gels increased with decreasing pH down to pH 4. The difference between the breaking stress of nonheated gels and that of gels reheated for **2** h at 90 °C became larger with increasing pH. Yamamoto and Cunha (2007) showed that failure stress increased at lower pH values, higher polysaccharide concentration, and with the application of heating before acidification. However, gels heated after acidification showed decreased failure stress. All the gels they examined were transparent and the water-holding capacity improved at higher concentrations and pH values or at lower pH values and gellan concentrations. Picone and Cunha (2011) examined the effect of pH (3.5, 5.3, and 7.0) on the formation of deacylated gellan gels by small and large deformation rheology and structural observation and found that formation and mechanical properties of gellan gels were directly dependent on the solution pH. An increase in pH led to the formation of a reduced number of junction zones and a decrease in molecular aggregation, reducing gel rigidity. Thus, they found that gels formed at neutral pH were more fragile and deformable than acid gels.

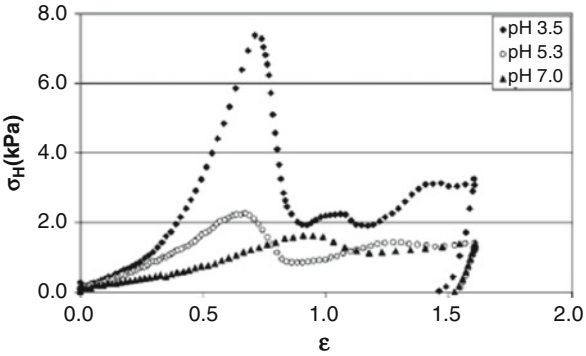

Fig. 7 Stress–strain curves of 1.5 (w/w) gellan gels at pH 3.5, 5.3, and 7.0 (Picone and Cunha 2011)

Gelation of gellan can also be induced by reduction in pH. Initial acidification from neutral pH to pH 3.5 causes a large increase in break stress as shown in Fig. 7 (Picone and Cunha 2011). They attributed it to a decreased formation of junction zones at higher pH values due to greater electrostatic repulsion between the gellan molecules, and thus, gels formed at neutral pH were more fragile and deformable than acid gels. However, by further decrease in pH to 2, the gels became extremely weak and turbid and showed phase separation of polymer and solvent (Moritaka et al. 1995). Indeed, precipitation by acid can be used as a method for isolation and purification of gellan (Sanderson 1990).

3.2.5 Effect of Sugars

Addition of sugars promotes conformational ordering and gelation of gellan. One obvious way in which high concentrations of sugars can promote association of polymer chains is by replacing most of the solvent. Much of the increase in the coil-to-helix transition temperature with increasing concentration of sugar could be explained in this way, but the value of the transition temperature for mixtures of gellan with the disaccharides (sucrose and trehalose) and glucose was still substantially higher than for equivalent concentrations of gellan alone (Miyoshi and Nishinari 1999b). The enhancements observed with sucrose, trehalose, and glucose were ascribed to sugar–water associations in competition with interactions between water and gellan.

Addition of sucrose increases Young's modulus, fracture stress, and fracture strain (i.e., with the gels becoming stronger and less brittle) at the compression tests as the concentration of sucrose is raised to 40 wt.%. On further increase in sucrose concentration to 60 wt.%, however, there is decrease in Young's modulus and a massive increase in fracture strain, with the gels remaining intact up to ~65 % strain. Figure 8 shows fracture stress and strain for gellan with no added sugar and in the presence of 60 wt.% sucrose (Kawai et al. 2008). The reduction in brittleness on incorporation of 60 wt.% sucrose is evident from the much greater deformation at break (stretch ratio 1.6~1.7) in comparison with the sample with no added sugar (stretch ratio 1.10~1.17). At high concentration of sugar, formation and aggregation of gellan double helices is inhibited probably due to "condensation" of sugar molecules around the polymer chains (Morris et al. 2012).

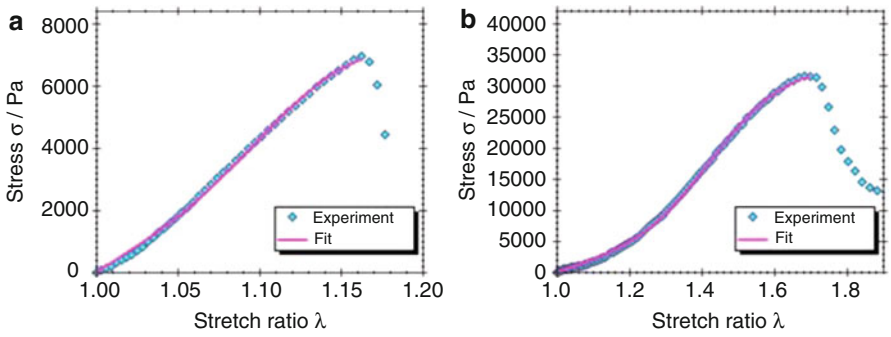

Fig. 8 Stress–strain curves of gellan gels obtained by experiments are plotted together with the calculated curves of a modified reel-chain model. (**a**) Gellan gel with the gellan concentration of 1.2 % without sucrose. (**b**) Gellan gel with 0.8 % gellan and 60 % sucrose (Kawai et al. 2008)

3.2.6 Effect of Acyl Substituents

The native gellan has an L-glyceryl substituent on O(2) of the 3-linked glucose residue of the tetrasaccharide sequence and, in at least some of the repeat units, an acetyl group at O(6) of the same residue (Kuo et al. 1986). In normal commercial production, both types of substituents are removed by treatment of the fermentation broth with hot alkali. Partial removal can be achieved by limiting the amount of alkali used. When hydrolysis is carried out in this way, at elevated temperature with the polymer in the disordered form, glyceryl substituents are liberated somewhat more rapidly than acetyl groups. Thus, it is possible to obtain samples differing widely in the proportion of repeat units carrying residual acetyl or glyceryl substituents.

High acyl gellan gives gels with lower modulus and lower hardness than those formed by commercial gellan gum, but the elasticity is much greater, and the "brittleness" values (strain at break) are also much greater (typically around 65 %, in comparison with ~30 %). In contrast to the ease with which gellan gum gels release fluid, the gels formed by high acyl gellan show no syneresis (Sworn 2009). As described below, the high acyl and deacylated forms also differ substantially in their response to changes in temperature and ionic environment. The conformational transitions that accompany formation and melting of high acyl gellan gels (1.0 wt.%) show no detectable thermal hysteresis, either in water or in 100 mM NaCl (Morris et al. 1996), and there are only small variations in melting temperature of the gels and in transition midpoint temperature from DSC heating scans (Mazen et al. 1999) on varying concentration of NaCl between 10 and 100 mM, in contrast to the massive changes in melting temperature observed for deacylated gellan over the same range of NaCl concentrations.

High acyl gellan shows higher transition temperatures in the DSC cooling scans (Morris et al. 1996) in comparison with deacylated gellan under the same conditions. The other noticeable difference is that the DSC peak is substantially wider for the high acyl form, indicating that the transition is less cooperative. Heating curves

Table 1 Physicochemical properties of gellan gum samples (Noda et al. 2008)

		GG1	GG2	GG4	GG6
Acyl content (HPIC)	Glycerate (%)	10.0	9.1	7.2	5.6
	Acetate (%)	3.0	2.8	2.7	2.5
	Total (%)	13.0	11.9	*9.9*	8.1
Cation content (ICP-OES)	Na (μmol/g)	5 %	600	674	622
	K (μmol/g)	0.82	ND[a]	ND[a]	ND[a]
	Ca (μmol/g)	4.25	3.75	7.5	5.75

Data are presented as means of duplicate
[a]Not detected

for the same samples, recorded in the same investigation but reported separately (Baird et al. 1992), were essentially equal and opposite to the cooling curves with again no indication of thermal hysteresis.

Noda et al. (2008) performed the rheological and structural studies using gellan samples with different acyl contents (Table 1). The temperature dependence of G' and tanδ is shown in Fig. 9. The thermal hysteresis between the sol-to-gel and the gel-to-sol transitions was observed, which became more apparent with decreasing acyl content from 13.0 % to 8.1 %. This is in good agreement with the previous finding that the conformational change from coil to helix and the subsequent intermolecular associations between the helices show detectable thermal hysteresis upon cooling and heating in the presence of gel-promoting cations (Morris et al. 1999b; Gunning et al. 1996). The sol-to-gel transition shifted to lower temperatures with decreasing acyl content – 65 °C, 62 °C, 60 °C, and 54 °C for GG1, GG2, GG4, and GG6, respectively – which agreed well with the result found in the absence of added cations (not shown), emphasizing the function of acyl groups, particularly glycerate, to stabilize the helical structures. At 20 °C, G' increased with decreasing acyl content, which was opposite to the result found in the absence of added cations (not shown). The unexpectedly large values of tanδ for GG6 at lower temperatures may be due to the slippage between the geometry and sample, which is difficult to avoid in rheological measurements of some gelling agents with relatively hard and brittle textures (e.g., deacylated gellan gum, kappa carrageenan, agar, etc.), showing syneresis when forming gel matrixes. Even if so, G' for GG6 was still much larger than those for the others, indicating highly developed gel structures.

Mixtures of high acyl and deacylated gellan give gels with textures that lie between the extreme brittleness of the deacylated form and the extreme extensibility of the high acyl form. There is no indication of the discontinuity in properties that would be expected from phase separation, which again strongly indicates an interpenetrating network structure (Morrison et al. 1999).

On cooling from high temperature, the mixtures show two regions of steep increase in G' (Kasapis et al. 1999), the first coincident with the sol–gel transition of high acyl gellan at high temperature and the second with the corresponding transition of the deacylated polymer at much lower temperature. Then, Kasapis et al. proposed a phase separation model for a 1 % mixed solution (0.5 % HA gellan and 0.5 % LA gellan) with NaCl at concentrations higher than 50 mM.

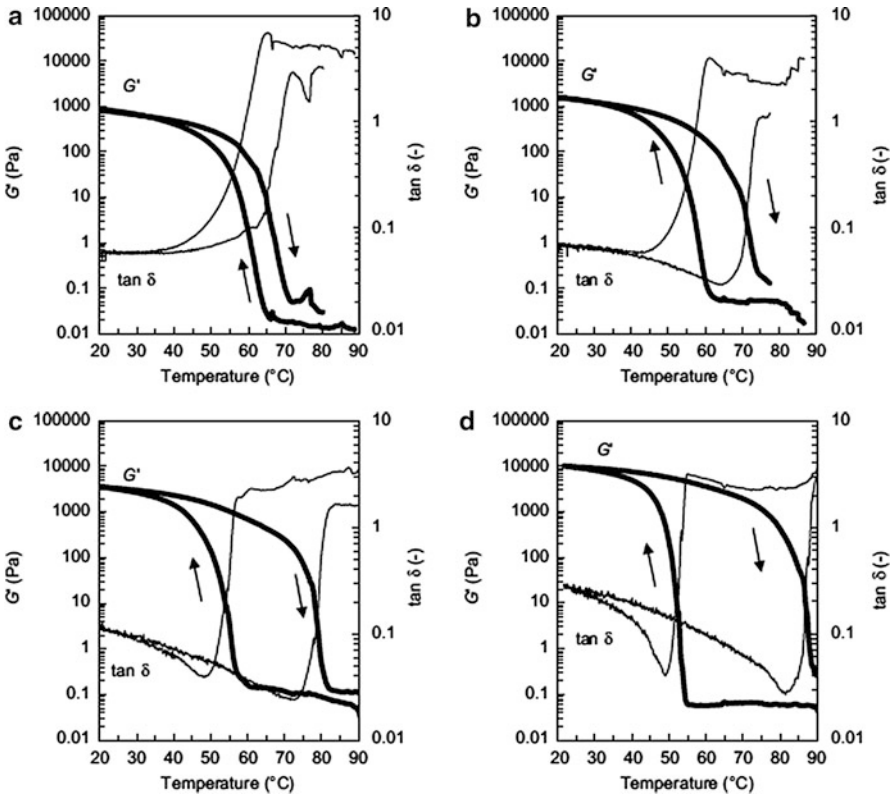

Fig. 9 Temperature dependence of dynamic viscoelasticities G' and tan δ for aqueous solutions of gellan gum in the presence of potassium. Aqueous solutions of each gellan gum sample were prepared at 1.0 w/w% in the presence of 0.1 M KCl. The solutions were placed into a double-wall Couette geometry as hot solutions at 90 °C. The geometry was preheated at 90 °C to avoid gelation in sample loading. After holding at 90 °C for 10 min, temperature decreased from 90 to 20 °C at a constant rate of 0.2 °C/min and then increased from 20 to 90 °C at the same rate. (**a**) GG1; (**b**) GG2; (**c**) GG4; (**d**) GG6. There was no difference in data from repeated measurements (Noda et al. 2008)

Matsukawa and Watanabe (2007) observed the good miscibility and transparency and no phase separation for the mixed solution of HA and LA without added salt. Therefore, they suggested that high acyl gellan chains show high preference for double-helix formation of the same kind of gellan chains in the homogeneous solution of mixed gellan species.

3.3 Mixture

3.3.1 Blending Gellan Gum with Other Polymers

Interactions between two different polymers can be classified as "associative" if they are thermodynamically more favorable than interactions between the individual

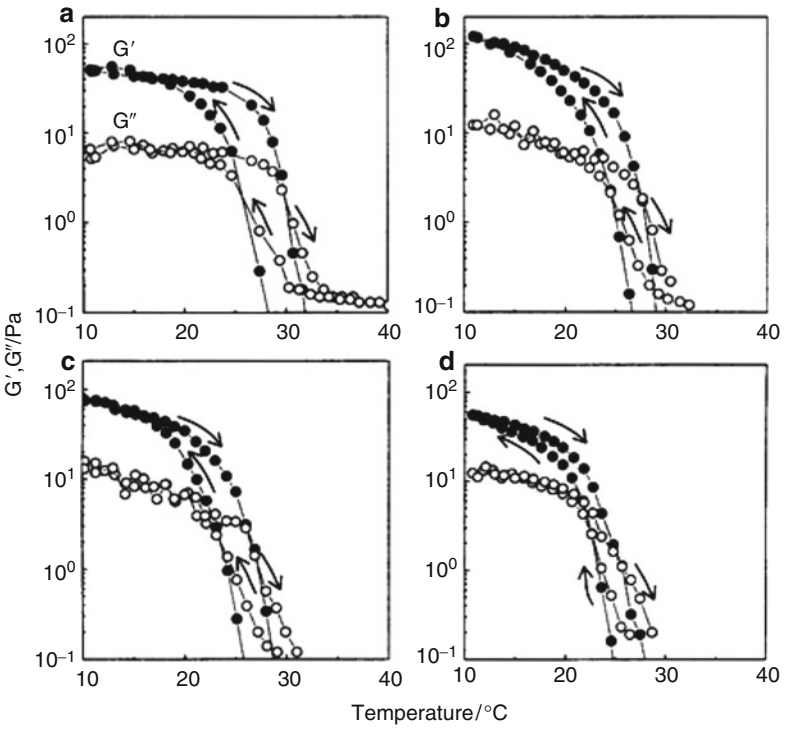

Fig. 10 Temperature dependence of storage and loss moduli of 1 % mixture of TSX and gellan on cooling and subsequent heating at 0.5 C/min and at 1.26 rad/s. TSX content: (**a**) 0.9, (**b**) 0.6, (**c**) 0.6, (**d**) 0.4 (Nitta et al. 2003)

polymers of each type and "segregative" if they are less favorable. The most common mechanism of association is electrostatic attraction between polyanions, such as negatively charged polysaccharides, and polycations, such as proteins below their isoelectric point. Segregative interactions can cause separation into a dispersed phase containing most of one polymer surrounded by a continuous phase containing most of the second polymer. The two phases may, however, run through one another, giving a bicontinuous structure. Alternatively, the two polymers may remain intimately mixed (without being confined to only part of the total volumes as in phase-separated bicontinuous networks) and form two separate gels permeating through one another, to give an IPN structure (Morris 1986, 1990).

Mixtures of Na^+ gellan with konjac glucomannan (KGM, MW950 kD) at a total polymer concentration of 0.8 wt.% in water were observed (Miyoshi et al. 1996a; Nishinari et al. 1996a) to give a sharp maximum in G' at a mixing ratio of 3:5 (i.e., 0.3 wt.% gellan plus 0.5 wt.% KGM) after cooling to low temperature (0 °C).

Tamarind seed xyloglucan (TSX) has the same backbone geometry as KGM, but the monosaccharide and disaccharide side chains confer the solubility on TSX. Mixtures of TSX with Na^+ gellan were examined (Fig. 10) (Nitta et al. 2003) at a

total polymer concentration of 1.0 wt.%, where the individual polysaccharides are nongelling. The mixtures converted from solutions to gels on cooling. Therefore, the gel was thought to be formed by a synergistic interaction. The temperature at the critical gel point increased with increasing content of xyloglucan, from 21 °C at 0.6 wt.% gellan gum plus 0.4 wt.% xyloglucan to 25 °C at 0.3 wt.% gellan gum plus 0.7 wt.% xyloglucan (Nitta et al. 2003; Nitta and Nishinari 2005).

Mixtures of K^+ gellan with kappa carrageenan, also in the K^+ salt form, were found to give two separate transitions in DSC, one at about the same temperature as for carrageenan alone and the other at higher temperature, about the same as for gellan gum alone, which seems to exclude the possibility of association between the two polymers (Nishinari et al. 1996b). This was attributed to phase separation, with consequent increase in the effective concentration of each polymer when confined to only part of the total volume. However, values of η* obtained for solutions prepared at a total polymer concentration of 0.25 wt.% with varying proportions of gellan gum and kappa carrageenan were substantially higher than those of the individual polymers, reaching a maximum at the ratio gellan gum/carrageenan = 2:8. At higher total concentrations of polymer (2–5 wt.%), where gels were formed, reaching minimum values at gellan gum/carrageenan = 1:1 can again be explained by phase separation, with the weaker network forming the continuous phase and the stronger gel confined to dispersed particles (Nishinari et al. 1996b). Unfortunately, the samples used for dilute solution studies and gel studies are not the same, and therefore, whether the occurrence of the synergistic interaction or phase separation depends on the difference in concentration or difference in the cation contents or not is not clear in this study and needs further clarification.

Phase separation has also been reported (Papageorgiou et al. 1994) for mixed gels of commercial gellan (Kelcogel) with calcium alginate. Incorporation of moderate concentrations of gellan gum (0.1–0.3 wt.%) increased the hardness (yield stress) of the gels formed by 2.0 wt.% alginate, but the breaking strain was unaffected, indicating that the brittle gellan gum network was distributed as dispersed particles embedded in a more elastic calcium alginate matrix. At higher concentrations of gellan gum (above 0.7 wt.%), however, the rheological response to addition of Mg^{2+} cations suggested a bicontinuous (phase-separated) network structure, although the possibility of phase inversion to a continuous gellan network surrounding dispersed particles of calcium alginate gel could not be eliminated conclusively.

Mixtures of K^+ gellan with agarose (Nishinari et al. 1994) showed separate DSC transitions for the two polysaccharides, as was also seen (Nishinari et al. 1996b) for kappa carrageenan–gellan mixtures, and a phase-separated network was again proposed. A subsequent study by (Amici et al. 2000), however, found no evidence of the large increase in turbidity expected to occur on phase separation or of biphasic structures in images obtained by confocal microscopy or TEM. Instead, the TEM micrographs showed a homogeneous distribution of both polymers, and rheological analysis was entirely consistent with interpenetrating networks of gellan and agarose.

Mixtures of Na^+ gellan with sodium and calcium salts of hyaluronic acid were studied by Mo et al. (2000). Gelation of mixtures of Na^+ gellan with Ca^{2+} hyaluronate

can, of course, be explained, at least in part, by interaction between the gellan component and Ca^{2+} cations from the hyaluronate, but substantial further research would be required to unravel the complexity of other possible macromolecular and ionic interactions in this system.

Mixtures of commercial gellan (Gelrite) with a wide range of starches (native and chemically modified) were studied (Sanderson et al. 1988). Gellan (0.5 wt.%) had little effect on the maximum viscosity generated by 4.5 wt.% starch during heating, in contrast to other hydrocolloids such as xanthan and carboxymethylcellulose which can give undesirably high values of peak viscosity. The starch pastes formed in the amylograph were cooled overnight and then sheared. The viscosities of the pastes containing gellan were reduced significantly by shearing, but remained higher than those of the corresponding pastes of starch alone, suggesting that gellan could be useful in preparation of a variety of starch-based fillings, which are generally prepared by cooking, stored in bulk, sheared during filling into their final containers, and allowed to recover viscosity on standing.

Direct association between gellan gum and gelatin by electrostatic attraction ("complex coacervation") was explored by Chilvers and Morris, using commercial gellan gum (Gelrite). The gelatin was obtained from pig skin by extraction with acid, giving an isoionic point of pH 8. Mixtures were prepared at a temperature (60 °C) well above the sol–gel transition temperatures of the individual polymers. Coacervate droplets (1–100 μm diameter) were formed at pH values in the range 3.5–5.0, where the gelatin had a net positive charge and the gellan gum a negative charge. The droplets gelled on cooling (Chilvers and Morris 1987).

The potential use of gellan gum–gelatin coacervates to encapsulate oils or solid particles was demonstrated by adding model substrates (paraffin oil, sunflower oil, aluminum powder, or beads of ion-exchange resin) to the biopolymer mixtures in the solution state, reducing pH into the range required for coacervation, and cooling to gel the coacervate layer that had formed around the core material. The resulting beads were then fixed with glutaraldehyde, washed, and dehydrated with isopropanol to yield concentrated slurries or free-flowing powders.

Bioartificial polymeric materials in the form of hydrogels were prepared starting from blends of PVA with gellan gum, using a procedure based on freeze–thawing cycles. Some results indicated that gellan gum favors the crystallization process of PVA allowing the formation of a material with a more homogeneous and stable structure than that of pure PVA hydrogels. Both the PVA melting enthalpy and the elastic modulus increased with increasing gellan gum content in the hydrogels; in addition, the higher the gellan gum content in the samples, the lower was the amount of PVA released. Gellan gum/PVA hydrogels were able to release human growth hormone (GH), and the release was affected by the content of the biological component. The amount of GH released was within a physiological range (Cascone et al. 2001a).

3.3.2 Films of Gellan Gum and the Mixtures with Other Polymers

Dried films cast from mixtures of commercial gellan (Kelcogel) with KGM (MW 1,320 kD) were studied (Xu et al. 2007). Maximum tensile strength was

observed when the KGM content of the films was around 70 %, which is in reasonable agreement with the mixing ratio of 3:5 gellan/KGM found (Miyoshi et al. 1996a; Nishinari et al. 1996a) to give maximum gel strength. The proposed interpretation was that KGM enhances the strength and thermal stability of gellan networks by binding to the surface of aggregated assemblies of double helices.

It was suggested that gellan gum–KGM films incorporating an antimicrobial agent (nisin) could be used in "active packaging" of food products to extend their shelf life (Xu et al. 2007).

Homopolymer films of gellan gum and PVA and blended films can be prepared by a solution casting method from a polymer solution in water of gellan and PVA. A single melt isotherm for the blends and the shift of onset, melt temperatures of the blends and a change in glass transition temperature were observed. A shift in the major signals of IR for OH absorption and with varied intensities in the FTIR spectra indicated cross-linked interaction by the polymers. The blended films had tensile strengths of 3.5–5.6 MPa and tear strengths of 15–30 g. The range of properties, both intermediate from the component polymers and extending beyond either individual components, suggests molecular interaction and cross-linking within the blends (Sudhamani et al. 2003).

Composite membranes based on gellan gum and poly(*N*-vinylimidazole) were prepared by using the method of solvent evaporation. The FTIR spectra evidenced ionic interactions between the two polymers, which contribute to the stabilization of the interpolymeric network in acid medium. The swelling behavior in media of various pH values was much dependent on the membrane compositions. This property would suggest the possibility of designing membranes with performances that might be anticipated by changing only the ratio between the two polymers (Alupei et al. 2006).

The membranes are soluble in basic medium regardless of their composition. The properties of the membranes indicate their potential application as biomaterials, especially as biodegradable materials or as supports for drugs in view of their controlled-release supports for including and releasing drugs in media of certain pH values (Alupei et al. 2006).

3.4 Texture, Syneresis, Flavor Release

3.4.1 Texture of Gellan Gels

In the texture profile analysis, five parameters are often examined: modulus, hardness, brittleness, elasticity, and cohesiveness. These parameters of gellan gels were also investigated and compared with gels formed by other gelling agents (Sanderson 1990; Gibson and Sanderson 1997). Hardness, which is defined as the maximum force generated in resistance to the compression and is usually equivalent to break stress, increased initially and then decreases on progressive reduction in pH or progressive addition of Na^+, K^+, Mg^{2+}, or Ca^{2+}. The brittleness value, which is identical to strain at break, decreased systematically with cation concentration (i.e., the gels become more brittle as more salt was added). The modulus, hardness, brittleness, elasticity, and cohesiveness of agar in traditional Japanese

food products could be matched well using gellan at much (two to three times) lower concentrations, although agar itself is a very efficient gelling agent (Sanderson et al. 1988).

Other gelling agents like agar and kappa carrageenan give firm and brittle texture, while gelation and the "synergistic" gels formed by mixtures of xanthan with plant polysaccharides such as locust bean gum (LBG) or konjac glucomannan give soft and flexible texture. The gels formed by commercial deacylated gellan (gellan gum) typically have brittleness values of around 25–30 %, giving firm and brittle texture. However, high acyl gellan gels give soft and flexible texture.

3.4.2 Syneresis

Gellan gels are comparatively stable when stored quiescently at ambient temperature or under refrigeration, but there may be some release of fluid (syneresis), particularly at polymer concentrations below ~0.2 wt.% (Gibson and Sanderson 1997). Syneresis is undesirable in most food products, and it may be necessary to add thickeners to prevent it.

Kubo et al. (2012) examined the syneresis of 0.15 % (w/w) deacylated potassium-type gellan gels in the presence of calcium ions from 1.6 to 25 mM and found that the syneresis increased from 2.4 % to 7.4 % with increasing calcium content. Syneresis can also cause problems of slippage when gellan networks are investigated by rheological measurements under shear (Morris et al. 1999b).

3.4.3 Flavor Release

It is well known in the food industry that perceived intensities of flavor and taste are lower for gelled products than for fluid products (such as sauces and drinks) incorporating the same objective concentrations of flavor compounds or "tastants" such as sugar or salt.

It is not well understood which factor governs the sweetness or saltiness of solid foods. Morris (1993) has proposed based on the study on gels with different rheological properties that the sweetness intensity increases with decreasing fracture strain because solid foods with low fracture strain are broken into small fragments and thus the total area is increased leading to the more contact of fragmented foods with taste buds. Clark (2002) showed a good correlation between the sweetness intensity with fracture stress and found two exceptions; gelatin gels which show a higher sweetness intensity in comparison with other gels with the same fracture stress because gelatin gels melt in the mouth and so showed a higher sweetness, and the other exception was a cohesive gel consisting of high acyl gellan/xanthan/locust bean gum, which showed a lower sweetness intensity because this mixed gel was not broken into small fragments, and thus Clark's explanation was not contradictory with Morris' proposal but rather complementary.

Gellan causes less suppression than most other gelling agents, or, as more commonly expressed, it has outstanding "flavor release" properties. One possible explanation (Gibson and Sanderson 1997), suggested by release of fluid when gellan gels are compressed, is that water is released from the gels during mastication, carrying with it flavor and taste compounds.

Moritaka et al. (1998) studied the relation between the texture and sweetness and milk flavor intensity of gellan jellies containing Pal Sweet (aspartame and maltose syrup) and milk powder. They found that sensory-evaluated hardness increased while smoothness decreased with increasing content of gellan gum. The sensory-evaluated milk flavor and sweetness increased with increasing content of sweetener and evaluated as higher for softer milk jellies. A jelly which was evaluated to have the strongest milk flavor contains less milk powder but with a higher content of sweetener. This phenomenon could be interpreted as the interaction between taste and aroma; sweetener intensified the milk flavor. Moritaka et al. (1999) also studied the relation between the texture and sweetness and lemon flavor intensity of gellan jellies containing Pal Sweet (aspartame and maltose syrup), citric acid, and lemon powder. Sensory-evaluated lemon flavor and sourness decreased with increasing sensory-evaluated hardness, which was well correlated with instrumentally determined hardness (fracture stress). It was observed that lemon flavor was evaluated higher for jellies with higher citric acid content. This may be again the result of the taste–aroma interaction; the sourness intensified the lemon flavor.

This cross-modal interaction between taste and flavor was recently reported by experimental psychologist groups. Hort and Hollowood (2004) showed that the sweet taste of sucrose solution intensified the banana flavor (isoamyl acetate, IAA) in the sensory evaluation based on the time–intensity method by varying the mixing ratio of sucrose solution, IAA solution, and water. Djordjevic et al. (2004) observed that the strawberry flavor intensified the sweetness of sucrose solutions and that the soy sauce flavor intensified the saltiness of salt solutions. They observed that this intensification occurred only by the imagination of the flavor of strawberry or soy sauce by the panelists without giving these flavors to their nasal cavity. They concluded that this cross-modal interaction occurred in the brain and not in the oral or nasal cavity.

Bayarri et al. (2001) investigated the influence of structure and rheological properties of gels of κ-carrageenan and gellan gels on the diffusion and perception of sweetener molecules. They found that the hydrocolloid used had an effect on the diffusion of sucrose and aspartame. The observed diffusion coefficients were higher in κ-carrageenan gels than in gellan gels. They attributed it to the incipient melting of κ-carrageenan gels. Bayarri et al. (2002) showed that fracture stress increased while the fracture strain decreased with addition of sucrose for concentrated gellan gels. Bayarri et al. (2005) also compared the influence of sucrose on gellan, *κ*-carrageenan, and *κ*-carrageenan/locust bean gum gels. They found that sucrose addition increased the fracture stress of all these gels, but this effect depended on the type and concentration of hydrocolloids. Addition of sucrose to soft gels did not influence the fracture strain, while sucrose addition to gels with higher hydrocolloid concentrations led to a slight decrease in the fracture strain.

Wang et al. (2014) examined the sucrose release from agar gels mainly based on uniaxial compression of cylindrically molded gels. The fracture stress increased with increasing agar concentration, while the concentration dependence of the fracture strain was different at different compression speeds. The number and total surface area of fragments increased with decreasing agar concentration.

Increasing the compression speed led to an initial increase followed by a monotonous decrease of both the number of fragments and their total surface area. The gel texture affected in a similar fashion both the sucrose release ratio and the total fragment surface area. The sucrose release ratio decreased with increasing agar concentration.

Sala et al. (2010) examined the effect of serum release on sweetness intensity in mixed whey protein isolate/gellan gum gels. With increasing gellan gum concentration, the size of the pores present in the protein network, the permeability, and the serum release increased, as well as the Young's modulus, the fracture stress, and the fracture strain. Increasing the sugar concentration induced an increase of the pore size, but resulted in a decrease of permeability and serum release. They found that serum release significantly boosted sweetness intensity; the sweetness scores for gels with 12 % serum release were found to be the same as for gels with 2 % serum release but with 30 % higher sugar concentration.

Sala and Stieger (2013) investigated the influence of the breakdown behavior on sweetness intensity of gelled model foods, emulsion-filled gelatin/agar gels with different fracture strains. They found that the sweetness intensity of the most brittle gel was almost twice as high as the sweetness intensity of the least brittle gel. They stated that the time to reach the sweetness intensity is important, i.e., the shorter the time, the higher the sweetness intensity. They suggested that the velocity of formation of new surfaces of the food in contact with the taste buds influences sweetness intensity in addition to the total surface which is generated during breakdown of the food.

4 Chemical/Physical Modifications of Gellan Gum and Their Effects

Gellan gum has found extensive applications in food and pharmaceutical industry because of their peculiar gelation, easy availability, biocompatibility, biodegradability, and cost-effectiveness. To improve its functional properties, a number of chemical and physical modification methods have been employed.

4.1 Comparison of Physical Gels and Chemical Gels

Physical gellan gum hydrogels, prepared with mono- or divalent cations, are used for the preparation of tablets, beads (Agnihotri et al. 2006), or microspheres in drug delivery systems. Chemical hydrogels of gellan gum are usually prepared via chemical cross-linking of the preformed physical networks, in order to enhance their mechanical properties and to obtain slower drug release profiles (Bhakoo et al. 1991). A novel gellan gum chemical hydrogel was obtained by cross-linking the polymer chains with L-lysine ethyl ester moieties and compared with the gellan gum physical hydrogels (Matricardi et al. 2009). The physical networks were much stronger than the chemical ones due to the formation of very tight junction zones

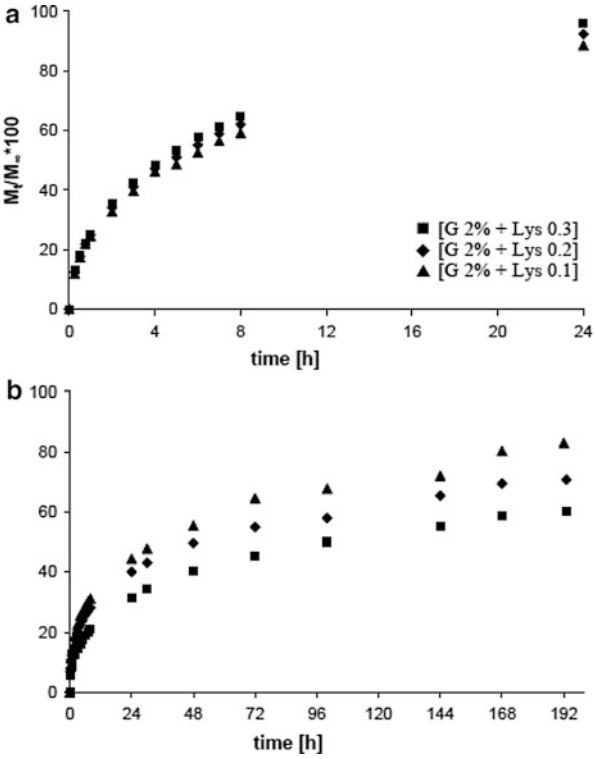

Fig. 11 Release profiles (37 °C, SIF) of DexFluo70: (**a**) from physical hydrogels; (**b**) from chemical hydrogels (in the legend G = Gellan) (Matricardi et al. 2009)

whose strength depends on the L-lysine ethyl ester content. On the other side, the chemical gels were weaker because of the applied cross-linking procedure: in fact the cross-linker was added when the polymeric chains were in a disordered conformation and therefore junction zone formation was forbidden. As a final effect the elastic modulus was lower of two orders of magnitude in comparison to those recorded for the physical networks. Of course, within the same kind of gel, physical or chemical ones, the storage modulus increased with the increase of the L-lysine ethyl ester content. In the physical gels the role of L-lysine ethyl ester was to decrease the electrostatic repulsions among the chains, and therefore the number of the junction zones was a function of the L-lysine ethyl ester content. In the chemical gel, obviously, the degree of cross-linking increases with the increase of L-lysine ethyl ester content. According to this model, also the yield stress and the compressibility parameters were affected by the kind and the degree of linkage among the polysaccharide chains (Matricardi et al. 2009).

The structural differences also influenced the delivery profiles in SIF (simulated intestinal fluid, phosphate buffer, pH = 7.4), in particular when high molecular weight model molecules were used. L-Lysine ethyl ester content does not affect significantly the delivery rate from the physical hydrogels, regardless of the steric hindrance of the guest molecules, due to the temporary nature of the junction zones (Fig. 11a). On the other side, for the chemical gels, quite different profiles were

recorded that were deeply influenced by the steric hindrance of the model molecule as well as by the L-lysine ethyl ester content, and a significant amount of the model molecule with the highest molecular weight still remains entrapped within the network even in 8 days (Fig. 11b) (Matricardi et al. 2009).

Injectable hydrogels are hydrogels capable of gelling in situ due to chemical and/or physical cross-linking development after injection. Because of the advantages over their conventional counterparts, injectable hydrogels have been found a great number of applications in tissue engineering, drug delivery, and cell and protein transplantation (Redouan et al. 2010; Jin et al. 2009; Bhattarai et al. 2010; Singh et al. 2009). The most attractive advantages are that they are easily deliverable by minimally invasive techniques and are capable of filling native or potential cavities by conforming to different shapes, which may be difficult to prefabricate (Aliyar et al. 2005).

Most recently, gellan gum-based physical hydrogels have been explored in tissue engineering because of their high biocompatibility, biodegradability, and injectability (Redouan et al. 2010; Oliveira et al. 2009a; Smith et al. 2007; Wang et al. 2008). However, substantial problems remain before the gellan gum hydrogels can become genuinely injectable for practical biological use – its gelation temperature is much higher than human body temperature (37°C) (Gong et al. 2009) and it gels in seconds in PBS medium, leaving no window of time for manipulation (Oliveira et al. 2009a). In addition, gellan gum physical hydrogels induced by cations tend to become weaker in physiological conditions due to the exchange of divalent cations by monovalent ones (Annaka et al. 2000).

An injectable, in situ physically and chemically cross-linkable gellan gum hydrogel is synthesized via gellan thiolation (Du et al. 2012). A chemical cross-linking was developed between gellan gum chains while the thiol groups underwent air oxidation in physiological conditions, giving a stable chemical hydrogel. The thiolation does not alter the gellan gum's unique 3D conformation but leads to a lower phase-transition temperature under physiological conditions and stable chemical cross-linking (Fig. 12). The possible mechanism behind the lower gelation temperature is due to the reduced cation binding sites on the gellan gum backbone and the steric effect of the attached small molecule. This gellan gum with decreased gelation temperature exhibited both physical gelling and chemical cross-linking ability, making it desirable for in vivo applications in tissue engineering (Du et al. 2012).

4.1.1 Carboxymethylation

Carboxymethylation of gellan gum was reported to improve its aqueous solubility, and so that the gelation of gellan can be controlled (Miyamoto et al. 1996). Carboxymethylation of gellan gum was found to increase its degree of crystallinity and surface roughness and diminish the cation-induced gelation and increase the solubility in water. Furthermore, carboxymethyl gellan gum showed 2.71-fold higher mucoadhesive strength than gellan gum. Evaluation of ex vivo ocular tolerance using chorioallantoic membrane of hen's egg and cytotoxicity screening on Vero cells using resazurin assay revealed that carboxymethyl gellan gum is

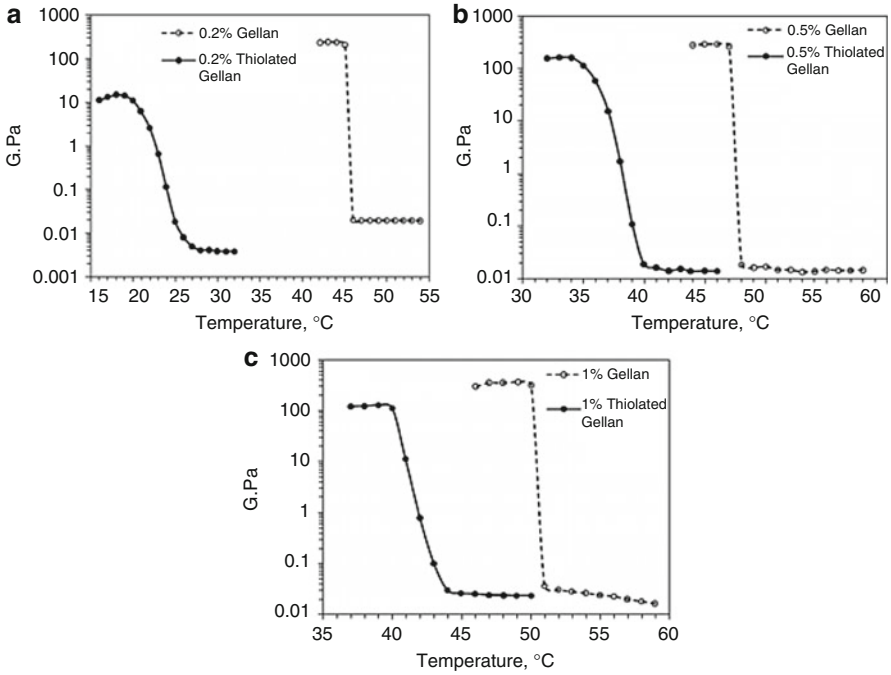

Fig. 12 Sol–gel transition of gellan (*dashed line*) and thiolated gellan (*solid line*) solutions in PBS at (**a**) 0.2 %, (**b**) 0.5 %, and (**c**) 1.0 % (w/v) upon cooling. Gellan solutions show high transition points (gelation temperatures) and narrow transition temperature ranges, while thiolated gellan solutions displayed decreased transition points and increased transition temperature ranges (Du et al. 2012)

non-irritant and biocompatible. Ionotiropically gelled beads of carboxymethyl gellan gum formulated using metformin as the model drug and calcium chloride as the cross-linking agent showed ex vivo bioadhesion of 100 % over 24 h. Further, it was observed that carboxymethyl gellan gum beads released metformin at a rate faster than gellan gum (Ahuja et al. 2013).

4.1.2 Oxidization

By regiospecific oxidation at C6 with NaOCl in the presence of 2,2,6,6-tetramethyl-piperidine-1-oxyl radical (TEMPO) and NaBr in aqueous solution at pH 10, the C6 primary hydroxyl groups of deacylated gellan gum were completely converted to carboxylate groups(Redouan et al. 2010). This original carbohydrate rhamnoglucuronan might find use as surrogates of ulvan, a sulfated cell wall polysaccharide extracted from green seaweeds (Ulva species), in pharmaceutical and medical areas (Redouan et al. 2010).

For tissue engineering purposes, gellan gum-based hydrogels need to be modified in order to meet the requirement of encapsulating living cells while maintaining their injectability, because the gelling point of this temperature-dependent gel is too

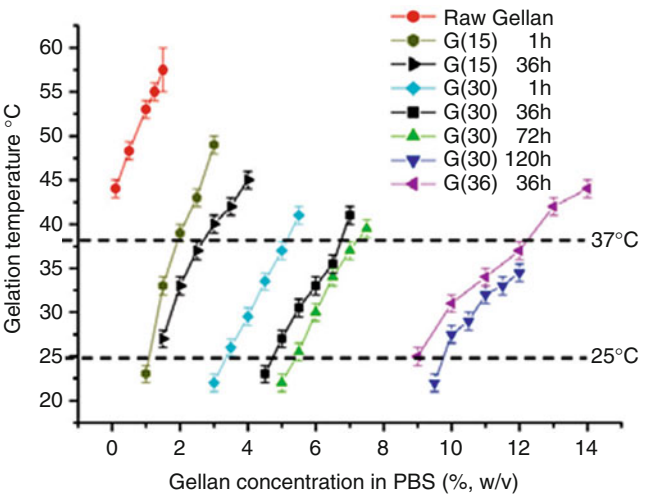

Fig. 13 Gelation temperatures of oxidized gellan with different reaction conditions (Gong et al. 2009).

high (above 42°C). Chemically scissoring (via oxidative cleavage) the gellan gum backbones can optimize the gelation temperature for injection as a result of downregulating their molecular size (Fig. 13) (Gong et al. 2009). Chondrocytes were then seeded into the modified gellan gum gels. Notably, chondrocytic constructs based on modified gellan gum gel were kinetically monitored for 150 days in comparison with those based on agarose gel, showing superiority for long-term cartilaginous development in terms of many aspects such as cell proliferation and specific matrix formation.

4.1.3 Amidation

Biomaterials have been designed as vehicles for cell transplantation in order to enhance cell survival after transplantation. In order to better mimic the extracellular matrix (ECM), biomaterials have been modified with several peptide sequences (Luo and Shoichet 2004; Tong and Shoichet 2001; Yu and Shoichet 2005) to influence biological processes, such as cell adhesion, growth, and development (Adams and Watt 1993; Schwarz et al. 1990). Cells transplanted in a material that mimics both the mechanical and chemical properties of native tissue have been shown to be more efficacious after transplantation (Cooke et al. 2010; Kim et al. 2012). Hydrogels are particularly appealing for soft tissue applications because they can be designed to match the mechanical properties and water content of these tissues. The gellan gum hydrogel is compelling because it can be injected in a minimally invasive way to form a gel in situ. But gellan gum hydrogel is a relatively bio-inert material. To enhance cell adhesion, the gellan gum (GG) was modified using Diels–Alder click chemistry with a fibronectin-derived synthetic peptide (GRGDS) (Silva et al. 2012). The GG–GRGDS had a profound effect on

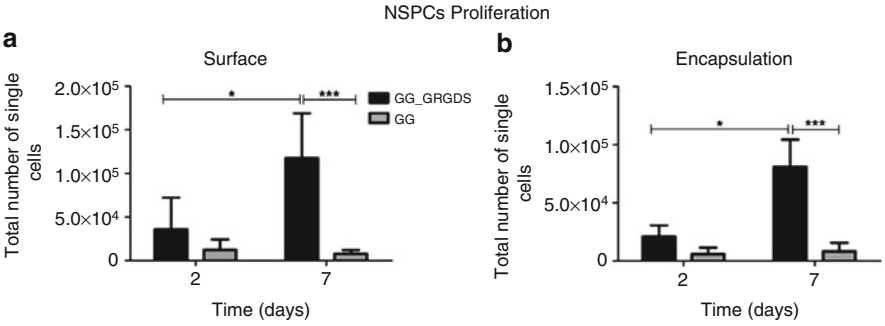

Fig. 14 Bioactivity of the GG–eGRGDS hydrogel. Proliferation analyses of NSPCs cultured either (**a**) on the surface of or (**b**) in the gel of the GG–eGRGDS (*black bars*) versus unmodified GG (*gray bars*) showed that on the 7th day a significantly higher number of single cells were found in the GG–eGRGDS. Moreover, only in the GG–eGRGDS was a significant increase in cell number observed from day 2 to day 7 (Silva et al. 2012)

neural stem/progenitor cell (NSPC) morphology and proliferation, distinct from that of NSPCs in GG alone, demonstrating the importance of GRGDS for cell–GG interaction (Fig. 14) (Silva et al. 2012).

4.2 Hydrophobization

In recent years, self-assembled nanohydrogels based on hydrophobically modified polysaccharides have been extensively studied due to their wide potential applications as drug delivery systems. As applied to drug delivery systems, natural polysaccharides have the advantages of their abundance in nature, low cost, safety, general biocompatibility, biodegradability, and high stability. Among all natural polysaccharides, gellan gum is a promising candidate for biomedical applications because of its peculiar physicochemical properties and its biocompatibility (Miyoshi et al. 1996b; Dreveton et al. 1996; Matricardi et al. 2009). Herein, gellan gum nanohydrogels (NHs) were prepared by self-assembly of the polymer chains, after an appropriate hydrophobic chemical derivatization with cholesterol and prednisolone moieties (D'Arrigo et al. 2012). The prednisolone moiety, linked to the polymer chains by means of a short hydrocarbon chain, simultaneously acts as a pharmacological agent and a promoter of NH formation, whereas cholesterol was chosen as a moiety able to promote the formation of reference NHs with no pharmacological activity. A slight decrease in cell viability was observed both for Ge–Pred and prednisolone treatments; at low concentrations, Ge–Pred showed a lower toxicity. However, it must be pointed out that in no case was cell viability reduced to less than 80 % (Fig. 15). This approach allows us to combine the advantages of nanotechnology, such as high stability, high carrier capacity, and feasibility of different routes of administration with an immobilized form of prednisolone that displays improved bioavailability, thus increasing its therapeutic effects and reducing its dosing frequency.

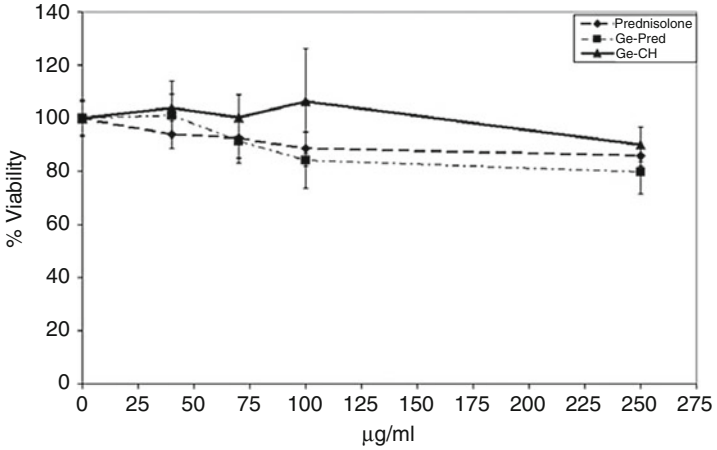

Fig. 15 Cell viability expressed as a function of the concentration of administered Ge–Pred (-), Ge–CH (:), or Pred (A). The viability of the control cells (without treatment) was set at 100 %. Each point is the mean SD of 16 replicates reaction scheme of the gellan gum–prednisolone synthesis (D'Arrigo et al. 2012)

4.3 Cross-linking

Over the past decades, great attention has been focused on biopolymer-based hydrogels for use as potential carriers in controlled drug delivery (Van Tomme et al. 2008; Coviello et al. 2007). Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids (Peppas et al. 2000), resembling biological tissues. Because of this property, great interest was devoted to these systems for biomedical applications. Indeed, the physicochemical properties of the hydrogels can be tuned by varying the cross-linking degree (physical and/or chemical), thus making these networks suitable devices for a modulated drug delivery.

Recently, gellan gum has been investigated as a candidate material for biomedical engineering because of its biocompatibility and low cytotoxicity (Oliveira et al. 2010a; Silva-Correia et al. 2011). Although the polysaccharide can be cross-linked with Ca^{2+} ions, the mechanical properties of polysaccharide are fragile and less malleable (Ichibouji et al. 2009). When implanted, tissue calcification occurs, which limits the biomedical application of Ca^{2+}-cross-linked gellan gum. In addition, chemical cross-linkers can be cytotoxic due to dosage responses and cross-linker residues (Powell and Boyce 2006).

To develop a nontoxic method of cross-linking gellan gum that can be applied in biomedicine is the main purpose of these research works. Cross-linking via the photodimerization of polymeric systems has been utilized in various applications. So, a new photocrosslinkable gellan gum molecule that contains a cinnamate moiety can be designed and may be used for medicinal purposes (Lee et al. 2012). The reaction does not require the addition of a light-sensitive initiator. Cinnamate

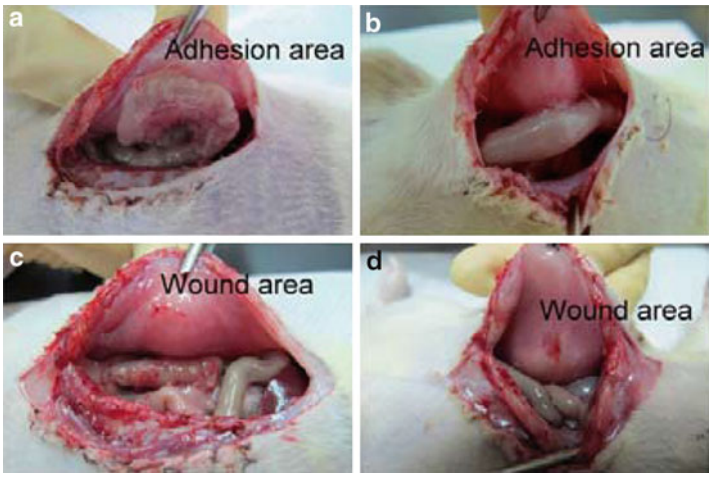

Fig. 16 Repair of the injured sites between the cecum and the peritoneal wall in the operated rats 3 and 7 days after surgery: (**a**) and (**b**) adhesion formation (control group) and (**c**) and (**d**) treated with gellan gum–cinnamate film without adhesion formation (Lee et al. 2012)

derivatives are natural compounds that possess anti-inflammatory and non toxic properties (Ballabeni et al. 2010). So, cinnamate functions not only as a cross-linking agent but also as an anti-inflammatory drug. The anti-adhesion films prepared from gellan gum–cinnamate polymers exhibited high gel contents (88 ± 2 %) and suitable mechanical properties. When implanted into rats, the gellan gum–cinnamate film exhibited the most promising anti-adhesion potential and anti-inflammation (Fig. 16). The gellan gum–cinnamate film thus has potential in clinical applications (Lee et al. 2012).

4.4 Graft Copolymerization

A graft copolymer is a macromolecular chain with one or more species of block connected to the main chain as side chain(s). Thus, it can be described as having the general structure, where the main polymer backbone, commonly referred to as the trunk polymer, has branches of another polymeric chain emanating from different points along its length (Zohuriaan-Mehr 2005). Graft copolymerization of synthetic polymers onto the polysaccharide backbone offers one of the best ways to use polysaccharides for controlled-release delivery. Graft copolymerization is an easier method to modify the structure of natural polymers and thus makes them attractive biomaterials in controlled-release applications since native polysaccharides may not be suitable in controlled drug delivery systems due to their substantial swelling and rapid enzymatic degradation in biological fluids (Soppimath et al. 2002). Acrylamide-grafted-gellan gum (AAm-g-GG) was synthesized using microwave-assisted ceric (IV) ion-induced grafting method (Fig. 17a). The acrylamide is toxic in nature, but from the toxicity study in mice, it is revealed that there is no morbidity

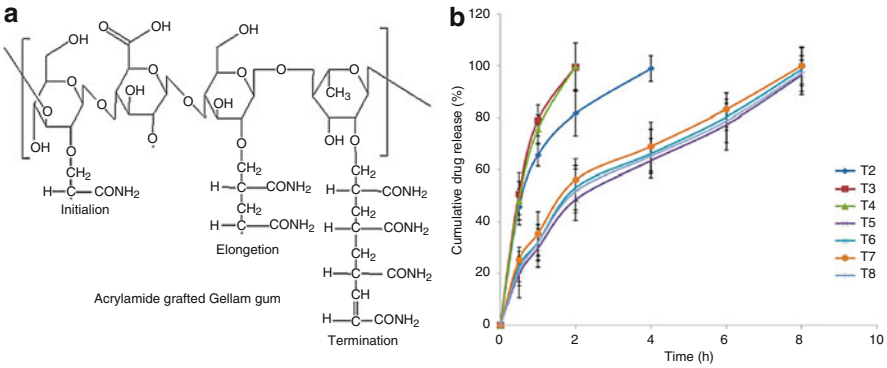

Fig. 17 (**a**) Acrylamide-grafted-gellan gum (AAm-g-GG), (**b**) Release profile of MTF from the tablet formulation containing different batches of AAm-g-GG (Vijan et al. 2012)

or mortality case during the LD50 study at a dose of 2,000 mg/kg body weight of AAm-g-GG. Sustained release tablet of metformin hydrochloride (MTF) was developed by using this grafted gum as a rate-controlling polymer. The release data could be fitted as a function of time $\sim t^n$. Cumulative release profiles of MTF from various formulations are shown in Fig. 17b, and the exponent n was found to be 0.363–0.556, thus indicating the release mechanism is Fickian or square root of time kinetics. Thus, microwave-assisted ceric (IV) induced graft copolymerization is an easy, efficient, less time-consuming, and reproducible (due to the supply of fixed energy form microwave) method for the development of graft copolymer which can be used as a rate-controlling polymer for the development of sustained release dosage form (Vijan et al. 2012).

Furthermore, a series of gellan gum unsaturated esters by addition of free radical polymerizable groups, which can be polymerized under mild conditions to design biodegradable three-dimensional networks having hydrogel properties, were synthesized by esterification under various conditions (Fig. 18). Different degrees of functionalization were obtained either homogeneously in water with acrylic acid or heterogeneously with acryloyl chloride and maleic anhydride in organic solvents. Maleic anhydride evidences a higher reactivity than the acryloyl chloride and acrylic acid, especially when the reactions of gellan gum esterification are carried out in DMF (Hamcerencu et al. 2008). Preliminary copolymerization experiments performed with acrylic monomers such as *N*-isopropylacrylamide and unsaturated gellan gum esters lead to thermo- and pH-stimulable hydrogels with adjustable cross-link density. Early results revealed that modified gellan gum-/*N*-isopropylacrylamide-based hydrogels are potential carriers in the design of controlled drug delivery systems.

4.5 Dual Interpenetrating Network

Hydrogels are promising candidates for tissue engineering scaffolds due to their high water content, high permeability to small molecules, biocompatibility, and

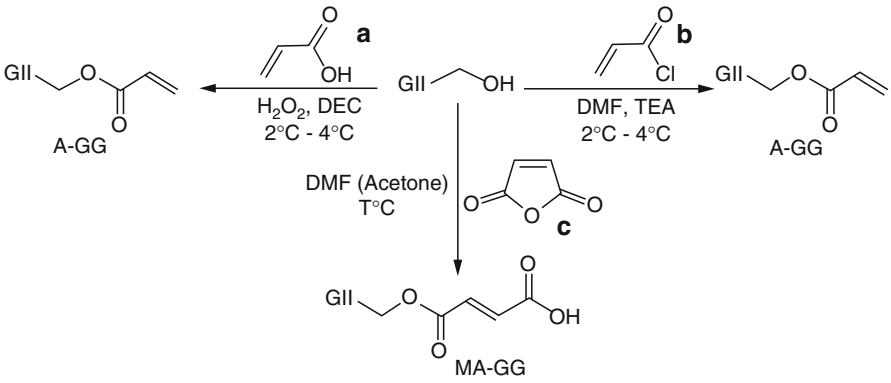

Fig. 18 Strategies for modification of gellan gum: (**a**) acrylic acid, (**b**) acryloyl chloride, (**c**) maleic anhydride (Hamcerencu et al. 2008)

mechanical properties which resemble natural tissues (Gong 2010; Lee and Mooney 2001; Peppas et al. 2006). However, hydrogels are often too soft and weak to be applied for a number of tissue engineering applications that require extensive load-bearing behavior. Therefore, developing hydrogels with high mechanical strength is a critical challenge for expanding the range of applications of hydrogels for tissue engineering scaffolds. Double-network (DN) hydrogels have attracted a great deal of attention for their high fracture toughness and high fracture stress (Gong et al. 2003; Nakayama et al. 2004). In the case of DN hydrogels, the first network is stiff and brittle, whereas the second is soft and ductile. In this scheme, the rigid network sustains the stress throughout the material, and the ductile network dissipates energy near the crack tip, preventing the fracture of gels (Na et al. 2004).

Gellan gum has been approved by the FDA as a food additive and has been recently receiving attention for tissue engineering applications (Gong et al. 2009; Oliveira et al. 2009b, 2010a). Gelatin is denatured collagen, which is a major constituent of the extracellular matrix. Due to the natural cell binding motifs, such as Arg-Gly-Asp (RGD), gelatin exhibits great biological properties such as cell adhesion and cell elongation, which makes it an attractive material for tissue engineering applications. So, both polymers can be modified into photocrosslinkable gellan gum methacrylate (GGMA) and gelatin methacrylamide (GelMA) and fabricated into DN hydrogels with high strength that can encapsulate cells (Fig. 19). In the DN hydrogels, GGMA is the rigid and brittle first network, and GelMA is the soft and ductile second network. As compared to single-network hydrogels, DN hydrogels exhibited higher strength, which approaches closer to the strength of cartilage. There is an optimal range of the cross-link density of the second network for high strength of DN hydrogels. DN hydrogels with a higher mass ratio of GelMA to GGMA exhibited higher strength, which shows promise in developing even stronger DN hydrogels in the future. Three-dimensional (3D) encapsulation of NIH-3T3 fibroblasts and viability test showed the cell

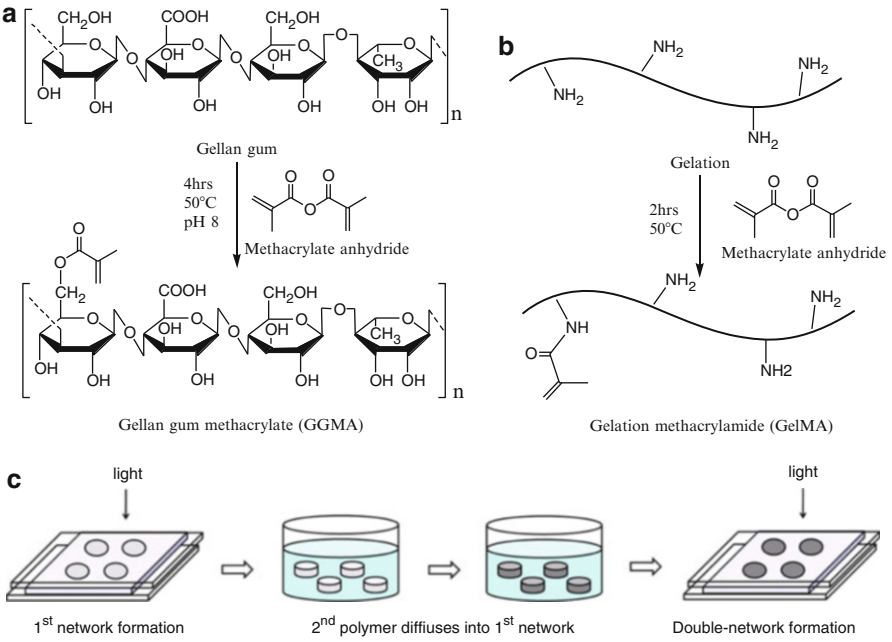

Fig. 19 Synthesis scheme of (**a**) gellan gum methacrylate (GGMA) and (**b**) gelatin methacrylamide (GelMA). (**c**) Fabrication of DN hydrogels through a two-step photocrosslinking process (Shin et al. 2012)

compatibility of the DN formation process. Given the high strength and the ability to encapsulate cells, the DN hydrogels made from photocrosslinkable macromolecules could be useful for the regeneration of load-bearing tissues (Shin et al. 2012).

Diltiazem hydrochloride is widely used in the treatment of angina pectoris and hypertension. It has a short half-life (3–4 h) and is administered three to four times daily (Shivkumar et al. 2006). The ion-exchange resins can be bound with diltiazem hydrochloride to form reversible complex. The advantages of using diltiazem–resin complex include reducing the bitter taste of drug, facilitating development of controlled-release dosage form, providing uniform drug absorption, and increasing stability by protecting the drug from hydrolysis. In addition, entrapping the diltiazem–resin complex within IPN matrix can modify the release rate of drug (Junyaprasert and Manwiwattanakul 2008). Diltiazem hydrochloride was bound to Indion 254, a cation exchange resin, and the resulting drug–resin complex was entrapped within IPN microcapsules of gellan gum and egg albumin prepared by ionotropic gelation and covalent cross-linking method (Fig. 20). The pure drug diltiazem showed rapid and complete dissolution within 60 min, while drug release from drug resinate was extended for 3 h and that from IPN microcapsules was still slower. The ionically cross-linked microcapsules were capable of releasing drug up to 9 h, and that from dual cross-linked microcapsules was extended up to 15 h. The microcapsules which were prepared with higher concentration of glutaraldehyde

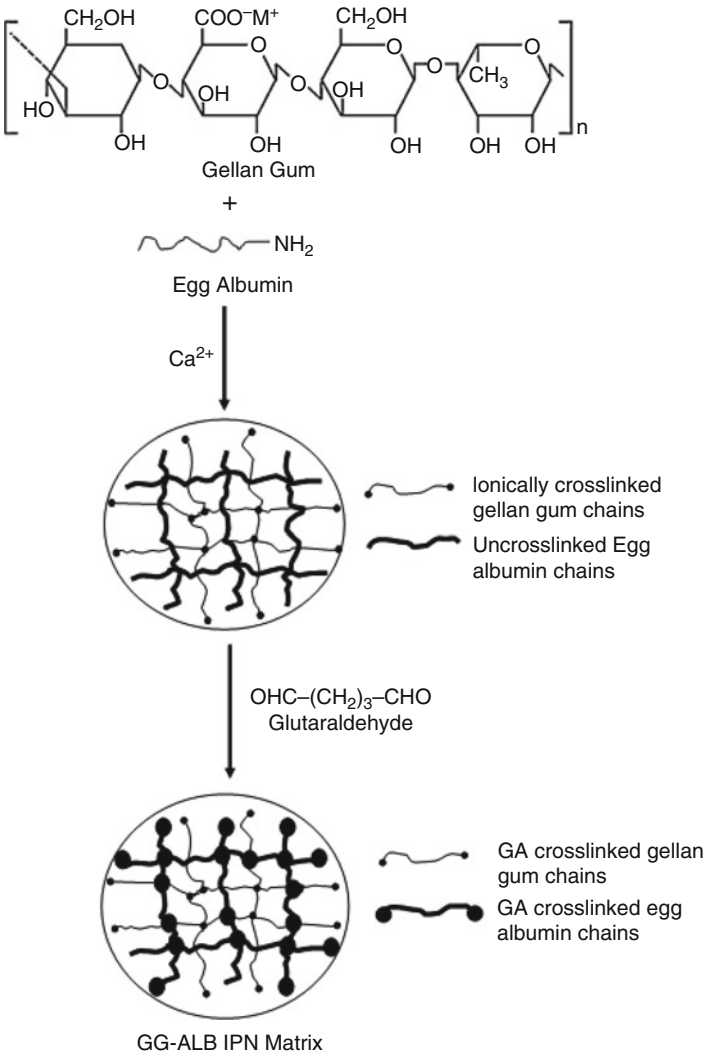

Fig. 20 Schematic representation of gellan gum–egg albumin IPN matrix (Kulkarni et al. 2011)

released the drug more slowly. The release data were fitted to an empirical equation to determine the transport mechanism (Kulkarni et al. 2011).

Gellan gum is a candidate material for cartilage tissue engineering, but its application is limited by the high gelation temperature and lack of mechanical strength (Coutinho et al. 2010). A double-network complex hydrogel with low gelation temperature ($T_{gelation}$) and high mechanical properties was prepared by mixed oxidized gellan gum with carboxymethyl chitosan (Fig. 21). The $T_{gelation}$ was lowered from 42°C to below physiological temperature by oxidation and further reduced by complexing with carboxymethyl chitosan. The complex hydrogel

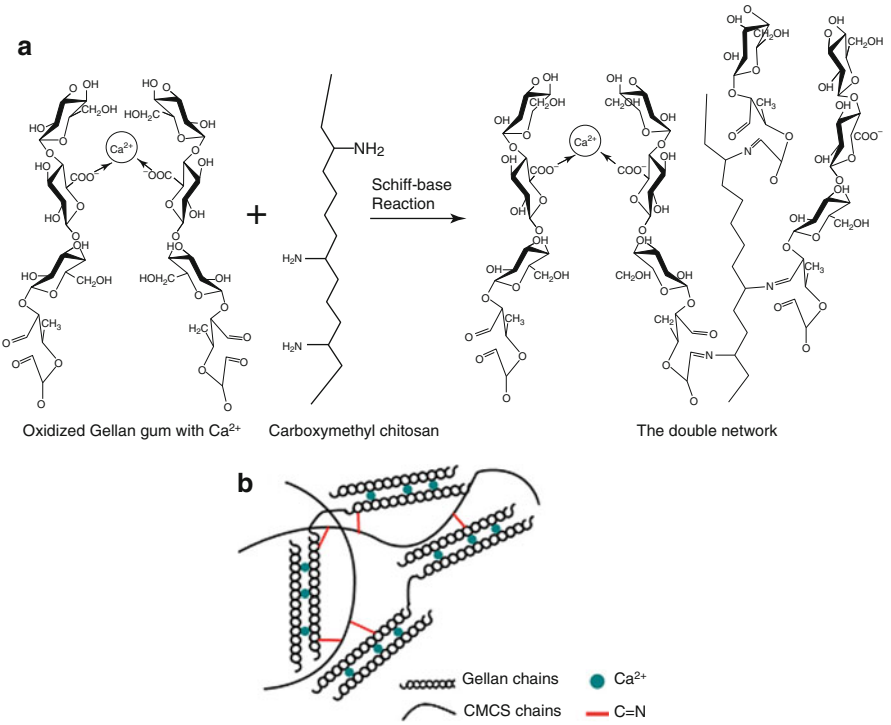

Fig. 21 (**a**) Schiff-base formation between amino groups of CM-chitosan and aldehyde groups of oxidized gellan gum. (**b**) The cross-linking mechanism of complex hydrogel (Tang et al. 2012)

showed an increased compressive modulus of 278 kPa and an ability to return to the original shape after release of the compressive load. In vitro chondrocyte encapsulation and proliferation experiments found that the complex hydrogel significantly enhanced the viability of the cells. These results suggest that the complex gel of gellan gum and carboxymethyl chitosan is a promising material for cartilage tissue engineering (Tang et al. 2012).

5 Applications of Gellan Gum and Its Modified Materials in Biomedicine

5.1 Applications in Food

Typical food applications of gellan are summarized in Table 2, which is taken directly from the CP Kelco booklet available online at the web address (http://www.appliedbioscience.com/docs/Gellan_Book_5th_Edition.pdf). Gellan is used as gelling agent for water-based dessert gels. In addition gellan is used to improve heat stability of other gelled products like aspic in which gelatin is usually used as gelling agent. Gellan can be an alternative for high methoxyl pectin, the gelling

Table 2 Typical food applications of gellan. http://www.appliedbioscience.com/docs/Gellan_Book_5th_Edition.pdf

Major food area	Typical products
Confectionery	Starch jellies, pectin jellies, fillings, marshmallow
Jams and jellies	Reduced calorie jams, imitation jams, bakery fillings, jellies
Fabricated foods	Fabricated fruits, vegetables, meats
Water-based gels	Dessert gels, aspics
Pie fillings and puddings	Instant desserts, canned puddings, precooked puddings, pie fillings
Icings and frostings	Bakery icings, canned frostings
Dairy products	Ice cream, gelled milk, yogurt, milkshakes, low-fat spreads, dips
Beverages	Fruit, milk-based, soy, and carbonated drinks
Films/coatings	Batters, breadings, coatings, adhesion systems

agent of general fruit jams. High methoxyl pectin requires high sugar level for gel formation. Reducing amounts of sugar is desired for jams and gellan can be used for this purpose. By applying gellan to starch jellies, the set time of starch jellies can be reduced. Gibson and Sanderson (1997) show many sample recipes for a low-solid fruit spread, a fruit pastille-type gel, a dessert jelly, and a milk jelly based on gellan gum.

For meat products, fat reduction is required from the viewpoint of preventing metabolic syndrome. On the other hand, fat plays an important role for taste and texture in meat products. Gellan was investigated whether it is possible to replace fat into carbohydrate polymers in meat products (Lin and Huang 2003; Totosaus and Pérez-Chabela 2009). Mixed gels of konjac and gellan were incorporated into reduced-fat (18%) frankfurters and compared with reduced-fat and high-fat (28%) controls for physicochemical, textural, and sensory properties and storage stability (Lin and Huang 2003). Water-holding capacity, sensory scores (firmness and juiciness) was improved by adding konjac and gellan compared to reduced-fat controls without added mixed gels (Lin and Huang 2003). Gellan was incorporated into low-fat and sodium-reduced meat batters with dicationic salts (Totosaus and Pérez-Chabela 2009). Inclusion of magnesium chloride showed better performance compared to calcium chloride in instrumental texture characteristics, which was attributed to differences of gellan gel structures (Totosaus and Pérez-Chabela 2009).

Among various polysaccharides, mainly cellulose derivatives methyl cellulose and hydroxypropylmethyl cellulose have been used to reduce the fat content in fried foods (Varela and Fiszman 2011). Some other trials to use gellan to reduce oil uptake during frying have been carried out. Addition of gellan to a traditional Indian deep-fat fried product *sev* consisting mainly of chickpea flour decreased oil content of final products without textural change (Bajaj and Singhal 2007).

In acidic condition, addition of gellan improves the stability of casein gels; sedimentation is suppressed. The effect of gellan on the structure and stability of a yogurt-based Iranian drink was investigated (Kiani et al. 2010). Incorporation of low concentration of gellan (0.01, 0.03, and 0.05 wt.%) reduced serum phase generated by sedimentation and inhibited further separation at longer times

(up to ~1 month). Low concentration of gellan was thought to form "weak gel" and gellan–casein associations (Kiani et al. 2010).

In beverages and pourable dressings, a "fluid gel" shown in the section 3.2.1 "Rheology of Gellan Solutions and Gels" is used to give a suspension at a low viscosity. Gellan can form a heat-resistant "fluid gel" so that it can be subjected to heating for pasteurization, while carrageenan and agar form only a non-heat-resistant "fluid gel" (Oomoto et al. 1999). Gellan gum is also advantageous because it is acid-resistant which is often required for drinks and beverages.

5.2 Drug Controlled Release

Natural polymers are preferred over synthetic polymers in controlled drug delivery systems because of their biodegradability, low cost, easy availability, and nontoxicity (Bhardwaj et al. 2000). However, they also possess certain drawbacks like uncontrolled hydration, microbial contamination, drop in viscosity during storage, etc. These limitations can be reduced following modification by cross-linking, blending, interpenetrating polymer network formation, etc.

The investigation of the material science of polymeric encapsulation systems has been a focused subject recently in interdisciplinary fields, such as food, drug, pharmaceutical, and environmental science (Vandegaer 1974). In particular, composite capsules are very promising and functional devices, in which the polymer matrices have been designed for protection and sustained release of the encapsulated substances (Kondo et al. 1991).

Methods to prepare the capsule include (a) complex coacervation (Das and Palchowdhury 1989; Iso et al. 1985), (b) phase separation (Iso et al. 1985; Hildebrand and Tack 2000), and (c) in situ polymerization (El-Samaligy et al. 1986; Wood and Whateley 1982). The preparative method via (a) is based on the polyionic complexation through the electrostatic interactions between cationic and anionic polymers, resulting in the formulation of insoluble spherical beads or capsules.

The advantage of this method in material science is its simple procedure, mixing a polyelectrolyte aqueous solution with a countercharged polyelectrolyte solution. In many cases the complexation of polyelectrolytes is performed in an aqueous solution, allowing the encapsulation of biological components, including enzymes or peptides, as their biological activities can be retained (Kabanov 1973).

True spherical capsules are formed by electrostatic polysaccharide interaction between chitosan and gellan gum via polyion complex (PIC) formation in aqueous solutions (Fig. 22). Dropwise addition of a chitosan solution into the gellan solution gave spherical capsules whose outside surface was gellan and whose inside was chitosan (chitosan^{in}–gellan^{out} capsule). Conversely, the addition of gellan into chitosan yields chitosan^{out}–gellan^{in} capsules. The capsules thus obtained were mechanically and chemically stable under given physiological conditions. The mechanical stability of the capsules is probably due to the fibrous network spread along the capsule membrane (Ohkawa et al. 2004).

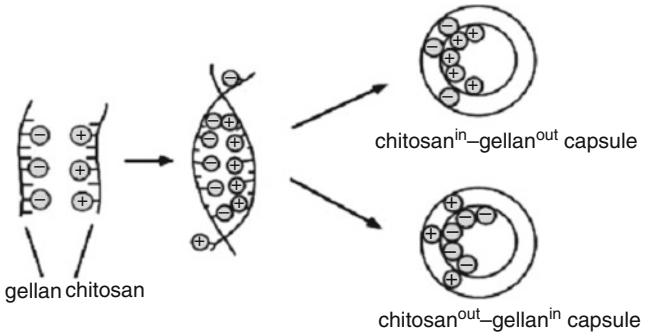

Fig. 22 Illustrative drawing of PIC capsule formation (Ohkawa et al. 2004)

Table 3 Molar mass dependence of releasing behavior of substances from chitosan–gellan capsules (Ohkawa et al. 2004)

		Release	
		%	
Substances	$\overline{M}_W$	Chitosan^{in}–gellan^{out}	Chitosan^{out}–gellan^{in}
Acetylsalicylic acid	180	29.0	48.4
Penicillin G potassium	372	25.7	45.9
Gramicidin D	1,880	16.9	34.0
Insulin	5,700	3.8	30.8
Trypsin	24,000	2.6	13.7
α-Chymotrypsin	25,000	2.4	14.0
Bovine serum albumin	57,000	1.7	12.4
Lactoferrin	86,000	1.1	12.0

Drugs and proteins could be easily loaded into the capsule by mixing the substances with the inside component then by forming the capsule. Comparison of the releasing characteristics of the two kinds of PIC capsule, i.e., chitosan^{in}–gellan^{out} and chitosan^{out}–gellan^{in} capsules, afforded several insights for controlling the release kinetics of small substances. The charge-dependent release control could be achieved by the inverse of the inside and outside composition of the PIC capsule. The M_w-dependent releasing properties of the chitosan^{in}–gellan^{out} and chitosan^{out}–gellan^{in} capsules were also examined (Table 3). The selectivity of permeation was rather steep for the chitosan^{in}–gellan^{out} capsule as compared to that of the chitosan^{out}–gellan^{in} capsule. The results described here, proved the usefulness of the chitosan–gellan capsule as a drug-carrier material having the property of a controlled-release mechanism (Ohkawa et al. 2004).

A polymer drug system based on gellan and PVA interpenetrated network (IPN) was prepared (Dumitriu et al. 2004). The drug (cephotaxime, CEF) has been included through a diffusion process, from an aqueous solution into a high swelling degree hydrogel. The amount of included drug, as well as the rate of its inclusion, depends on the network cross-linking degree and its water swelling characteristics,

respectively. The kinetics of CEF release from the hydrogels used is typical to diffusional processes, a maximum rate being recorded in the first moments, followed by its stabilization at a practically constant value, over a wide time interval. The release rate of CEF became slower with increasing cross-linking degree of the interpenetrated network (Dumitriu et al. 2004).

Novel interpenetrating polymeric network microspheres of gellan gum and PVA were prepared by the emulsion cross-linking method. The prepared IPNs demonstrated a better mechanical property as compared to pure gellan gum, indicating the suitability of the IPNs for microsphere preparation. The cross-link density was significantly affected by the content of glutaraldehyde and gellan gum in the formulations. Carvedilol was successfully entrapped into the IPN matrix. Microspheres were spherical with smooth surfaces. The release of carvedilol was found to depend upon the extent of cross-linking of the matrix as well as the amount of gellan gum present in the matrix. Higher release rates were observed for microspheres with lower cross-link density and lower amounts of gellan gum in the matrix. No significant difference was observed in the diffusion coefficients in the simulated gastric and intestinal fluids (Agnihotri and Aminabhavi 2005).

Considering relatively fast drug release rate of Ca^{2+}/gellan beads in phosphate buffer solution, a novel glipizide-loaded bead system was developed through ionotropic gelation of gellan with trivalent Al^{3+} ions and covalent cross-linking with glutaraldehyde (GA) (Maiti et al. 2011). Following GA treatment, spherical Al^{3+}/gellan beads contracted leaving wrinkles on bead surface. A maximum of 97.67 % drug entrapment efficiency was achieved; however, GA treatment reduced the efficiency by 11.89 %. All the beads released only 10 % drug in acidic medium in 2 h; however, it was found to be 38–47 % for Al^{3+}/gellan beads and only 15 % for GA-treated beads in weakly alkaline medium. The drug release did correlate well with their swelling behaviors. An exponent value of n greater than 0.85 in Korsmeyer–Peppas equation signifies super case II transport mechanism (Ritger and Peppas 1987). The anomalous drug transport mechanism for GA-treated beads shifted to super case II transport since the value of diffusion exponent n was 1.27, where the polymer relaxation phenomenon was dominant. The drug was relatively stable, amorphous in the beads. Thus, both GA-treated and -untreated Al^{3+}/gellan beads could be useful carriers for the controlled oral delivery of glipizide.

In recent years, controlled drug delivery formulations and the polymers used in controlled drug delivery systems have become much more sophisticated, with the ability to do more than simply extend the effective release period for a particular drug. For example, intelligent or smart polymers play an important role in drug delivery since they may dictate not only where a drug is delivered but also when and with which interval it is released (Soppimath et al. 2002). So, a novel class of unsaturated gellan derivatives/*N*-isopropylacrylamide stimuli-responsive hydrogels was synthesized by free radical polymerization. Gellan gum was partially functionalized by esterification with maleic anhydride under various conditions. By copolymerization of this maleate polysaccharide with *N*-isopropylacrylamide, a known temperature-sensitive precursor, water-swollen hydrogel with interpenetrating polymer networks was obtained. Depending upon composition

and the nature of the base polysaccharide, the hydrogels showed different response rates to the external changes of temperature as well as pH. By changing the feed composition ratio of precursors and cross-linking agent (β-cyclodextrin acrylate or *N*,*N*′-methylenebisacrylamide, respectively), the phase-transition temperature (lower critical solution temperature) could also be adjusted near to the body temperature for biomedical and biotechnological applications (Hamcerencu et al. 2008).

Polymeric implants are one of the attractive devices for targeted drug delivery (Dash and Cudworth 1998). The polymeric matrix can protect drugs from conditions that could degrade or render the drug in the body inactive. Therefore, various polymers have been investigated in order to obtain an ideal drug delivery system that would allow ease of incorporation of drugs without affecting their bioactivity and delivery to the target site at a desired rate and exhibit biocompatibility when in contact with the tissue. An implant controlled-release system for methotrexate (MTX) delivery based on a polyion complex composed of chitosan and gellan was investigated (Kumar et al. 2008). Multilayered implant was prepared by using PVA, gellan, and chitosan. Two chitosan layers sandwiched the PVA–gellan layer, which acted as a methotrexate reservoir. Both in vitro and in vivo results indicate that MTX release is slow from implants. The results of the in vitro release of MTX from the implants showed that percent release rate decreased with increasing $CaCl_2$ concentration, which was attributed to the increase in cross-link density in the network. Chitosan coated PVA–gellan multilayered implants can represent an effective delivery system for the sustained release of MTX. Moreover, the potential for toxicity is low because of the extended period over which the release occurs. Also the implants showed good biocompatibility, which is essential for implant-based sustained delivery systems (Kumar et al. 2008).

5.3 Tissue Engineering

Modified gellan gum can be of potential of a suitable material for tissue engineering applications though some challenges still remain in the aspects of toughness, extensibility, and appropriate mechanical characteristics. A lot of work has been done taking advantage of the excellent characteristics of gellan gum in tissue engineering (Ferris et al. 2013a).

5.3.1 The Advantage of Gellan Gum for Tissue Engineering Applications

Gelation of GG, as described previously, is preceded by a conformational transition from coil to double helix, and association of these helices in junction zones is facilitated through either monovalent or divalent cations. Consequently, GG hydrogels may be formed at low concentrations of divalent cations or even in the presence of monovalent cations alone (Morris et al. 2012). This could be advantageous in tissue engineering applications, and it has been shown that GG can be cross-linked to form self-supporting hydrogel structures simply by the addition of

standard cell culture media with no added ions (Miyamoto et al. 1996). In addition, GG formed a gel on contact with tear fluid (Rupenthal et al. 2011), which is advantageous for ophthalmic drug delivery. GG in combination with hyaluronan was reported to be a candidate for short-term vitreous substitutes because of its transparency and similar wetting properties to that of vitreous (Suri and Banerjee 2006).

GG hydrogels are also stable during long-term culture in standard media and do not suffer from unwanted dissolution due to ionic exchange (De Silva et al. 2013). De Silva et al. (2013) found a steady mass loss during soaking GG gels for a much longer time of 28 days than 7 h; after that Hossain and Nishinari (2009) found a much greater chain release and erosion of GG gels at 10 °C although De Silva et al. examined the mass loss at the higher temperature (37 °C) of the surrounding medium. The difference in the period of the steady mass loss was due to the difference in cations: Hossain et al. used the potassium-type GG without further addition of cations, while De Silva et al. added calcium in the gel preparation. Taking into account the previous results reported by Hossain et al., De Silva et al. interpreted that there was a smaller amount of unassociated GG chains in their gels compared to that of the K–GG gels without added cations. Using murine dermal fibroblasts (L929), De Silva et al. examined the cell growth inhibition (CGI) of hydrogels. The percentage CGI was determined by the following equation:

$\mathrm{CGI} = (n_c - n_s)/n_c$, where n_s and n_c are the number of cells in the sample and media control dishes, respectively. Cell growth inhibition of hydrogels was found to be proportional to mass loss, that is, CGI values increased with increasing GG concentration in the degradation medium (De Silva et al. 2013).

In addition to these gelation properties, GG's excellent optical clarity could prove advantageous in analysis of encapsulated cells (Giavasis et al. 2000). It has been shown that GG scaffolds can be made porous using straightforward fabrication methods (Peña et al. 2010). Furthermore, GG appears not to inhibit polymerase chain reaction (PCR) analysis (Rath and Schmidt 2001) and is suitable as an injectable material (Du et al. 2012; Ferris and in het Panhuis 2009; Oliveira et al. 2010b).

A further attractive characteristic of GG for tissue engineering is the mechanical similarity to the elastic moduli of common tissue. The mechanical characteristics of GG depend on type (low acyl GG (LAGG) or high acyl GG (HAGG)) and concentration as well as the type and amount of physical cross-linker. Other strategies involve methacrylation of the GG chain followed by physical and/or chemical cross-linking (Shivkumar et al. 2006). These approaches can be used to tune the elastic modulus of GG hydrogels to that comparable with a wide range of human soft tissues such as the muscle, liver, and cartilage (Discher et al. 2005).

Finally the degradation behavior of GG can be controlled. There are a number of human enzymes such as lysozyme, amylase, and trypsin which are known to degrade common polysaccharides (Garrett and Grisham 2007). These enzymes are commonly found in tears, mucus, milk, and the stomach and degrade polysaccharides through hydrolysis. It has been reported that the enzymatic degradation of GG containing hydrogels resulted in a mass loss of 20 % and 30 % over 7 days with lysozyme and trypsin, respectively (Suri and Banerjee 2006).

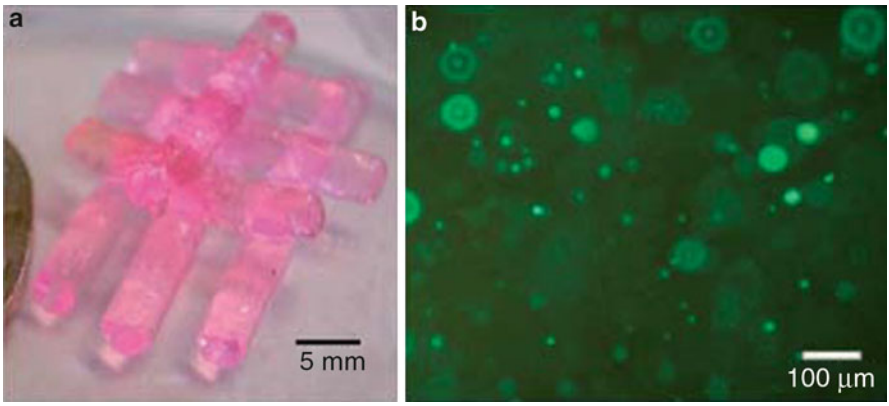

Fig. 23 (**a**) Gellan hydrogel cylinders produced by extruding 1 % w/v gellan solution into culture medium. (**b**) Calcein-stained rat bone marrow cells in gellan hydrogel after 10 days in culture (Smith et al. 2007)

5.3.2 Gellan Gum as Tissue Engineering Material

GG hydrogels could be used to encapsulate viable mammalian cells (Smith et al. 2007). GG could be cross-linked by the addition of cell culture media alone, owing to the gelation of GG at millimolar concentrations of divalent cations which are present in most media formulations, to form self-supporting hydrogels (Fig. 23a). This enabled a mild encapsulation process for rat bone marrow cells (Fig. 23b), which remained viable in the GG hydrogels for 21 days in culture.

Chondrocytes remain viable when encapsulated in GG hydrogels (Oliveira et al. 2010a) and exhibited extracellular matrix (ECM) production when implanted subcutaneously in nude mice (Oliveira et al. 2009a). Injectable delivery of these chondrocyte-laden gels was also investigated, taking advantage of the ability of GG to form a gel under physiological conditions (Oliveira et al. 2009b). GG hydrogels were also used to encapsulate oligodendrocyte-like cells within the center of a tubular structure fabricated by 3D extrusion printing of starch (Silva et al. 2010). GG can be methacrylated to introduce the possibility of photo-initiated cross-linking (Coutinho et al. 2010), thus enhancing the range of mechanical and degradation properties that can be tailored in GG hydrogels (Fig. 24). These materials have been studied for application as cellular or acellular artificial nucleus pulposus implants in the treatment of intervertebral disk degeneration (Silva-Correia et al. 2011, 2012; Pereira et al. 2011).

Physical parameters of GG hydrogels for cartilage applications can be optimized by blending low acyl and high acyl GG (Lee et al. 2011). Increasing the HAGG/LAGG ratio resulted in a decrease in the gel's stiffness, and that gels of 2 % (w/v) LAGG were most suitable for fibrocartilage applications. Blending GG with other types of biomolecules (e.g., polysaccharides and enzymes) for tissue engineering applications has also been considered (Ciardelli 2005; Bertoni et al. 2006; Cascone et al. 2001b; Patil et al. 2011; Coutinho et al. 2012; Barbani et al. 2012).

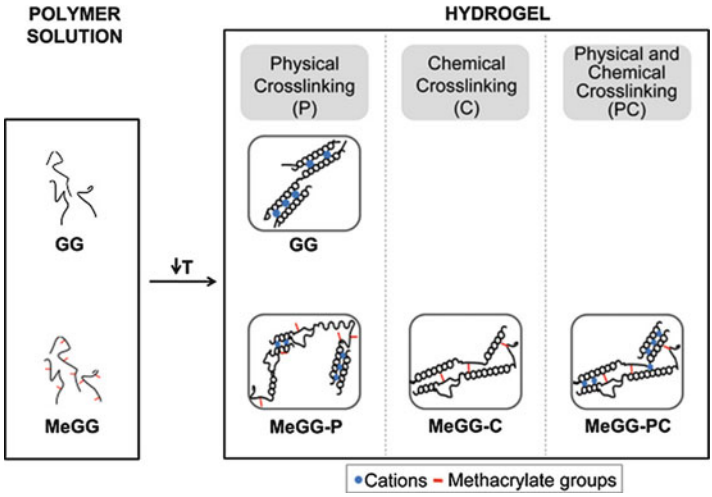

Fig. 24 Possible cross-linking mechanisms for GG, low-MeGG, and high-MeGG hydrogels (Coutinho et al. 2010)

GG is a relatively bio-inert material. This has been demonstrated through the lack of cell infiltration and angiogenesis observed when implanting GG hydrogels in vivo (Silva-Correia et al. 2012) and through evaluation of the behavior/response of anchorage-dependent cells encapsulated in GG hydrogels (Coutinho et al. 2010; Silva et al. 2010). Thus, application has so far been largely limited to anchorage-independent cells like chondrocytes (Gong et al. 2009; Oliveira et al. 2010a). Therefore, in order to function as a useful artificial ECM for anchorage-dependent cell types, GG must be modified. Previously, GG microspheres produced by a water-in-oil emulsion process have been covalently functionalized with gelatin through redox-mediated cross-linking to encourage the attachment of human dermal fibroblasts and human fetal osteoblasts (Fig. 25) (Wang et al. 2008). Photocrosslinkable variants of both GG and gelatin have also been combined in a novel double-network hydrogel with enhanced mechanical properties (Shin et al. 2012). More recently, GG hydrogels were modified with RGD-containing peptides to enhance interaction with encapsulated neural stem/progenitor cells (Fig. 26) (Silva et al. 2012). Another interesting development is the modification of GG with surfactants to function as a bio-ink for cell printing applications (Ferris et al. 2013b).

Although it is straightforward to prepare GG hydrogels with elastic moduli similar to that of tissue, matching the toughness and load tolerance of mammalian tissue is not. For example, LAGG and HAGG gels can be prepared with elastic moduli (kPa range) similar to that of the liver, fat, muscle, or cartilage (Coutinho et al. 2010; Discher et al. 2009). But the compressive stress at failure of these gels (kPa range) is orders of magnitude lower than tissues such as the cartilage (MPa range) (Coutinho et al. 2010; Kerin et al. 1998). In other words GG hydrogels are mechanically weak, which is a generally recognized drawback of hydrogel materials under consideration for tissue engineering.

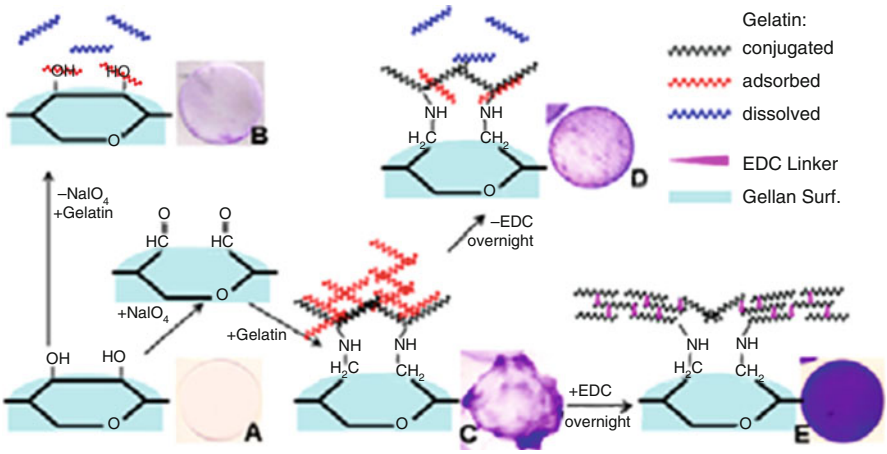

Fig. 25 Schematic diagrams of fabrication of gelatin-grafted-gellan (TriG) microspheres (Wang et al. 2008)

A number of strategies have been adopted to address the mechanical weakness and/or load intolerance. Hydrogels based on chemically/physically cross-linked methacrylated GG resulted in improvements in the magnitude of compressive stress at failure (up to 0.9 MPa) (Coutinho et al. 2010). It is not known if these gels would be able to recover from damage (load tolerance). Progress toward the latter has been made by building on the pioneering research on toughening gels by Gong et al. (Gong 2010; Gong et al. 2003; Haque et al. 2012). These gels exhibit excellent mechanical performance, for example, compressive stress at failure values of up to 60 MPa (Haque et al. 2012). The toughening of hydrogels is achieved using an interpenetrating polymer network approach which results in the so-called "double-network" (DN) hydrogels with mechanical properties that are significantly improved compared with either one of the parent networks (Gong 2010; Gong et al. 2003; Haque et al. 2012). The two polymer networks in the DN approach are chemically (covalently) cross-linked, and the toughening mechanism arises from efficient energy dissipation due to fragmentation of the first (brittle) network, thereby allowing the second (ductile) network to facilitate large deformations (Gong 2010; Haque et al. 2012). The DN approach has also been adopted for methacrylated GG in combination with methacrylated gelatin (Shin et al. 2012). The resulting DN gels exhibited compressive stress at failure values of close to 7 MPa. DN gels are extremely tough, but due to the irreversible, permanent fracture of the chemical cross-links (Gong 2010; Haque et al. 2012), DN gels are not able to recover from significant loading and have poor fatigue resistance.

Recently, it has been demonstrated that preparing IPN gels combining one network with reversible physical (non-covalent) bonds and one network with irreversible covalent networks results in gels that are tough but can recover from damage (Haque et al. 2011; Bakarich et al. 2012; Sun et al. 2012). For example, it was demonstrated that hydrogels consisting of ionically cross-linked LAGG and

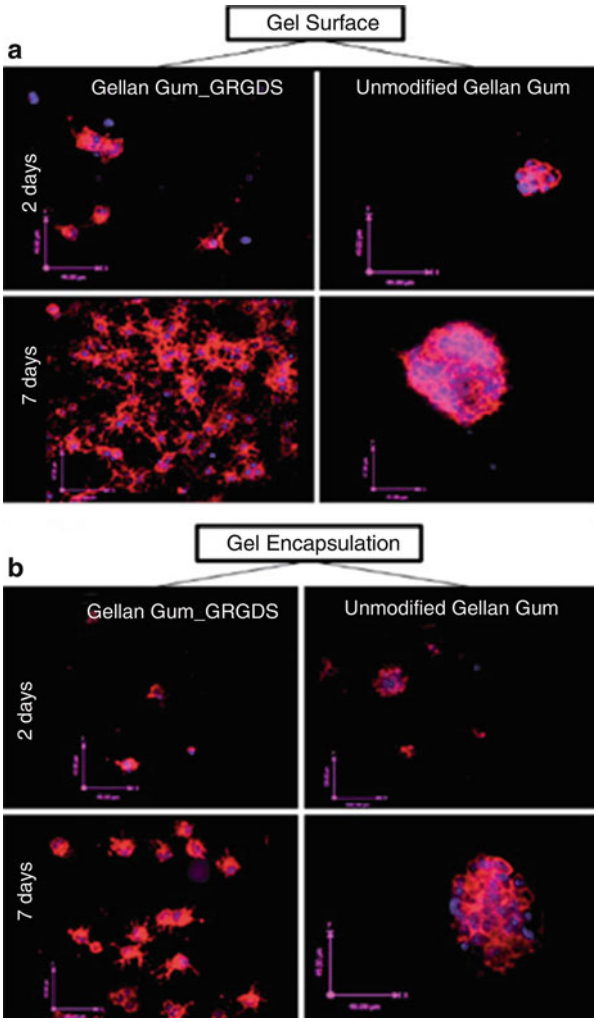

Fig. 26 Morphology and dispersion of neural stem/progenitor cells (NSPCs) on the gellan gum hydrogel modified with the cell-adhesive peptide (GG–GRGDS). Confocal analyses revealed substantial differences in NSPC morphology when cultured either (**a**) on the surface or (**b**) encapsulated within the GG–GRGDS versus unmodified GG gel. Cell spreading and visible cytoplasmatic extensions were only observed in the GG–GRGDS. In the unmodified GG, NSPCs proliferated as neurospheres. The cytoplasm was stained with the anti-F-actin/phalloidin (*red*) and nuclei counterstained with DAPI (*blue*) (Silva et al. 2012)

covalently cross-linked poly(acrylamide) exhibited double-network behavior, i.e., improved mechanical properties compared to their respective single-network hydrogels (Bakarich et al. 2012). These so-called ionic–covalent entanglement hydrogels exhibited self-recovery of 53 ± 4 % within 80 min from the first compressive cycle.

5.4 Other Applications in Biology and Environment

The dispersibility of carbon nanotubes (CNTs) in most common solvents is rather limited, but it is well known that this can be improved through the use of dispersants

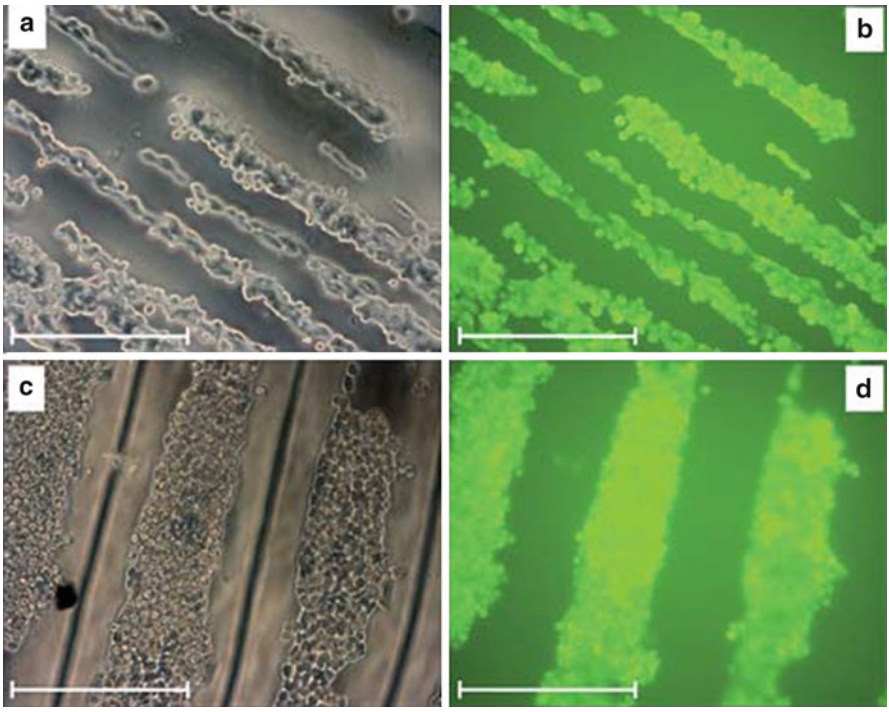

Fig. 27 Microscopy images of cells growing on optimal hydrogels (Ferris and in het Panhuis 2009). (**a**) and (**c**) Bright field microscopy images of adherent L-929 cells after 3 days of culture growing on hydrogels molded by VR1 and VR2 substrates, respectively; (**b**) and (**d**) fluorescent microscopy images of cells growing on hydrogels molded by VR1 and VR2 substrates, respectively, after 3 days of culture

such as surfactants and polymers (in het Panhuis 2006). Gellan gum is an extremely good dispersant for carbon nanotubes (CNTs) in aqueous solutions, able to disperse CNTs at concentrations as low as 0.0001 % w/v of gellan gum (in het Panhuis et al. 2007). Gellan gum hydrogels containing carbon nanotubes have been prepared. They are a promising candidate for biomaterials which are able to stimulate incorporated cells toward functional tissue formation. The ability to tailor the gelation temperature to be physiologically relevant suggests the possibility of cell-encapsulating or injectable scaffold systems. By altering hydrogel composition or incorporating topographical surface features, it demonstrated the ability to modify cell responses (Fig. 27). This could be implemented in the design of scaffolds for specific cell types. Furthermore, it demonstrated percolation of a CNT network in a gellan gum hydrogel, and it is hoped these composite systems will be developed further into a means by which electrical signals may be propagated through biomaterials (Ferris and in het Panhuis 2009).

It was demonstrated that these gellan gum/carbon nanotubes dispersions could be inkjet-printed onto flexible substrates to create transparent, conducting, thin films.

The resistance of the composite films under ambient conditions (12 MΩ) was found to be only one order of magnitude lower than that of gellan films (350 MΩ). Interestingly and contrary to most other water-soluble polymer composite materials, the resistance of these inkjet-printed gellan and gellan–CNT films decreased significantly (to 0.850 MΩ) in the presence of water vapor. In other recent works, the gel and carbon nanotube gel fibers prepared by polyelectrolyte complexation of gellan gum and chitosan exhibited similar behavior (Granero et al. 2008a, b). It can be explained as follows. Under ambient conditions only transport pathways through inter-nanotube junctions contribute to the current. Exposure to humid conditions enables transport through the additional polymer-dominated pathways resulting in the observed increased current. It was shown that cation mobility and polymer conformation play an important role in this water-sensitive behavior. In particular, it was suggested that the current contains an electrical contribution from electron transport through the nanotubes and an ionic contribution due to cations. It was shown that increasing the nanotube mass fraction increases the relative contribution to the current of the electron transport over ion mobility. Moreover, it was observed that there was a variable ionic contribution to the current rather than the expected ionic contribution being a constant value. The ionic contribution was found to scale exponentially with increasing nanotube mass fraction (in het Panhuis et al. 2007).

Printing is the deposition of a material (ink) onto a substrate in order to produce a desired structure. Specialist technologies are increasingly requiring advanced material properties from the printed structures, such as flexible, stretchable conductors and sensors (Siegel et al. 2010; Rogers et al. 2010; Yamada et al. 2011). There is a drive to develop new printing processes to further extend the sophistication and the capabilities of the printed structures. Extrusion printing (also known as direct writing and solid free-form fabrication) is a process that involves the pressurized delivery of an ink through a capillary onto a moving substrate (Lewis 2004; Calvert and Crockett 1997). Relative to other printing processes, extrusion printing offers greater flexibility in the ink materials and a large range in the printed structure's minimum feature sizes (or resolution), from the micro- to the macroscale (Calvert et al. 1997). Extrusion printing can also manufacture three-dimensional, structurally robust structures and embed material into substrates (Mire et al. 2011). This functionality makes up for some of the drawbacks (e.g., resolution) compared to other methods such as inkjet printing (Mire et al. 2011; Calvert 2001; Singh et al. 2010). Carbon nanotube networks in biopolymer solutions are explored as potential ink systems for the extrusion printing of conducting structures. The biopolymer gellan gum is found to act as an excellent dispersant of multiwalled carbon nanotubes and has the appropriate flow properties to act as a thickener for the controlled dispensing of carbon nanotube networks. Absorbing substrates are found to improve the resolution and the flexibility of the printed structures. These printed conducting carbon nanotube networks exhibit interesting mechanical and electrical characteristics, which are applied to demonstrate their actuating and strain gauging capabilities (Pidcock and in het Panhuis 2012).

Currently gold and silver nanoparticles are being explored for drug delivery and other biomedical applications; therefore, it is necessary to develop a novel and

green synthesis process for these nanoparticles. Recently, gellan gum has been employed as a reducing and stabilizing agent for the synthesis of gold and silver nanoparticles (Dhar et al. 2008, 2011, 2012). These nanoparticles display greater stability to electrolyte addition and pH changes relative to the traditional citrate and borohydride-reduced nanoparticles. Furthermore, these gellan gum capped/reduced gold nanoparticles were further conjugated with sophorolipids which again were accessed through a biochemical transformation of a fatty acid. These sophorolipid-conjugated gellan gum reduced/capped gold nanoparticles showed greater efficacy in killing the glioma cell lines and more prominently glioma stem cell lines. The cytotoxic effects became more prominent once the doxorubicin hydrochloride was also conjugated to these gold nanoparticles (Dhar et al. 2011).

Hazardous waste compounds such as gasoline cause a serious pollution of the aquifer, and bioaugmentation, the introduction of microorganism which can degrade these harmful compounds, has been expected to be a promising solution to solve the problem of contamination of groundwater. To ensure the viability of microorganism and transport to a wide area, the microencapsulation method is used. Moslemy et al. (2002) encapsulated an enriched bacterial consortium in gellan gum microbeads (16–53 μm) and dispersed encapsulated cells within the porous soil matrix of saturated soil microcosmos and found that gasoline hydrocarbons were degraded effectively.

Heavy metals released into the environment have posed a significant threat to the environment and public health because of their toxicity and persistence in environment and, therefore, must be removed from water. Various conventional processes (precipitation, electrochemical processes, and/or membrane processes) have been employed to treat industrial effluents (Gavrilescu 2004). However, these applications are often limited by technical and economical issues.

The integrated process, which couples magnetic separation with surface complexation adsorption and ionic exchange, is especially effective in the remediation of heavy metals (Ngomsik et al. 2006; Yantasee et al. 2007). This system has several advantages that the process does not generate secondary waste and the materials involved can be recycled and facilely used on an industrial scale, and furthermore that the magnetic particles can be tailored to fix specific metal species in water, waste, or slurries (Ngomsik et al. 2006; Li et al. 2008). So, a composition of gellan gum and Fe_3O_4 nanoparticles was employed as a unique adsorbent for heavy metal removal in aqueous solutions. Adsorption kinetics analysis showed that the adsorption capacities were in an order of $Pb^{2+} > Cr^{3+} > Mn^{2+}$. Different experimental parameter studies indicated that adsorbent dosage, initial metal concentration, temperature, and initial pH played important roles in adsorption process (Wang et al. 2009).

6 Conclusions

Gellan gum has been one of main bacterial polysaccharides with various applications such as additives in food and cosmetic industry. According to current reports, chemically modified gellan gum offers great opportunities as a material for tissue

engineering though its toughness and extensibility, appropriate mechanical characteristics, suitable degradation behaviour should be further improved. Many further advances will be made before gellan gum comes close to realising its full potential. These will undoubtedly include identification of new areas of application, and formulation of novel or improved products. An additional approach, however, might be informed manipulation of acyl content, by variant cultures or controlled deacylation, to tailor the properties of gellan to the requirements of specific applications. Although the feasibility of this approach has already been demonstrated, it has not yet been exploited on a commercial scale.

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Bioactivity and Applications of Polysaccharides from Marine Microalgae

54

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Contents

Abstract

Marine microorganisms have been under research for the last decades, as sources of different biocompounds, each with various applications. Polysaccharides (**PSs**) are among these chemicals being produced and released by marine microalgae. These are very heterogeneous, including cyanobacteria and eukaryotic microalgae from several divisions/phyla, each of which with different characteristics. The **PSs**, sulfated or not, that they produce have already proved to be promising agents in various fields, such as food, feed, pharmaceutical, and biomedical. They can also be applied in wastewater and/or soil treatment and in some engineering areas, as naval engineering.

After a brief introduction on the general types of biopolymers produced by marine microalgae and cyanobacteria, this chapter starts by presenting the species of these microorganisms and the types of **PSs** they produce, as well as

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the respective chemical composition; goes into the production of **PSs** and the effect of specific compounds; and focuses on the physicochemical properties of these **PSs** and their composition and structure, approaching the rheological properties relevant for their functions and behavior. The bioactivity of **PSs** and their applications are, next, presented, including therapeutic applications based on their antiviral and antibacterial activities, antioxidant properties, anti-inflammatory and immunomodulatory characteristics, antitumoral activity, and antilipidemic and antiglycemic properties, among others. The potential use of **PSs** from marine microalgae as it is or incorporated in health foods is also considered. The mechanisms behind their antiviral and antibacterial activities are explained. Toxicological and safety issues are also disclosed, and there is a brief mention of the bioavailability of **PSs** from microalgae. The chapter ends by listing some preclinical studies with this type of polymers.

Keywords

Marine microalgae • Polysaccharides • Sulfate (exo)polysaccharides • Health foods • Bioactivity-Antioxidant • Antiviral • Antitumoral • Immunomodulators • Toxicity

Abbreviations

arab	Arabinose
CaSp	Calcium spirulan
CB	Cyanobacterium(a)
EC_{50}	The molar concentration of a drug that produces 50 % of the maximum possible response for that drug
ED_{50}	In vitro or in vivo dose of drug that produces 50 % of its maximum response or effect
fru	Fructose
fuc	Fucose
GAG	Glycosaminoglycan
gal	Galactose
galAc	Galacturonic acid
glcAc	Glucuronic acid
glc	Glucose
IC_{50}	The molar concentration of a drug which produces 50 % of its maximum possible inhibition
ID_{50}	In vitro or in vivo dose of a drug that causes 50 % of the maximum possible inhibition for that drug
man	Mannose
MW	Molecular weight
NaSp	Sodium spirulan
NO	Nitric oxide
PS	Polysaccharide
rham	Rhamnose

sEPS	Sulfated exopolysaccharide
sPS	Sulfated polysaccharide
xyl	xylose

1 Introduction

Polysaccharides (**PSs**) and oligosaccharides can be synthesized by microorganisms; some of them are even secreted out into the environment (or culture medium), from which they are easily extracted. **PSs** have been studied for a long time due to their characteristics, especially their conformation, which is reflected in their chemical behavior and, therefore, in their wide range of applications. However, the knowledge on their complete composition and structure is still taking the first steps, and despite the applications that might appear after a solid understanding of their structure and conformation, these applications could even be extended to the medicine field. As a matter of fact, there is some difficulty on studying these polymer chains because of the diversity and distribution of simple sugars (mono-, di-, and oligosaccharides) along the **PSs** chains and whether these chains are linear or ramified. Therefore, the analysis of the **PSs** structures and, consequently, their applications is a real challenging task. Thus, the analysis of these polymer chains has been limited to oligosaccharides obtained by hydrolysis of high molecular polymers and to *X-ray* diffraction studies of **PSs** gels (Eteshola et al. 1998).

Among the **PSs** produced by microalgae and cyanobacteria (thereafter referred to as microalgae), especially marine species, only the **sPS** from *Gyrodinium impudicum* is a homopolymer of **gal** (Yim et al. 2007) and perhaps a cell wall **PS** from *Chlorella vulgaris*, a β-(1,3)-glucan (Nomoto et al. 1983), composed of D-**glc**; the **PSs** from all the other marine unicellular algae are heteropolymers, mostly constituted of **xyl**, **gal**, and **glc** in different proportions, but some other neutral sugars can also be constituents of the **PS**, as it is the case of **rham**, **man,** or **fuc**, and also some methyl sugars (Table 1). But in spite of this similarity in monosaccharide composition, the types of sugar themselves and the glycosidic bonds between each molecule are two of the characteristics that establish all the differences between the properties of **PSs** found in microalgae. Either the composition of monosaccharides and their distribution or the percentage in sulfate and uronic acids greatly determines the rheological behavior of the **PSs**, whose aqueous solutions can be highly viscous, as it happens with the **EPSs** from the marine red microalgae, or they may not present any apparent viscosity, as it is the case of the glucuronorhamnan from *C. vulgaris*.

2 Marine Sources

In the last decades the interest in products that have a marine origin, mainly seaweeds and microalgae, and also in the compounds they produce is growing rapidly. Nevertheless, microalgae have an advantage over macroalgae: they are easy to grow and manipulate, and harvesting does not depend on the climate or

Table 1 Marine species of microalgae producing **PS**s

Microalgae/ cyanobacteria	Group	Type of polysaccharide	Main neutral sugars	References
Microalgae *Cylindrotheca closterium* *Navicula salinarum* *Phaeodactylum tricornutum* *Haslea ostrearia* *Nitzschia closterium* *Skeletonema costatum* *Chaetoceros* sp. *Amphora* sp.	Diatoms	**sPS** **sPS** **sEPS** **EPS** **EPS** **EPS** **EPS**	xyl, glc, man, rham glc, xyl, gal, man glc, man, xyl, rham	Staats et al. 1999; Pletikapic et al. 2011 Staats et al. 1999 Guzman et al. 2003; Ford and Percival 1965a, b Rincé et al. 1999 Penna et al. 1999 Chen et al. 2011
Chlorella stigmatophora *C. autotrophica* *C. vulgaris* *Dunaliella salina* *Ankistrodesmus angustus* *Botryococcus braunii*	Chlorophytes	**sPS** **sPS** **PS** β-(1,3)-glucan **EPS** **EPS** **EPS**	glc, xyl, **fuc** rham, gal, arab 2-*O*-methyl-rham glc gal, glc, xyl, fru gal, **fuc**, glc, rham	Guzman et al. 2003 Guzmán-Murillo and Ascenci 2000 Ogawa et al. 1997, 1999; Nomoto et al. 1983 Mishra et al. 2011 Chen et al., 2011 Allard et al. 1987 Allard and Casadeval 1990
Tetraselmis sp.	Prasinophyte	**sPS**		Guzmán-Murillo and Ascencio 2000
Isochrysis sp.	Prymnesiophyte/ haptophyte	**sPS**		Guzmán-Murillo and Ascencio 2000
Porphyridium sp.	Rhodophytes	**sPS**	xyl, gal, glc	Geresh and Arad 1991; Dubinsky et al. 1990; Arad 1988
P. cruentum *P. purpureum* *Rhodella reticulata* *R. maculata*		**sPS** **sPS** **sPS**	xyl, gal, glc, 3-*O*-methyl-xyl xyl, rham 3-*O*-methyl-rham 4-*O*-methyl-	Garcia et al. 1996; Kieras 1972; Raposo et al. 2014; Gloaguen et al. 2004; Geresh et al. 2002a; Dubinsky et al. 1992 Radonic et al. 2010 Geresh and Arad 1991; Dubinsky et al. 1992 Evans et al. 1974;

(*continued*)

Table 1 (continued)

Microalgae/ cyanobacteria	Group	Type of polysaccharide	Main neutral sugars	References
			gal xyl, gal, glc 3-*O*-methyl-xyl	Fareed and Percival 1977
Cochlodinium polykrikoides *Gyrodinium impudicum*	Dinoflagellates	**sPS** **sPS**	man, gal, glc gal	Hasui et al. 1995 Yim et al. 2007
Cyanobacteria *Aphanothece halophytica*	Cyanophytes	**EPS**	glc, **fuc**, man, arab	Li et al. 2001
Arthrospira platensis *Anabaena*, *Gloeothece*, *Nostoc* *Aphanocapsa*, *Phormidium*, *Synechocystis*, *Cyanothece*		**EPS** s-Spirulan **sPS**	gal, xyl, glc, fru rham, **fuc**, glc 3-*O*-methyl-rham	Radonic et al. 2010; Hayashi et al. 1996b; Martinez et al. 2005 Hayashi et al. 1996b; Senni et al. 2011; Lee et al. 2000 Senni et al. 2011

Adapted from Raposo et al. (2013)

season. Marine microalgae do not need much for culturing: a simple medium of seawater, with a source of nitrogen, phosphate, iron, magnesium, and some minor salts, is the only requirement to produce them. Their culture can be easily controlled and, hence, the properties and physicochemical characteristics of the biocompounds they produce, such as the polysaccharides, can be maintained all over different cultures.

2.1 Marine Unicellular Algae Producing PSs

Some marine/brackish species are already produced commercially, as it is the case of *Arthrospira* (*Spirulina*) *platensis*, *Dunaliella salina*, *Isochrysis galbana*, *Nannochloropsis salina*, *Phaeodactylum tricornutum*, and *Porphyridium cruentum* (Fig. 1), either for their biomass and/or extracts or the compounds they produce. In addition, many other species are known to produce and secrete out **PSs** into the culture medium (Table 1), **EPSs**, which can be, or not, sulfated polysaccharides (**sPSs**) these **EPSs** show properties that go from application as antiviral agents to inclusion in health foods. But these marine microalgae are so diverse (Table 1) that it seems useful to locate their taxonomic positions and present some of their characteristics.

All diatoms belong to the class Bacillariophyceae, which includes organisms with round cells (Centrophycidae) and organisms with elongated cells (Pennatophycidae). *Chaetoceros* and *Skeletonema* belong to the first group; *Amphora*, *Cylindrotheca*, *Haslea*, *Navicula*, *Nitzschia,* and *Phaeodactylum* (Fig. 1-V) are included in the second group. The main characteristic of these unicellular organisms is the presence of a silicate ornamented two-piece frustule surrounding the protoplast. Their brownish color comes from the large quantities of xanthophylls (fucoxanthin, diatoxanthin, diadinoxanthin, neoxanthin), but they also possess chlorophylls a and c and α- and β-carotenes. Their main reserves are lipids, leucosin (or chrysolaminarin, a β-(1,3)-linked and β-(1,6)-linked glucose polymer) being the second main reserve.

Isochrysis (Fig. 1-IV) is a flagellated organism belonging to the class Prymnesiophyceae (or Haptophyceae). These golden-colored unicellular algae also have chlorophylls a and c, β-carotene, and xantophylls fucoxanthin, diatoxanthin, diadinoxanthin, and echinenone. They usually present two flagella and one smooth haptonema (hence the name of the class). Their main reserve compound is leucosin. Prymnesiophyceae and Bacillariophyceae are two classes of the phylum Chromophyta.

Another diverse group of algae is phylum Chlorophyta. As it happens with the plants, the microalgae members of Chlorophyta are green in color due to the high quantities of chlorophylls a and b. But α- and β-carotene and xantophylls (neoxanthin, lutein, violaxanthin, and zeaxanthin) are also present. Their reserve is mainly starch. This is a very diverse group, including macro- and microalgae. Only two classes are referred to in this chapter: Prasinophyceae, to which *Tetraselmis* belongs, and Chlorophyceae, the latter includes *Chlorella* (Fig. 1-II), *Ankistrodesmus*, and *Botryococcus braunii* (Fig. 1-I), all of them being Chlorococcales.

Porphyridium and *Rhodella* are two genera from the phylum Rhodophyta. This is the group that includes red macro- and microalgae. The main photosynthetic pigments are chlorophylls a and d, but their red color is associated mostly to the phycobiliproteins phycocyanin, allophycocyanin, and phycoerythrin. Lutein is the main xanthophyll. *Porphyridium* belongs to the class Porphyridiophyceae, order Porphyridiales, and family Porphyridiaceae; *Rhodella* is included in the class Rhodellophyceae, order Rhodellales, and family *Rhodellaceae*. However, there are still some organisms with different scientific names, such as *Dixionella grisea*, *Rhodella reticulata*, and *Porphyridium purpureum* and *P. cruentum* (Fig. 1-VI).

Both *Cochlodinium* and *Gyrodinium* are dinoflagellates that belong to the phylum Pyrrophyta, class Dinophyceae, and order Gymnodiniales. The pigments that characterize dinoflagellates are chlorophylls a and c2, β-carotene, and xantophylls piridinine, dinoxanthin, diadinoxanthin, diatoxanthin, and neodinoxanthin; fucocyanin is the main pigment responsible for the brownish color of pyrrophytes; starch and lipidic droplets are the main reserve substances. Dinoflagellates are very particular organisms, many of them produce highly toxic compounds (dinotoxins), the most known being saxitoxins and gonyautoxins (paralytic shellfish toxins or PST), two groups of carbamate alkaloid neurotoxins, brevetoxins (another group of neurotoxic shellfish toxins or NST), and the diarrheic shellfish toxin okadaic acid (Camacho et al. 2007; Wang 2008). These toxins affect all marine organisms' and also humans' lifes as

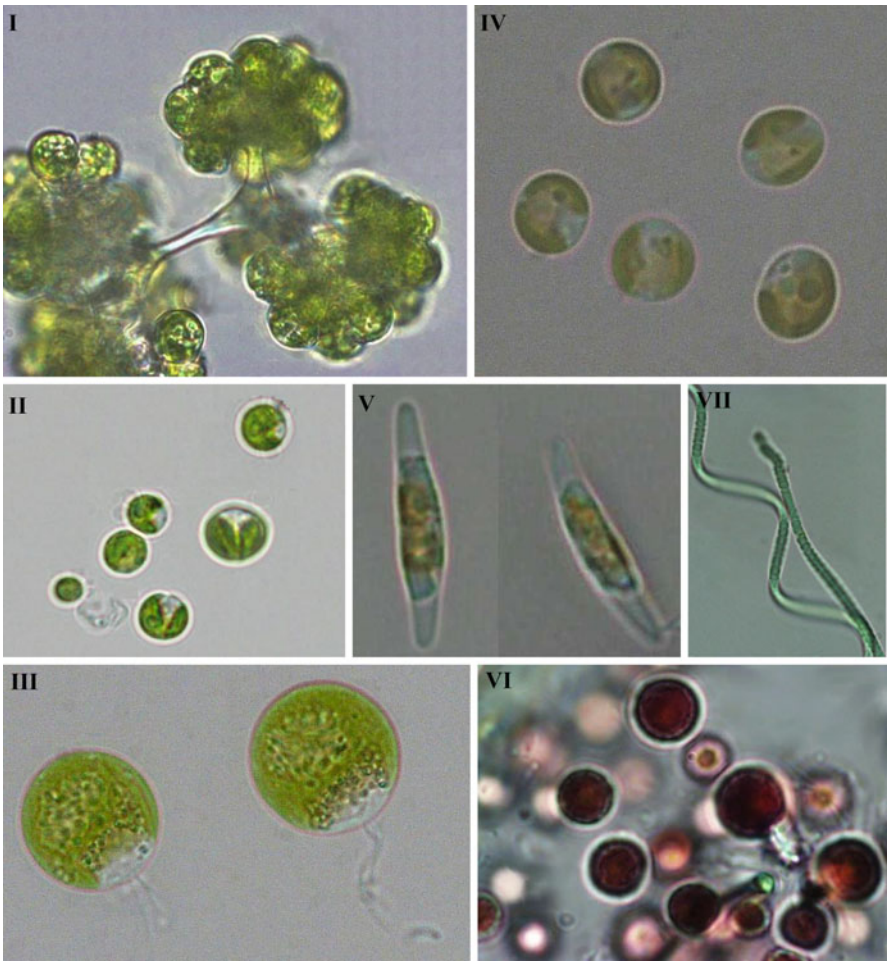

Fig. 1 Some of the microalgae cited in this chapter: **I**, *Botryococcus braunii*; **II**, *Chlorella vulgaris*; **III**, *Dunaliella salina*; **IV**, *Isochrysis galbana*; **V**, *Phaeodactylum tricornutum*; **VI**, *Porphyridium cruentum*; **VII**, *Arthrospira platensis*

seafood consumers. The red toxic tides are due to a high accumulation (or bloom) of flagellated dinophyceae, the red color coming from the remarkable accumulation of carotene.

Cyanophyta is a group of prokaryotic organisms that is most of the times studied along with microalgae (eukaryotic organisms). Cyanophytes are unicellular, solitary, or colonial organisms. This phylum includes a class, Cyanophyceae, with either filamentous or nonfilamentous structures, distinction of subclasses being based on hormogonia formation. *Aphanocapsa*, *Aphanothece*, *Cyanothece*, *Gloeothece*, and *Synechocystis* do not form hormogonia and, therefore, they are included in the subclass Coccogonophycidae, order Chroococcales; *Anabaena*,

Table 2 Percentage of sulfate, protein, and uronic acids in polysaccharides from different marine microalgae

Microalgae/ cyanobacteria	Sulfate (%)	Protein (%)	Uronic acids (%)	References
Microalgae				
Porphyridium sp.	4–14.6	1–5.5	7.8–18	Geresh and Arad 1991; Sun 2010; Arad et al. 1985, Gloaguen et al. 2004; Raposo et al. 2014
Rhodella sp.	8	6	5–7.8	Geresh and Arad 1991; Badel et al. 2011a
B. braunii			24	Fernandes et al. 1989
C. stigmatophora	7.8–9.4		3.7–9.0	Guzman et al. 2003
C. vulgaris	–	–	14	Ogawa et al. 1999
P. tricornutum	7.5–13.3		1.4–6.3	Guzman et al. 2003
C. closterium	0–10.9	7.7–9.2	4.8–21.0	Staats et al. 1999
N. salinarum	6.3–11.5	0.5–4.9	7.7–8.0	Staats et al. 1999
C. polykrikoides	7–8		(a) presence	Hasui et al. 1995
G. impudicum	10.3		2.9	Yim et al. 2007
Cyanobacteria				
A. platensis	5–20	6	7–14.4	Lee et al. 2000; Trabelsi et al. 2009
(s-Spirulan)	3.24–5.7		15–16.5	Hayashi et al. 1996b; Lee et al. 1998, 2000
A. halophytica	–	–	14	Li et al. 2001

Adapted from Raposo et al. (2013)

Arthrospira (Fig. 1-VII), *Nostoc*, and *Phormidium* belong to the subclass Hormogonophycidae, order Nostocales/Oscillatoriales, whose filaments are not ramified or, if they present branches, these are false. Only one chlorophyll (chlorophyll a) and phycocyanin are their main pigments, but they also contain carotenes and phycoerythrin (Table 2).

2.2 Production of the Polysaccharides: Influence of Specific Compounds

The production of **EPSs** and their composition depend on the algal species; on the strain; on the composition and nutrient status of the culture medium, namely, the N source (Banerjee et al. 2002), the N/P ratio, and the deficiency in silicon (for diatoms); and on the culture growth phase. Some microalgae produce large amounts of **EPSs** during the stationary phase, but some others increase the yield and continue to release even during the exponential phase of growth (Ramus and Robins 1975), when synthesis of biocompounds is more active, or even during both the growth phases, depending on the culture conditions (Penna et al. 1999).

Glyoxylate is one of the compounds that can positively influence the production of **EPSs** (Bergman 1986). In *A. cylindrica*, *C. capsulata*, and *Scenedesmus obliquus*, the addition of glyoxylate to the culture medium enhanced the yield of **EPSs**. One explanation could be the metabolization of glyoxylate into serine via glycine, a process associated to photorespiration. Glyoxylate, thus, induced some changes in the metabolism of carbon, increasing its relative yield and, therefore, increasing intracellular **PSs** and the release of soluble **EPSs** (Bergman 1986; De Philippis et al. 1996; Liu et al. 2010). However, in some microalgae, the **PS** is secreted out into the culture medium only when N metabolism is not affected. As a matter of fact, after being exposed for a short period to glyoxylate, the concentration of **EPSs** produced by *C. capsulata* increased by 43 % (De Pilippis et al. 1996). Nevertheless, nitrogen starvation had also proved to induce an overproduction of carbohydrates, via an alternative pathway, with the consequent release of **PSs**, not only in other cyanobacteria but also in microalgae (de Phillipis et al. 1993; Arad et al. 1992). In addition to glyoxylate, some other substances can interfere with the production and release of **PSs**. For example, an increase of **EPS** can be induced by a magnesium shortage (de Phillipis et al. 1991; Raposo et al. 2014) or by higher ion concentrations (Raposo et al. 2014), depending on the culture medium and species of microalga. The ratio N/P and a deficiency in silicon also influence the production and release of **PSs** – while a high ratio N/P induces an increase in the **EPS** from *N. closterium*, *S. costatum* and *Chaetoceros* produce high quantities of **EPS** under low N/P ratios (Penna et al. 1999).

3 Biochemical Composition and Physical Properties

Carbohydrates represent the major group of compounds synthesized by microalgae and include some of the substances under research for the last decades because of their physicochemical and biological properties and promising applications, even in medicine. But it is well known that the biological activities of **PSs** are closely related to the chemical composition and structure of the polymers, these factors being also the reason for their physicochemical behavior. Some of the characteristics that must be taken into account are their molecular weight, as large molecules are difficult to transfer across membranes in order to carry out their specific functions, and their sulfate and uronic acid content (or other constituents that can give the polymers their anionic and acidic properties), as these components seem to have great influence on their biological activity and applications. The number of monosaccharides, the type of linkages and distribution in the molecule, the conformation and type of chains (linear or ramified), and the existence of some other chemical groups (such as amino acids, proteins, or nucleic acids) that can be (non)covalently linked to the **PS** chains are other features that are worthy to be evaluated, along with the rheological properties and resistance to digestion, either acidic or enzymatic.

The composition of the **PSs** may differ due to the method used for the extraction and hydrolysis. Sometimes a single strong step of acidic hydrolysis is used; some other times the sugar profile is obtained by means of a multistep hydrolysis

associated to an ion exchange fractionation (Dubinsky et al. 1992). The fractionation and centrifugation can help to obtain different polymers, which can be separated from the initial **PS**, as they have different sedimentation coefficients (Kieras and Chapman 1976). In *Porphyridium*, for example, one of the fractions obtained by the elution with urea showed to be xylan, **xyl** and **glcAc** being the main constituents (75 % and 13 %, respectively) (Geresh et al. 1992). In *R. reticulata*, a similar fraction was obtained by the same technique, **xyl** and **glcAc** also being the predominant constituents (Dubinsky et al. 1992). Cleavage of the **PS** molecule can also be attained by enzymatic action by **PS**-lyases (EC4.2.2.-) and **PS**-hydrolases (EC3.2.1.-), endo-and exoenzymes, but the process can be time-consuming (Badel et al. 2011b) due to all the techniques that have to be employed. These researchers, however, developed a new promising method by adapting the Biofilm Ring Test® (or BRT®) technique used to degrade the **PS**. They applied the BRT in microplate assays and associated the BRT® to the biofilm index (BFI), which corrects some of the discrepancies between images of the former technique, before and after the magnetic treatment of particles (Badel et al. 2011b).

3.1 Structure

Within the group of **PSs**, not only the intracellular and the cell wall **PSs** but also and mainly the exo- or extracellular polysaccharides (**EPS**) will be focused in this work. Among all these polymers, only the **sPS** of *G. impudicum* is a homopolymer of **gal** (Yim et al. 2007), a galactan, and perhaps the cell wall **PS** of *C. vulgaris*, a *β*-(1,3)-glucan, composed of **glc**; the **EPSs** from all the other marine microalgae are heteropolymers of **gal**, **xyl**, and **glc** in different proportions. Other sugars can also be constituents of the **PSs**, such as **rham**, **fuc**, **fru**, and some unusual methyl sugars. The types of glycosidic linkages are described only for some of the **PSs** produced by microalgae. This is the case of the **EPS** from *A. halophytica* – most of the linkages are 1,3-type (1,3-linked **glc**, 1,3-linked **fuc**, 1,3-linked **arab**, 1,3-linked **glcAc**), but 1-linked **glc** and 1-linked **glcAc** (Fig. 2a) can also be found, as well as 1,2,4-linked **man** and 1,3,6-linked **man** (Li et al. 2001). In the **CaSp** of *A. platensis*, the linkages and monosaccharides of the backbone structure are usually 1,3-linked **rham** and 1,2-linked 3-*O*-methyl-**rham** (acofriose) (Lee et al. 1998). 2,3-di-*O*-methyl-**rham** and 3-*O*-methyl-**xyl** are the monosaccharides in the nonreducing end. Besides D-**xyl**, D-**glc,** and L- and D-**gal**, the main neutral sugars, the **EPS** from *P. cruentum* has also small amounts of 3-*O*-methyl-**xyl**, 3-*O*- and 4-*O*-methyl-**gal**, and 2-*O*-methyl-**glcAc** (Percival and Foyle 1979). This type of monosaccharide is also part of the glucuronorhamnoglycan, or glucuronorhamnan (White and Barber 1972; Ogawa et al. 1999) of *Chlorella*, as 2-*O*-methyl-L-**rham** and 3-*O*-methyl-L-**rham** (or acofriose) (Ogawa et al. 1997); these methylated **rham** sugars seem to appear only in some green algae. 2-*O*-methyl-L-**rham** was firstly reported by Ogawa et al. (1997) to be part of the **PS** of *Chlorella*, but, in fact, it was also identified in *A. platensis* (Collins and Munasinghe 1987). Despite seeming to be a characteristic of *Chlorella* (Ogawa et al. 1997), 3-*O*-methyl-L-**rham** was also identified in *B. braunii* and *A. platensis* (Lee et al. 1998;

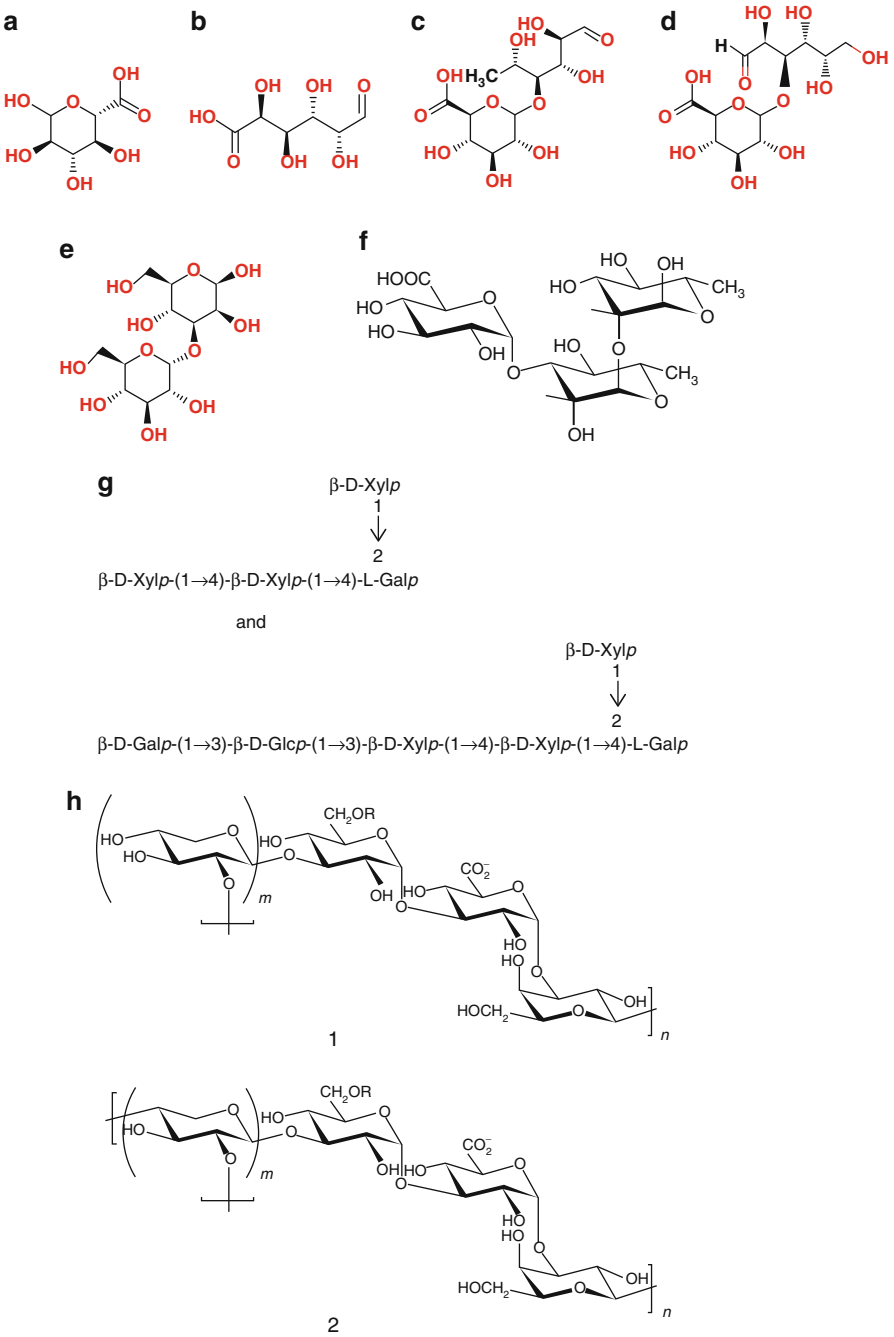

Fig. 2 Some of the components already identified for the **PSs** produced by marine microalgae: (**a**) D-glucuronic acid; (**b**) D-galacturonic acid (http://www.chemspider.com; accessed on 07-04-14);

Collins and Munasinghe 1987). *B. braunii* also present other less common methyl sugars: 3-*O*-methyl-**fuc** and 6-*O*-methyl-hexose besides **fuc** (Banerjee et al. 2002). Besides **rham** (52.3 %), the main neutral sugar, and other minor monosaccharides (**fuc**, **glc**, **arab**), **CaSp**, another **PS** of *A. platensis*, presents small amounts of 2,3-di-*O*-methyl-**rham** and 3-*O*-methyl-**xyl** (Lee et al. 1998). The positions of sulfate in the exocellular glycan of *P. cruentum* were identified (Archibald et al. 1981) in the **glc** and **gal** residues, as D-galactopyranose 6-sulfate, D-glucopyranose 6-sulfate, and D-galactopyranose 3-sulfate.

If the composition of monosaccharides is indicated for most of the **PSs** by several researchers, further information for higher levels of organization of the polymers is scarce and only for a couple of microalgae. Only some di- and oligosaccharides are described, some of them are characteristic for the microalgae from which the **PS** was obtained. White and Barber (1972) and Ogawa et al. (1998, 1999) advanced in the structure of the glucuronorhamnan of *Chlorella* with the identification of the disaccharide 3-*O*-*α*-D-glucopyranuronosyl-L-rhamnopyranose (or glucoronosyl-**rham**) (Ogawa et al. 1998) (Fig. 2c) and the acidic trisaccharide *α*-D-glucopyranuronosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-rhamnopyranose (or glucoronosyl-rhamnosyl-**rham**, $C_{18}H_{30}O_{15}$, Ogawa et al. 1999) (Fig. 2f), whose molecular weight was found to be 73 kDa, and with the determination of the respective structures. Besides the aldobiuronic acid 3-*O*-(*α*-D-glucopyranosyluronic acid)-L-galactopyranose (Fig. 2d), two different heterosaccharides were found to be part of the structure of the **EPS** from *P. cruentum*. These two oligosaccharides were identified after digestion of the **EPS** (Pignolet et al. 2013), whose complete structure was established by Gloaguen et al. (2004) (Fig. 2g, h), who also found the monosaccharide composition and distribution, their absolute configuration in the molecules, and that the oligosaccharide 1 was part of the oligosaccharide 2. Hence, they found the unusual presence of both L- and D-**gal** isomers in the **EPS** from *P. cruentum*, a common characteristic of the red algae **PS**, besides having confirmed the presence of the aldobiuronic acid. **EPS** from *P. cruentum* can, eventually, present two other aldobiuronic acids (3-*O*-(2-*O*-methyl-*α*-D-glucopyranosyluronic acid)-D-galactopyranose and (3-*O*-(2-*O*-methyl-*α*-D-glucopyranosyluronic acid)-D-glucopyranose) (Heany-Kieras and Chapman 1976), but signals of OMe groups should be confirmed (Geresh et al. 1990). This acid-type was also found in other species of *Porphyridium* and in *Rhodella reticulata* (Geresh et al. 1990). However, a lot of work is still necessary in order to determine whether these oligosaccharides are repeating building blocks of the **EPS** and whether there is

Fig. 2 (continued) (**c**) glucuronyl-**rham** (or glucuronosyl-(1,4)-L-**rham**), (**d**) 3-*O*-*α*-D-glucopyranuronosyl-L-**gal,** and (**e**) *O*-D-glucopyranosyl-(1,3)-*O*-D-mannopyranose are aldobiuronic acids found in the acidic **PSs** produced by microalgae (**c** and **e** http://www.chemspider.com; **d** adapted from http://www.chemspider.com and http://www.ebi.ac.uk; accessed on 07-04-14); (**f**) *α*-D-glucuronosyl-*α*-L-rhamnosyl-*α*-L-**rham**, an acidic trisaccharide found in *Chlorella* (Ogawa et al. 1999); (**g**) oligosaccharides I and II from *Porphyridium* (Gloaguen et al. 2004); (**h**) models 1 or 2 for the possible acidic repeating unit in polysaccharide II, from *Porphyridium* sp., according to Geresh et al. (2009); R = H, SO_2O, terminal **gal** or terminal **xyl**, m = 2 or 3

any kind of alternate distribution. Another microalga, whose **sPSs** (**CaSp** and **NaSp**) have an aldobiuronic acid-type disaccharide, the *O*-hexunorosyl-**rham**, and *O*-rhamnosyl-acofriose, another disaccharide, is *A. platensis*. But as far as we know, the "hexose" is still waiting to be identified. Further, Lee et al. (2000) referred the structure as consisting alternately of a uronic acid and a **rham**. The *O*-rhamnosyl-acofriose, *O*-rhamnosyl-(3-*O*-methyl-**rham**), is the other repeating di-unit identified as being part of **CaSp** and **NaSp** (Lee et al. 2000; Kaji et al. 2004) constituted by alternating molecules of **rham** and acofriose (Lee et al. 2000). Besides these disaccharide units, an oligosaccharide is also part of the **CaSp** – the trisaccharide *O*-rhamnosyl-acofriosyl-**rham** (Lee et al. 2000). A tetrasaccharide was also identified: it is composed of two units of an aldobiuronic acid; in other words, two uronic acids alternating with two **rham** monosaccharides make up that tetrasaccharide. The di-unit uronic acid-**rham** can eventually be repeated some more times along the **CaSp** glycan, forming some more repeated units of the aldobiuronic acid-type disaccharide (Lee et al. 2000).

Despite being almost "forgotten," some knowledge on the structure of the **PS** produced by *P. tricornutum* came into light some decades ago (Ford and Percival 1965a, b). At that time, these researchers found that the **PS** from this diatom is a hetero-, ramified polymer, one of the products obtained by acid hydrolysis being a sulfated glucuronomannan, composed of β-(1,3)-linked **man**. The aldotriuronic acid *O*-D-glucopyranosyluronic acid-(1,3)-*O*-D-mannopyranosyl-(1,2)-*O*-D-mannopyranose (Fig. 2e) was found to be a constituent of the side chains. As a matter of fact, an aldobiuronic acid was also identified as being *O*-D-glucopyranosyluronic acid-(1,3)-D-mannopyranose. Another derived product, a glucan, seems to be the other constituent of the crude **PS**, comprising of β-(1,3)-linked **glc** units.

3.2 Rheology

In order to fully understand the several uses and applications of (**E**)**PSs**, the physicochemical characteristics must be taken into account. Rheological properties and the molecular weight are some of the most important parameters as they seem to be relevant to their functions and behavior.

Most **PSs**, especially **EPSs**, are polymers of high molecular weight (220–2.9 × 10^3 kDa; Kaji et al. 2004; Hayashi et al. 1996b; Hasui et al. 1995; Li et al. 2001; Pignolet et al. 2013; Mishra et al. 2011), negatively charged (anionic) and sulfated. Sulfated half-ester groups and uronic acids (mostly **glcAc** and **galAc**), along with the carboxyl groups, are responsible for the acidic and anionic characteristic of **PSs**.

Unfortunately, information on the rheological properties and behavior is scarce and described only for *A. platensis* and a couple of red marine unicellular algae.

Qualitatively, solutions of **sPSs** from red microalgae, such as *Porphyridium* and *Rhodella*, are characterized by their pseudoplastic properties (Sun 2010; Geresh and Arad 1991) and thixotropic characteristics (i.e., exhibit a stable form at rest but become more fluid when under some agitation) (Eteshola et al. 1998) these characteristics can be evaluated quantitatively by their viscosity, elasticity, shear rate, shear strain, and shear stress (www.vilastic.com). It is well known that hydrocolloid

solutions of **sPSs** produced by red microalgae and the cyanobacterium *A. platensis* present a non-Newtonian behavior, as their viscosity depends negatively on the shear strain rate, i.e., decreases with the increase of this last parameter (Raposo et al. 2014; Geresh et al. 2000, 2002a; Eteshola et al. 1998; Badel et al. 2011a; Ginzberg et al. 2008), thus showing to be a pseudoplastic compound (Geresh and Arad 1991) with a strong shear-thinning behavior (Eteshola et al. 1998). However, Sun et al. (2009b) showed that fragments of **EPSs** from *Porphyridium* have a different rheological behavior, depending on the degree of degradation, sometimes exhibiting even the typical characteristics of Newtonian fluids. In addition, elasticity, viscosity, and intrinsic viscosity decrease when high temperatures (>90 °C) are applied in the drying process of the **EPS**, as these high temperatures cause significant modifications in the conformation of the polymer chains (Ginzberg et al. 2008). Another reason supporting the idea of **EPS** from *Porphyridium* having weak-gel characteristics is the fact that the elasticity (G') values are higher than the viscosity (G") ones (Raposo et al. 2014) after small deforming oscillatory forces are applied to the **EPS**, which was previously dried at temperatures below 140 °C (Geresh et al. 2002a; Ginzberg et al. 2008). These properties prevailed also when the polysaccharide was obtained from cultures grown under different concentrations of sulfate (Raposo et al. 2014). The decrease in viscosity with the application of higher shear rates was suggested to be related to the dissociation of the strong hydrogen bonds that exist between polymer chains (Ginzberg et al. 2008). In this study, Ginzberg and coworkers described well the influence of several factors on conformational modifications and also highlighted the effects caused by drying on the interactions between the polymer and its non-covalently linked glycoprotein. Raposo et al. (2014) also found that the viscosity of the **EPS** from *P. cruentum* decreases with the increase of $MgSO_4$ concentration in the culture medium. The reason for this behavior can be associated with the decrease in the dimensions of the chains as a result of the intrachain electrostatic repulsions. An explanation of the similar behavior of the **EPS** was given by Eteshola et al. (1998) when NaCl, at different concentrations, was added to the aqueous solutions of **EPSs**. Another mechanism that can explain the gel-forming characteristic of polymers might be associated with hydrophobic and ionic forces, as it was referred for the anionic colloids of *Amphora*, *P. tricornutum* (diatoms), and *Ankistrodesmus* (chlorophyte) (Chen et al. 2011). Hence, some of these extracellular polymers behave as fluid-dynamic polymers, as it is the case of the **EPS** from *Porphyridium*, giving place to highly viscous solutions at very low concentrations, in a wide range of pH and temperatures, showing rheological properties similar to the ones of industrial **PSs** (Arad and Levy-Ontman 2010; Patel et al. 2013). Viscosity indexes, or the degree of polymerization, also seem to be closely associated with the culture growth temperature, since solutions with similar concentrations of the polymer presented higher viscosities when the cultures had been grown at the optimum temperature (Lupi et al. 1991). Therefore, growth of the culture at the optimum temperature might induce polymers with a higher polymerization degree.

Further, Eteshola and coworkers (1998) presented a fairly complete study on the rheology of the **EPS** produced by the red microalgae, including X-ray diffraction

techniques, referring as well the viscoelastic properties of the **EPS** by using dynamic mechanical spectra. They observed, as well, an increase in the G' modulus for temperatures above 60 °C, suggesting that heat promoted polymer self-association in an aqueous solution.

However, as far as we know, some **PSs** only jellify when dropped into $FeCl_3$ solutions, and some others are not viscous at all. In fact, the somewhat turbid solutions of the glucuronorhamnan produced by *C. vulgaris* do not have the ability to form gels, and hence the solutions do not show any viscosity (Ogawa et al. 1999), and the calcium-rich **PS** produced by *A. halophytica* has the capacity to jellify when some drops of the **EPS** aqueous solution (1 % w/v) are put in contact with $FeCl_3$ solutions (0.05 M), the jellified beads formed being stable for over 1 month (Li et al. 2001).

Furthermore, the rheological behavior of the **EPS** released by both *C. capsulata* and *P. cruentum* seems to be similar, the viscosity decreasing with the increase of the shear rate. Also, there were no significant differences between the **EPS** behaviors when the microalgae were subjected to different culture media (De Philippis et al. 1996; Raposo et al. 2014). This might indicate that the molecular weights of **EPSs** are stable, showing no major differences no matter the changes to the growth media and conditions, even under nutrient starvation (Gasljevic et al. 2008; Geresh and Arad 1991).

The high molecular weight is also an important requirement for the **PSs** to be good drag-reducing polymers, as polymers with higher molecular weights are usually more efficient as drag-reducing agents (Gasljevic et al. 2008). Therefore, **EPSs** from microalgae are promising candidates, as their **PSs** have similar, if not higher, molecular weights than industrial polymers, as xanthan gum, for example.

4 Bioactivity and Applications

Below is a list of some potential applications of the polysaccharides produced by microalgae:

- Drugs or nutraceutical carriers in the pharmaceutical industry, to slow and control the release of the substances; for bacterial vaccines, to improve nonspecific immunity (Mishra et al. 2011);
- Thickeners and gelling agents in food industries, to improve quality and texture (Mishra et al. 2011);
- In soils and water treatment, to act as nutrient carriers to fertilize soils (Raposo and Morais 2011); to improve the aggregation of soils and sand particles (Paterson 1989; Sutherland et al. 1998), influencing the stability and cohesiveness of sediments and improving water holding capacity of soils (Mishra et al. 2011); to act as soil conditioners (Kroen and Rayburn 1984; Metting and Rayburn 1983) and to improve sludge settling and dewatering (Subramanian et al. 2010); to be used in wastewater treatment (Raposo et al. 2010), in detoxification acting as metal chelators due to the presence of uronic acids (Kaplan et al. 1987), and in bioremediation to remove toxic metals from polluted waters (Otero and Vincenzini, 2003); and to act as growth promoters for crops (Pignolet et al. 2013).

4.1 Therapeutical Applications

Antiviral, antibacterial, anti-inflammatory, immunomodulatory, antilipidemic, antiglycemic, anti-adhesive, antioxidant and free radical scavenging, and prevention and treatment of tumors are some of the therapeutical applications of **PSs**.

4.1.1 Antiviral Activity

Many studies have already highlighted that the polysaccharides released (or not) into the culture medium by some marine microalgae present antiviral bioactivity against different kinds of viruses, either mammalian or otherwise (Table 3); the interest increased after some experiments were conducted on HIV (Hayashi et al. 1996a; Hasui et al. 1995). Radonic et al. (2010) and Chen et al. (2010) have recently reviewed the antiviral effects of several **sPS** on different host cell lines. To date, **PSs** from *Arthrospira* and *Porphyridium* were the most studied anionic sulfonated polymers, exhibiting antiviral activity against a wide range of viruses, including *Herpes simplex* and *Varicella zoster* viruses (HSV AND VZV), human cytomegalovirus (HCMV), measles, mumps and Flu-A viruses, and *vaccinia virus*, a variola-related virus. In fact, the **PS** TK-V3 from *A. platensis* and **EPS** from *P. purpureum* proved to be active against *Vaccinia* and *Ectromelia orthopoxvirus* infection; in studies conducted with HEp-2 and Vero C1008 cells, the $\mathbf{IC}_{50}$ is significantly lower (0.78 and 0.65 μg/ml, respectively) than the response to dextran sulfate (1.24 μg/ml). Despite being slightly toxic, **PSs** can be safely applied for in vivo experiments, as they were also effective *in ovo*, by decreasing the VACV replication (Radonic et al. 2010). Besides, the **EPS** from *P. cruentum* has also demonstrated a significant inhibition against *Vesicular stomatitis* virus proliferation on HEL cells (Raposo et al. 2014), the response being higher for the **EPS** isolated from the culture medium enriched with 104 mM in sulfate. As a matter of fact, the antiviral activity of the **EPS** from *P. cruentum* depends not only on the culture medium, algal strains, and cell lines used for testing but also on the methodology and the degree of sulfation and uronic acid content of the **EPS** (Raposo et al. 2014; Huleihel et al. 2001, 2002). These acidic compounds, along with the half-ester sulfate groups and the carboxyl groups of the polymer, contribute to their anionic characteristics, making the **EPS** of *Porphyridium* a good agent to be used against viruses (Raposo et al. 2014). **CaSp**, an intracellular polysaccharide produced by *A. platensis*, inhibited the replication of several viruses in vitro by inhibiting the penetration of the virus into the different host cells used (Hayashi et al. 1996a, b). The experiments conducted by Hayashi and coworkers (1996b) also confirmed the importance of sulfate groups on the antiviral activity of **CaSp** from *A. platensis*, activity proved by studying the effect of calcium-free-Spirulan and another compound derived from **CaSp**, without sulfate, on the replication of HSV-1 and on the cytotoxicity in HeLa cells, the latter compounds showing a higher toxicity and a significantly lower antiviral capacity. However, the molecular configuration due to the chelation of the calcium ion with the sulfate groups might have a crucial role in the antiviral properties, as no antiviral effect was verified when the calcium-free compound was used despite the presence of sulfate groups (Hayashi et al. 1996b).

Table 3 Antiviral applications of **EPS** from marine microalgae (*in* Raposo et al. 2013)

Microalgae/ cyanobacteria	Virus strain	Family/group of virus	Cell lines	EC50/ED50 (μg/mL)	References
A. platensis; *A. maxima*	Vaccinia virus VACV and VACV-GFP; ectromelia virus (ECTV); HSV-1, HSV-2, human cytomegalovirus (HCMV), measles virus, mumps virus, HIV-1, Flu-A	*Orthopoxvirus*/Poxviridae; *Simplexvirus*/ Herpesviridae; *Morbillivirus*/ Paramyxoviridae; *Rubulavirus*/ Paramyxoviridae; *Lentivirus*/Retroviridae; *Influenza virus*/ Orthomyxoviridae	HEp-2 and Vero C1008; HeLa, HEL, Vero, MDCK, MT-4 (HIV-1)	0.78; 69; 0.92–16.5; 8.3–41; 17–39; 23–92; 2.3–11.4; 9.4–230	Radonic et al. 2010; Hayashi et al. 1996a, b; Hernandez-Corona et al. 2002
Porphyridium sp.	Herpes simplex virus HSV-1 and HSV-2; varicella zoster virus (VZV); murine sarcoma virus (MuSV-124) and MuSV/MuLV (murine leukemia virus)	*Simplexvirus*/ Herpesviridae; *Varicellovirus*/ Herpesviridae; *Gammaretrovirus*/ Retroviridae (type VI)	NIH/3T3	1–5 (in vivo, 100); 0.7; 10 and 5 (RT_{50})	Huleihel et al. 2001, 2002; Talyshinsky et al. 2002
P. cruentum	Hepatitis B virus (HBV); viral hemorrhagic septicemia virus (VHSV); African swine fever virus (ASFV), vaccinia virus (VACV), vesicular stomatitis virus (VSV)	*Orthohepadnavirus*/ Hepadnaviridae; *Novirhabdovirus*/ Rhabdoviridae; *Asfarvirus*/ Asfarviridae; *Orthopoxvirus*/Poxviridae; *Vesiculovirus*/ Rhabdoviridae	HEL	20, 200 (exocellular extracts); 12–56; 20–45	Huang et al. 2005; Fabregas et al. 1999; Raposo et al. 2014; Vieira and Morais 2008
P. purpureum	Vaccinia virus VACV and VACV-GFP, ectromelia virus (ECTV)	*Orthopoxvirus*/Poxviridae	HEp-2, Vero C1008	0.65	Radonic et al. 2010

(*continued*)

Table 3 (continued)

Microalgae/ cyanobacteria	Virus strain	Family/group of virus	Cell lines	EC50/ED50 (μg/mL)	References
R. reticulata	Herpes simplex virus HSV-1 and HSV-2, varicella zoster virus (VZV), murine sarcoma virus (MuSV-124), and MuSV/MuLV (murine leukemia virus)	*Simplexvirus*/ Herpesviridae; *Varicellovirus*/ Herpesviridae; *Gammaretrovirus*/ Retroviridae (type VI)	NIH/3T3	10–20; 8; 150 and 50 (RT_{50})	Huleihel et al. 2001; Talyshinsky et al. 2002
G. impudicum	Encephalomyocarditis virus; influenza A virus (Flu-A)	*Cardiovirus*/ Picornaviridae; Orthomyxoviridae	MDCK	0.19–0.48	Yim et al. 2004
C. polykrikoides	Flu-A and Flu-B, respiratory syncytial virus types A (RSV-A) and B (RSV-B), HIV-1, HSV-1, parainfluenza virus type 2 (PFluV-2)	Orthomyxoviridae; *Pneumovirus*/ Paramyxoviridae; Retroviridae; Herpesviridae; *Rubulavirus*/ Paramyxoviridae	MDCK, Hep-2, MT-4, HMV-2	0.45–1.1 and 7.1–8.3; 2.0–3.0 and 0.8; 1.7; 4.52–21.6; 0.8–25.3	Hasui et al. 1995

EC_{50}/ED_{50} is the concentration/dose at which 50 % of the population exhibit a response after being exposed to a certain compound

Other factors that are correlated to the inhibition of viral infection are the size of the molecules and the degree of sulfation (Ghosh et al. 2009); the composition of monosaccharides and the diversity of the linkage types also determine the specificity of **sPSs** and influence their functional properties. That is why tests for antiviral capacity should be carried out with a variety of virus and isolates, as wide as possible.

Unlike what happens with most of **sPSs**, the **PS** from *C. polykrikoides* showed to be effective against influenza virus type B (Flu-B), among other different viruses, including that of HIV-1. This sulfated polymer that does not contain proteins, aminoacids, or nucleic acids showed an inhibitory effect higher than that of dextran sulfate on Flu-B and MSLV, with no cytotoxic effects against the different cell lines used for testing concentrations up to 100 μg/ml (Hasui et al. 1995).

The antiviral activity is probably the most studied quality exhibited by sulfated polysaccharides of marine microalgae, especially the one produced by *Porphyridium*. The mechanisms for this activity are not yet completely understood. As happens with heparin, the anionic nature of **sPS** makes it a good candidate to protect against viruses. Several mechanisms have been proposed. Hayashi and colleagues (1996a, b) noted that **sPS** inhibited infection by different viruses through inhibiting the penetration of viral particles into host cells. But other mechanisms can also be involved, such as the inhibition of attachment/adsorption, or even replication during the early phases of the virus cycle (Martinez et al. 2005; Kim et al. 2012), without any toxicity to the host cells (Hasui et al. 1995).

4.1.2 Antibacterial Activity

The **PS** from *A. platensis* has antibacterial properties, the activity depending on the solvent used to extract the polymer. While water and methanolic extracts showed antimicrobial properties on both Gram-positive and Gram-negative bacteria, methanolic **EPS** extracts show a wider capacity to inhibit the growth of bacteria than aqueous extracts. However, methanolic extracts presented only a bacteriostatic effect against the strain NCIMB8166 of *Micrococcus luteus*, needing an MBC/MIC ratio >4, i.e., the minimum inhibitory concentrations (MIC) of **EPS** is considerably lower than the concentration needed to kill the organisms (MBC, minimum bactericidal concentrations) (Challouf et al. 2011). Ethanolic and some other solvent extracts did not show any antimicrobial activity. In fact, the **EPS** did not exhibit any activity against *E. coli* (strain ATCC25922) and *S. aureus* (ATCC25923). A similar explanation can be applied to the results obtained by Raposo et al. (2014), who tested the antimicrobial activity of the **EPS** from *P. cruentum* and reported that their ethanolic extracts did not show a significant inhibition on the growth of bacteria *E. coli* and *S. aureus*. Perhaps the bioactive portions of the molecules have different affinities to the solvents used, being highly influenced by mutual interactions (Basedow et al. 1980), and ethanol might not be adequate for the extracts to maintain the antibacterial active principle/ingredient of the **EPS**. Nevertheless, the ethanolic extract of the **EPS** from *P. cruentum* showed some activity against *S. enteritidis* (Raposo et al. 2014).

4.1.3 Antioxidant Activity and Free Radical Scavenging

As photoautotrophs, microalgae are highly exposed to oxidative and radical stresses, therefore accumulating effective antioxidative scavenger complexes to protect their own cells from free radicals (Pulz and Gross 2004). Oxidation of lipids by reactive oxygen species (ROS), like hydroxyl radicals, hydrogen peroxide, and superoxide anion, can affect the safety of pharmaceuticals and also decrease the nutritional quality of foods. Sulfated **PSs** produced and secreted out by marine microalgae may act not only as dietary fiber (Dvir et al. 2009), but have also showed the capacity to prevent the accumulation and the activity of free radicals and reactive chemical species, therefore acting as a protective system against these oxidative and radical stress agents (Table 4).

It was already demonstrated that the **sPS** from *Porphyridium* exhibited antioxidant activity against the autoxidation of linoleic acid and inhibited oxidative damage to 3T3 cells that might be caused by $FeSO_4$ (Tannin-Spitz et al. 2005). These researchers also proved that the bioactivity was dose dependent, correlating positively with the sulfate content of the **sPS**, and mentioned the possibility of the glycoprotein to contribute to the antioxidant properties. They even suggested that the antioxidant activity of this polymer relied on its ability to act as a free radical scavenger. Despite the various applications suggested for the **EPSs** from different species/strains of marine microalgae, when the biological activity involves crossing the cellular membrane of cells, the high molecular weight of the polymers can be a drawback to pursue their properties. This feature was confirmed by Sun et al. (2009b). These researchers submitted the **EPS** from *P. cruentum* to microwave, and the **EPS**-derived products (6.55, 60.66, and 256.2 kDa) showed different levels of antioxidant activity, a lower molecular weight (6.55 kDa) being a requisite for a stronger activity either by scavenging hydroxyl, superoxide anion, and $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical) free radicals or by inhibiting the (per)oxidation of lipids induced by $FeSO_4$ and ascorbic acid, thus giving better protection to mouse cells and tissues against oxidative damage. They found that the antioxidant ability is dose dependent; the same is true in relation to the inhibition of oxidation damage of both liver cells and tissue. The free radical scavenging of some of the **EPS** fragments was significantly higher at the same, or even lower, concentration than that reported for vitamin C (Xing et al. 2005). But strangely, they found no scavenging activity and no inhibition of oxidative damage in cells and tissues for the crude high molecular **sPS** from *Porphyridium cruentum* (Sun et al. 2009b).

The sulfated exopolysaccharide from *Rhodella reticulata* also has antioxidant activity, the effects being dose dependent (Chen et al. 2010). Unlike what happened with the **sPS** from *Porphyridium* (Sun et al. 2009b), crude **sPS** from *Rhodella* exhibited higher antioxidant properties than the polysaccharide-modified samples, these demonstrating lower radical scavenging activity (Chen et al. 2010). These researchers found that all the different samples of **sPS** from *R. reticulata* had a stronger ability than α-tocopherol against superoxide anion radical scavenging, the crude polysaccharide being twice as strong as α-tocopherol.

Besides the antibacterial properties, the methanolic extracts of **EPS** from *A. platensis* also exhibit a moderate antioxidant capacity (TEAC=0.27 mg/mL)

Table 4 Applications, other than antiviral uses, of **EPS** from marine microalgae (*in* Raposo et al. 2013)

Microalgae/ cyanobacteria	Applications	Cells/animals used for in vitro/in vivo studies	References
Porphyridium	Health foods, nutraceutical, and functional foods	Rats	Dvir et al. 2000, 2009
Rhodella, *Porphyridium*	Antioxidant and free radical scavenging	3T3; mouse liver homogenates and erythrocyte hemolysates, sarcoma 180 cells/mice	Sun 2010; Chen et al. 2010; Tannin-Spitz et al. 2005; Sun et al. 2009b
Porphyridium, *P. cruentum*; *R. reticulata*	Antilipidemic, antiglycemic	Rats/mice, chickens	Dvir et al. 2009; Arad 1999; Ginzberg et al. 2000; Huang et al. 2006
Porphyridium, *Chlorella stigmatophora*, *Phaeodactylum tricornutum*	Anti-inflammatory and immunomodulatory	Polymorphonuclear leukocytes/human dermal microvascular endothelial cells, humans; rabbits and sheep (bone joints); mice macrophages/mice and rats	Guzman et al. 2003; Sun 2010; Matsui et al. 2003; Arad and Atar 2007
Porphyridium, *R. reticulata*, *Gyrodinium impudicum*, *A. platensis*	Prevention of tumor cell growth	FD early myeloid cell line, 24-1 and EL-4T-lymphoma cell lines; Graffi myeloid cells; rats	Senni et al. 2011; Geresh et al. 2002b; Gardeva et al. 2009; Shopen-Katz et al. 2000
Phaeodactylum, *Tetraselmis*	Anti-adhesive	HeLa S3/sand bass culture cells	Guzmán-Murillo and Ascencio 2000; Dade et al. 1990
Porphyridium	Biolubricant (for bone joints)		Arad and Atar 2007; Arad et al. 2006
Porphyridium, *R. reticulata*	Ion exchanger		Lupescu et al. 1991
P. cruentum, *R. reticulata*, *R. maculata*	Drag reducers		Gasljevic et al. 2008; Ramus et al. 1989

using Trolox, a common antioxidant substance, while the ethanolic extracts presented lower antioxidant activity (Challouf et al. 2011). According to Mendiola et al. (2007) and Sun et al. (2009b), uronic acid contents are directly related to the radical scavenging properties of **PSs**, but other factors also seem to have influence on the antioxidant capacity, namely, low molecular weights (Chen et al. 2008; Sun et al. 2009a), and the structure and conformation of the polymer (Tao et al. 2007). The antioxidant properties of these **PSs** might be exerted by improving the activity of antioxidant enzymes, scavenging free radicals, and/or inhibiting lipid (per) oxidation (Sun et al. 2009a).

4.1.4 Anti-inflammatory and Immunomodulatory Properties

Polysaccharides from marine microalgae, like *Porphyridium*, *Phaeodactylum*, and *C. stigmatophora*, had already demonstrated to have pharmacological properties, such as anti-inflammatory activity and as immunomodulatory agents (Table 4). The **sPS** from both *C. stigmatophora* and *P. tricornutum* demonstrated a significant anti-inflammatory activity against paw edema induced by carrageenan injected as a sterile saline solution (0.9 %), with $\mathbf{IC}_{50}$ values of 2.25 and 2.92 mg/kg for *C. stigmatophora* and *P. tricornutum*, respectively, compared to the anti-inflammatory indomethacin, with an $\mathbf{IC}_{50}$ of 8.50 mg/kg. The anti-inflammatory efficacy was tested in vivo, by intraperitoneally injecting the crude **PS** in female rats and mice, and in vitro, the phagocytic activity being evaluated in macrophages from mice (Guzman et al. 2003). The direct stimulatory effect of *P. tricornutum* on immune cells was evidenced by the positive phagocytic activity tested either in vitro or in vivo, and the activity of the extract of **sPS** from *C. stigmatophora* showed immunosuppressant effects (Guzman et al. 2003). As reported for the polysaccharide from *Ulva rigida*, a green seaweed (Leiro et al. 2007), the **sPS** p-KG03 from the marine dinoflagellate *G. impudicum* also activates the production of nitric oxide and immunostimulates the production of cytokines in macrophages (Bae et al. 2006). On the other hand, inhibition of leukocyte migration seems to be related to the anti-inflammatory activity of the polysaccharides (Matsui et al. 2003). As leukocyte movement to the site of injury contributes to additional cytokine release and to the production of nitric oxide, therapeutics has to be effective against this over-inflammation. In fact, the **sPS** from *Porphyridium* seems to be a good candidate for this role as it inhibited the movement and adhesion of polymorphonuclear leukocytes in vitro and inhibited the development of erythema in vivo as well (Matsui et al. 2003).

Besides inhibiting tissue oxidative damage, **EPS** from *P. cruentum* can be used to inhibit the biomembrane peroxidation as well (Sun et al. 2009b) and to enhance in vitro immunomodulatory activity (Sun et al. 2012). These researchers have also explained the mechanism/pathway that is most probably involved in the immune response enhancement by **EPS** from *P. cruentum* – the stimulation of macrophages. They found that low molecular fractions of **EPS** can stimulate the proliferation of macrophages and the production of NO (nitric oxide). NO is a signaling free radical gas molecule that can be synthesized by phagocytes (monocytes, macrophages, and neutrophils) and is involved in the human immune system response. When studying the effects of **EPS**-derived products, Sun (2010) showed that **EPS** from *Porphyridium* presented immunostimulating activity in mice with S180 tumors by increasing both spleen and thymus index and also spleen lymphocyte index. Sulfated **PS**-derived products, with lower molecular weight, can also improve the production of NO in mouse macrophages. In his Ph.D. Thesis, Sun referred to the fact that sulfate content has a positive correlation with the immunomodulatory system. Furthermore, Namikoshi (1996) noted that **sPS** can stimulate the immune system by triggering cells and humor stimulation. This shows the capacity of marine unicellular algae **sPS** to directly stimulate the immune system.

Spirulan is a **GAG**-like **PS** (Senni et al. 2011). This means that spirulan from *A. platensis*, for example, is recognized as having similar properties as glycosaminoglycans (GAG), present in all animals. **GAG**s are sulfated (or not)

PSs composed of disaccharide repeating units, a uronic acid or a neutral monosaccharide, and an amino sugar (Senni et al. 2011) with anticoagulant properties, such as heparin and hyaluronic acid. By interacting with a vast range of proteins involved in many human body physiological and pathological responses, GAGs show several bioactivities associated either to inflammatory processes or to tissue repair (Gandhi and Mancera 2008; Mulloy and Linhardt 2001). Some sulfated **GAG**s can be covalently linked to proteins (proteoglycans); this is the case of the **sPS** from red marine unicellular algae *Porphyridium*, which has also some protein moieties non-covalently linked, but shows anti-inflammatory properties.

On the other hand, immunomodulators are response modifier biocompounds that present an enhancement or suppression of the immune responses, depending on a wide range of factors, such as dose, way, and time of administration, but also on the site of activity and the respective mechanism of action (Tzianabos 2000). β-(1,3)-Glucans, such as that of *C. vulgaris* (Nomoto et al. 1983), have already proved to exhibit several biological properties, including prevention of some infections and antitumor activity (Bleicher and Mackin 1995; Nomoto et al. 1983). These polymers stimulate the functional activity of macrophages (Burgaleta et al. 1978) and the proliferation of monocytes and macrophages, presenting also potent hematopoietic properties (Patchen and Lotzova 1980; Riggi and DiLuzio 1961).

4.1.5 Activity Against Tumors and Vascular Muscle Cell Proliferation

Sometimes, after suffering some kind of damage, vascular endothelial cells are not sufficiently repaired by their own cell type; there can be an invasion of platelets and/or macrophages or other blood cell types, which secrete cytokines and growth factors that can increase the proliferation of vascular smooth muscle cells, causing a hyperplasia of the arterial *intimae*. This atherosclerosis is one of the main causes of myocardium and cerebral infarction.

Some **PSs** that are used as anticoagulants have also some inhibitory activity against the proliferation of vascular smooth muscle cells. This is the case of heparin (Clowes and Clowes, 1987) and heparin sulfate (Kaji et al. 2004) and the sulfated fucoidan from some seaweeds (Vischer and Buddecke 1991). Nevertheless, spirulan (either Na- or Ca-) from *A. platensis* is a more potent inhibitor of cell proliferation, as it was demonstrated by Kaji and coworkers (2004) on bovine arterial smooth muscle cells. These researchers also demonstrated that it is not enough to be composed of sulfate for the **PSs** to show inhibitory activity against cell growth: while both **NaSp** and **CaSp** inhibited the proliferation of vascular smooth muscle cells, as it happened with heparin and heparin sulfate, the desulfated equivalent compounds did not show this effect. And, as depolymerized compounds (**PS**-derived products with lower molecular weights) of **NaSp** and **CaSp** maintained the inhibitory capacity against the proliferation of arterial smooth muscle cells, with MW $\geq$14,700, this suggests that spirulan (especially **NaSp**) is a particular polymer with a specific structural sequence and conformation maintained by the linkage of Na^+ to the sulfate groups, keeping, therefore, that strong inhibitory activity (Kaji et al. 2004); the effect is dose and time dependent. Furthermore, depolymerized **NaSp** inhibited the growth of vascular smooth muscle cells without inhibiting the growth of vascular endothelial cells (Kaji et al. 2002, 2004).

Spirulan is also capable of inhibiting pulmonary metastasis in humans and to prevent the adhesion and proliferation of tumor cells (Senni et al. 2011).

Other **sPSs** have also antiproliferative activity in cancer cell lines (in vitro) and inhibitory activity against tumor growth (in vivo). The **sPS** p-KG03 from *G. impudicum* is one of these polymers that prevents and suppresses tumor cell growth either in vitro or in vivo by activating NO production and by stimulating the innate immune system, increasing the production of cytokines interleukin-1 (or IL-1), IL-6, and THF-α in macrophages (Bae et al. 2006; Namikoshi 1996; Yim et al. 2005). This **PS** has immunostimulating properties in vivo as well (Yim et al. 2005).

Another candidate with potential to be used as an antitumor agent is the *β*-(1,3)-glucan from *C. vulgaris*, besides being considered an active immunostimulator (Laroche and Michaud 2007). Low molecular weight fragments (6.53–1,002 kDa) of the **sPS** from *P. cruentum* are also good immunostimulators as they all inhibited in vivo S180 tumors implanted in the peritoneal cavity of mice models by inhibiting the tumor cell proliferation and the growth of the tumor, increasing the spleen and thymus indexes and the number of spleen lymphocytes as well, enhancing the immune system in this way (Sun et al. 2012). However, nonmodified or higher molecular weight fragments of the same **sPS** showed no inhibition of tumor cell growth (Geresh et al. 2002b; Sun 2010). Nonetheless, in a recent study, Gardeva et al. (2009) reported the strong antitumor activity exhibited by the polysaccharide of *P. cruentum*. This sulfated polymer strongly inhibited Graffi myeloid tumor proliferation in vitro and in vivo, the activity being dose dependent, and the survival time of hamsters was increased by 10–16 days. Gardeva and coworkers (2009) also suggested that the antitumor activity could be related to the immunostimulating properties of the polymer. Therefore, it can be concluded that the reinforcement of the immune system induced by the **sPSs** is probably the main mechanism against tumor growth and respective effects (Sun et al. 2012; Zhou et al. 2004). However, other mechanisms, such as changes in the biochemical characteristics of the cell membrane, inducing tumor cell differentiation and apoptosis, and regulation of the cell signaling pathways, can also be involved (Zhou et al. 2004). Besides these mechanisms, the antimetastatic properties may be associated to the capacity of blocking the interactions between cancer cells and the basement membrane or inhibiting the adhesion of tumor cells to the substrates.

In addition, some years ago, it has already been demonstrated that high molecular weight oversulfated **EPSs** from *Porphyridium* inhibited neoplastic mammalian cell growth and that the biomass of this marine microalga could prevent the proliferation of colon cancer in rats (Geresh et al. 2002b; Shopen-Katz et al. 2000).

4.1.6 Antilipidemic and Antiglycemic Properties

Sulfated **PSs** from seaweeds and marine animal origin are potent inhibitors of human pancreatic cholesterol esterase, an enzyme that promotes its absorption at the intestinal level (Laurienzo 2010). These inhibitory effects are enhanced by higher molecular weights and degree of sulfation, as well as by the presence of 3-sulfate in the monosugar molecule (Laurienzo 2010). And most of the **PSs** from marine microalgae are naturally and highly sulfated with high molecular weights, making them non-readily absorbable and thus enabling them to be used as anticholesterolemic agents.

However, this area of research has not been sufficiently explored in what concerns microalgae (Table 4). When Ginzberg and coworkers (2000) fed chickens with biomass containing **EPS** from *Porphyridium*, they verified that cholesterol decreased either in serum or egg yolk of chickens, the fatty acid profile was modified, and the carotenoid content in the egg yolk was improved as well. Furthermore, in rats fed with *Porphyridium* and *R. reticulata* biomass, which **PSs** contain dietary fibers, there was a decrease in serum cholesterol and triglycerides; hepatic cholesterol levels were also improved and the levels of VLDL considerably lowered with no toxic effects noticed in the animals (Dvir et al. 1995, 2000, 2009). Also, either the biomass of *Rhodella* or the **sPSs** from *Porphyridium* were able to lower the levels of insulin and/or glucose in diabetic rodents (Dvir et al. 1995; Huang et al. 2006), causing no modifications in the pancreatic island cells and no fibrosis or hemorrhagic necrosis in cells (Huang et al. 2006).

These experiments suggest the strong potential of sulfated polysaccharides from unicellular algae to be used as hypolipidemic and hypoglycemic agents, but they are also promising substances in reducing coronary heart disease due to their hypocholesterolemic effects (Dvir et al. 2000, 2009).

Mechanisms focusing on the role of dietary fibers in lowering cholesterol are not yet completely understood, but Oakenfull (2001) proposed that it could be related to the increase in the viscosity of intestinal contents, which have influence on nutrient absorption, micelle formation, and decreasing of lipid absorption. The decrease in serum cholesterol levels and the increase in bile excretion, caused by the disruption of the enteropathic circulation of bile acids, were suggested as another possible explanation (Glore et al. 1994; Marlett 2001).

4.2 Other Biological Activities

4.2.1 Anticoagulant and Antithrombotic

There are several studies on the anticoagulant properties of the **PSs** isolated from seaweeds, presented in a recent review by Wijesekara and coworkers (2011). Carrageenans, for example, are **sPSs** that show potent anticoagulant activity, inhibiting platelet aggregation as well, probably due to the antithrombotic capacity, which, in turn, is associated to a high sulfate content (Prajapati et al. 2014). However, there are only a few references to microalgae. On one hand, it was stated that the anticoagulant activity is associated to the high sulfate content of the **PS**, which is a characteristic of most of the **PSs** with marine microalgae origin. But, this feature could be an inconvenience when considering their use for the treatment of virus-induced diseases, for example, as an anti-inflammatory. On the other hand, Hasui and colleagues (1995) found no anticoagulant activity in the **sPS** of *C. polykrikoides* in spite of the high contents in sulfate of this **PS**. This suggests that the anticoagulant properties of polysaccharides may not only depend on the percentage of sulfate residues but rather on the distribution/position of sulfate groups and, probably, on the configuration of the polymer chains

(Ginzberg et al. 2008; Pereira et al. (2002). Spirulan from *A. platensis* is one of the marine microalgae **PS** that strongly interferes with blood coagulation-fibrinolytic system and exhibits antithrombogenic properties (Hayakawa et al. 1996, 2000). Both **NaSp** and **CaSp** enhance the antithrombin activity of heparin cofactor II and the production of the tissue-type plasminogen activator in human fetal lung fibroblasts (Hayakawa et al. 1997), and, in addition, **NaSp** still enhances the secretion of urokinase-type plasminogen activator and inhibits the secretion of the plasminogen activator inhibitor type 1, sulfate being essential for these properties (Yamamoto et al. 2003). Therefore, spirulan is a promising antithrombotic agent in clot breakdown, but some care should be taken in relation to hemorrhagic strokes.

4.2.2 Biolubricant

This is one of the lesser known applications for **sPSs**, and very little has been published on this issue (Table 4). Nevertheless, the **sEPS** of *Porphyridium* has already shown good lubrication capacity due to its rheological properties (Arad and Weinstein 2003). Arad and coworkers (2006) have compared the lubricating properties of **sPSs** to the most used hydrogel lubricant, hyaluronic acid. They simulated efforts of joints, during both walking and running, and found a better quality of the **EPS** from *Porphyridium*. The explanation for these properties is associated to **EPS** rheological characteristics as they showed to be stable than most lubricants at higher temperatures, the viscosity of the latter decreasing along with a decrease of lubricity. A 1 % **PS** solution presented the best friction properties under high loads, and its viscosity did not suffer any significant change when incubated with hyaluronidase, with standing degradation by this enzyme (Arad et al. 2006). This experiment shows the potential of the **sPS** from *Porphyridium* to be an excellent candidate to substitute hyaluronic acid as a biolubricant. Another promising application could be as a substance to be part of a joint-lubricating solution, as it was demonstrated by injecting the **EPS** from *Porphyridium* into the joints of rabbits' knees (Arad and Atar 2007), thus mitigating degenerative joint disorders caused by arthritis.

4.2.3 Anti-adhesive

Sulfated **PSs** from marine microalgae revealed the ability to block the adhesion of pathogenic microorganisms, suggesting the hypothesis to be used in anti-adhesive therapeutics. In fact, several **sPSs** presented a higher inhibition of the adherence of both *Helicobacter pylori* to HeLa S3 cell line and three fish pathogens to spotted sand bass gills, gut, and skin cultured cells (Guzmán-Murillo and Ascencio 2000) (Table 4).

Infection by microorganisms appears usually after binding to the cell membrane. Carbohydrates were already demonstrated as recognition sites for decades (Ofek et al. 1978), heparan sulfate glycosaminoglycan being one of those receptors in the host cells (Ascencio et al. 1993). These researchers suggested that this interaction could be associated to the net charge and molecular stereochemistry of the polymer.

4.3 Health Foods, Nutraceuticals, Functional Foods

Nowadays, the implications of specific diets on health assume a relevant role in developed countries, and the pursuit for equilibrated diets, supported by considerable epidemiological evidences, is a major issue for the scientific community and consumer in general, who more and more look for natural food products. In this context, microalgae have great potential to be used in food and feed preparation due to their rich composition, including high protein content with balanced amino acid pattern, carotenoids, fatty acids, vitamins, polysaccharides, sterols, phycobilins, and other biologically active compounds (Gouveia et al. 2008). The commercial production of microalgae for human nutrition is already in practice, and they find many applications either as nutritional supplements, for instance, in the form of tablets and pills, or as functional foods, incorporated in food products, such as pastas and cookies. The health-promoting effects associated with microalgal biomass are probably related to several effects due to their phytochemical constituents.

Some **PSs** from microalgae may by themselves be of interest for industrial and commercial applications. **PSs** can find applications in the food industry as emulsifying and gelling agents and as flocculant and hydrating agents, emulsifiers, stabilizers, and thickening agents, i.e., food additives (Bernal and Llamas 2012), like agar E406, alginates E400-404, and carrageenan E407. Because of the presence of peptide/protein moieties and deoxysugars, such as rhamnose and fucose, some **PSs** from marine cyanobacteria and unicellular algae show a significant hydrophobic behavior, conferring them emulsifying characteristics (Flaibani et al. 1989; Shepherd et al. 1995). In addition, some **PSs** include fucose as a constituent, this deoxysugar being of high value in the chemical synthesis of flavoring agents (Lupi et al. 1991). The **sPS** from marine microalgae could also be used as nutraceuticals due to their fiber content, their ability of acid binding and cation exchange, and their property of fecal bulking, and they are also good candidates as prebiotics (Ciferri 1983) and in some cases with a strong bioactive potential as hypolipidemic and hypoglycemic agents (Gonzalez de Rivera et al. 1993; Dvir et al. 2000, 2009) similar to polysaccharides from seaweeds (O'Sullivan et al. 2010). The **PSs** from microalgae alone or in combination with other compounds have also great potential to be used in edible films and coatings of foods other than carriers of flavors, colorants, spices, and nutraceuticals (Marceliano 2009). **PSs** from microalgae also have the potential to be used in low-fat or fat-free food products, as fat replacers in mayonnaises (Franco et al. 1998; Raymundo et al. 1998), and salad dressings and other food emulsions (Raymundo et al. 2005).

4.4 Other Applications

Another little known field of application is as drag reducers. Only a few studies were conducted in order to determine whether polysaccharides had the potential of drag-reducing ability (Ramus et al. 1989; Gasljevic et al. 2008), in order to extend

their functionalities to engineering applications (Table 4), namely, naval engineering. It is known for some years that the efficiency of **PSs** drag-reducing properties is improved by the high molecular and linear structure of the polymers, associated with a strong resistance to mechanical degradation (Gasljevic et al. 2008). In fact, these researchers have already studied the potential of several marine microalgal **PSs** as drag reducers. *P. cruentum* and *R. maculata* were the ones whose polysaccharides showed the higher drag-reducing power at lower concentrations, followed by *Schizochlamydella* (former *Chlorella*) *capsulata*. However, the **PSs** of some of these microalgae proved to be more powerful than others. As a matter of fact, to have the same level of drag-reduction effectiveness, 25 % more **PS** of *R. maculata* is required in relation to *P. cruentum* and almost three times more than the polysaccharide of *S. capsulata* (Gasljevic et al. 2008). Thus, if applied to the hulls of the vessels, these high-molecular **EPSs** could reduce friction losses by reducing flow turbulence due to the elasticity of the polymers. Therefore, there could be a reduction in the fuel consumption and in the propelling power for a ship to achieve a certain velocity (Gasljevic et al. 2008).

Another promising and emerging application of microalgae might be associated to the production of nanofibers from the biomass of *A. platensis* to be used as extracellular matrices for the culture of stem cells in order to treat spinal cord injuries (Raposo et al. 2010).

Their gluing and adhesive capacities and also their strong cohesive and binding strength, allied to their nontoxic and nonirritating properties, make these bioadhesive **PSs** produced by marine microalgae good candidates as mucobioadhesives or glues for bone gluing and soft tissue closure after surgery, promising to be, in the near future, the substituents of metallic screws and traditional wound closure methods, respectively (Laurienzo 2010).

Other areas of application of the marine microalgal **sPSs** could be as diverse as cosmetics or as ion exchangers, due to their chemical composition, rheological characteristics, and ion affinity.

Finally, besides all these applications, the adhesion properties of the **sPSs** produced by microalgae seem to play an important role in either the locomotion of some algae (Wetherbee et al. 1998) or in the aggregation of soil and sand particles (Paterson 1989; Sutherland et al. 1998), influencing stability and cohesiveness of sediments.

5 Mechanisms of Action

5.1 Antibacterial Activity

Some researchers have found that some **PSs** have an inhibitory effect on some bacteria: the extract from *Chaetomorpha aerea* inhibited the growth of *S. aureus* (Pierre et al. 2011); **EPS** from *P. cruentum* inhibited the growth of *S. enteritidis* (Raposo et al. 2013); fucoidan from the brown seaweed *Laminaria japonica* inhibited *E. coli* (Li et al. 2010). This inhibitory effect might be explained by the

anti-adhesive properties of sulfated exopolysaccharides of some microalgae against the adherence of microorganisms. Several **sPSs** inhibited the adherence of both *Helicobacter pylori* to HeLa S3 cell line and three fish pathogens to spotted sand bass gills, gut, and skin cultured cells (Guzman-Murillo and Ascensio 2000). **PSs** may compete with carbohydrates as recognition sites to which microorganisms can attach to, this mechanism having already been evidenced for other carbohydrates in cell surfaces (Ofek et al. 1978). Heparan sulfate glycosaminoglycan was identified as a receptor in host cells, this interaction having been associated with the net charge and molecular stereochemistry of the polymer (Ascencio et al. 1993).

However, the same **PS** may also not show activity against other bacteria (*C. aerea* extract against *S. enteritidis*, Pierre et al. 2011; *P. cruentum* extract against *S. aureus*, Raposo et al. 2014). The different reactions of various bacteria to the biological extracts of **PSs** could be due to the composition of the bacterial cell wall, to the absence of a specific structure in the bacteria, or also to the ability of the bacteria to change the chemical structure of the extract (Michael et al. 2002). The antibacterial activity may also be related to the antibiofilm formation ability of the **EPS** (Bernal and Llamas 2012) and, therefore, with the anti-adhesive properties. Most evidence suggests that these molecules act by modifying the physical properties of biotic surfaces (Rendueles and Ghigo 2012). Gram-negative *E. coli* and Gram-negative *S. enteritidis* present different cell surfaces that might explain the differences found between the correspondent inhibitions by the **EPS** (Raposo et al. 2014).

Rendueles and Ghigo (2012) suggest that **PSs**, as surfactant molecules, may modify the physical properties of the bacterial cell surfaces. **PSs** from microalgae might act in a similar way as *E. coli* exopolysaccharides, which can inhibit the autoaggregation via adhesins of bacterial cells (Valle et al. 2006; Rendueles et al. 2011). Polysaccharides, as sugar polymers, have also the capacity to act as inhibitors of lectin, which, being mainly located on the surface of bacteria cells, facilitate the attachment or adherence of bacteria to host cells by binding to the glycan substrates present on the surface of those host cells (Esko and Sharon 2009). **PSs** compete with the sugar-binding domain of lectins and inhibit the lectin-dependent adhesion of pathogens and biofilm formation, therefore reducing the occurrence of infection.

5.2 Antiviral Activity

Several mechanisms have already been proposed to explain the antiviral activity of **EPSs**, either involving the inhibition of the virus penetration into the host cells (Hayashi et al. 1996b), by competing with the glycoprotein attachment sites of the membrane/envelope of the viruses (Damonte et al. 2004; Radonic et al. 2010; Rashid et al. 2009), or relating to the replication during the early phases of the virus cycle (Martinez et al. 2005; Kim et al. 2012).

The general mechanism of the antiviral activity of most **PS** against enveloped viruses could be based on shielding off the positively charged sites in the viral

envelope glycoprotein through ionic interactions between the anionic (mainly sulfate) groups in the polysaccharide and the basic amino acids of the glycoprotein (Damonte et al. 2004; Radonic et al. 2010; Rashid et al. 2009), in a similar way to what happens when a virus attaches to a cell through the cell surface heparan sulfate receptor (Witvrouw and De Clercq 1997). Therefore, the **EPS** could compete with the amino acids of the virus glycoprotein, blocking the viral adsorption process, in an identical way to what happens in relation to bacteria, already mentioned. There is evidence that carrageenan, a sulfated polysaccharide from a macroalgae, could directly bind to human papillomavirus capsid (Buck et al. 2006). The **EPS** from *P. cruentum* is a good candidate to protect against viruses (Raposo et al. 2013), since uronic acids, along with the half-ester sulfate groups and the carboxyl groups of the **EPS**, contribute to its anionic properties. Raposo et al. (2014) found that the **EPS** from a Spanish strain of *P. cruentum* revealed, in general, greater antiviral activity than the **EPS** from an Israeli strain, and this fact might be related to the higher degree of sulfation of the former, although they did not discard other factors.

6 Safety and Regulatory Aspects

The safety hazards of a **PS** as food or food ingredient is directly or indirectly associated to the microalgae which it was extracted or secreted from, respectively.

Some microalgae have been widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives, and some have the GRAS status being attributed by the FDA (Food and Drug Administration) of the USA (Table 5).

In the EU, several *Chlorella* species and *A. platensis* were on the market as food or food ingredient, and consumed to a significant degree, before 15 May 1997. Thus, in general, they are not subject to the Novel Food Regulation EC No. 258/97. However, in some EU Member States, specific legislation may restrict the placing of these products in the market, and it is recommended to check this issue with the national competent authorities.

Safety hazards related to algae may include allergens and toxins, heavy metals and pesticides, and pathogens (van der Spiegel et al. 2013). Allergenicity has been reported for the cyanobacteria *Phormidium fragile* and *Nostoc muscorum* (Sharma and Rai 2008) and the green algal genus *Chlorella* (Tiberg and Einarsson 1989). No toxins have been found in *Arthrospira* and *Chlorella*, but toxic microcystines were detected in other cyanobacteria (Kerkvliet 2001; Heussner et al. 2012). Extracts from *Aphanizomenon flos-aquae*, *Spirulina*, and *Chlorella*, or mixtures thereof, were cytotoxic (Heussner et al. 2012). Pheophorbides may be formed in *Chlorella*, which give rise to photosensitization in some humans (Kerkvliet 2001). *A. platensis* remarkably reduced the incidence of liver tumors and prevented DBN-induced hepatotoxicity in rats without causing any side effects or organ toxicity (Ismael et al. 2009). Microalgae may also accumulate heavy metals, depending on the conditions under which they are grown (Hung et al. 1996; Wong et al. 1996). The presence of pathogenic microorganisms is another crucial safety aspect that must be considered. If microalgae are cultivated in open tanks, this may result in

Table 5 Some food and feed applications of microalgae and safety aspects

Microalgae/cyanobacteria	Species	Safety aspect	Microalgae/cyanobacteria	Species	Safety aspect
Cyanophytes	*Arthrospira/spirulina*	GRAS	Diatoms	*Navicula*	NT
	Synechococcus	NT		*Nitzschia dissipata*	NT
Prasinophyte	*Tetraselmis*	NT		*P. tricornutum*	NT
Chlorophytes	*Chlamydomonas reinhardtii*	NT		*Thalassiosira pseudonana*	NT
	H. pluvialis	NT		*Odontella aurita*	NT
	Dunaliella	NT		*Skeletonema*	NT
	Chlorococcum	NT	Eustigmatophytes	*Monodus subterraneus*	NT
	Scenedesmus	NT		*Nannochloropsis*	NT
	Desmodesmus	NT	Haptophytes	*Isochrysis*	NT
	Parietochloris incisa	NT		*Pavlova*	NT
	Chlorella	GRAS	Dinoflagellate	*Crypthecodinium cohnii*	GRAS
Rhodophyta	*P. cruentum*	GRAS			

Adapted from Enzing et al. (2014)
NT no toxins known, *GRAS* generally recognized as safe

microbiological contamination from birds, insects, or rodents (Kerkvliet 2001). These safety problems might be eliminated by growing the microalgae in closed bioreactors (Amara and Steinbuchel 2013).

PSs from microalgae might be a valuable material for a wide potential range of uses, including food, feed, and biomedical applications due, in general, to the absence of or to no known toxicity problems. Most toxicological data from the **PSs** from microalgae are driven from in vitro tests. Raposo et al. (2014) found no cytotoxic effects of the **EPS** from *P. cruentum* for the concentration (100 μg/mL) and cell lines tested. Other studies with Vero cells indicated that the cytotoxic effect only occurred for concentrations higher than 250 μg/mL, and some other in vivo assays indicated that this type of polysaccharide does not show any cytotoxic effect at 2 mg/mL (Huleihel et al. 2001).

The treatment of aortic endothelial smooth muscle cultured cells with depolymerized sodium spirulan (NaSP), an **sPS** obtained from a hot water extract of *A. platensis*, resulted in a significant inhibition of the proliferation of the arterial smooth muscle cells, therefore preventing atherosclerosis without exhibiting any toxic effects on the integrity of the vascular endothelial cell layers (Yamamoto et al. 2006). Some **sPSs** may interfere with blood coagulation and, therefore, have the potential to be used in some biomedical applications and not in another (Raposo et al. 2013).

It should be remarked that any **PS** extracted from microalgae/cyanobacteria and refined to be used as food is considered a new product and, thus, falls under the Novel Food Law of EU. For biomedical applications the novel biological products, in the USA, must comply with FDA Biologics Control Act (21 CFR 600 Biological Products General, Subpart A – General Provisions, Sec.600.3 Definitions) (Whiteside 2011). In the EU the term "biological product" was first published in the Directive 2003/63/EC, amending Annex I of the Directive 2001/83/EC (Noffz 2011). This implies that companies have to provide information on the safety of the food product (including results of animal testing) to the EFSA or FDA, before commercialization is authorized. For instance, carrageenans, which are polysaccharides from seaweeds, not from microalgae, are used as a food additive (E-407), have very low toxicity, and have been shown not to be teratogenic (Necas and Bartosikova 2013). However, the poligeenan, formerly referred to as degraded carrageenan, is not a food additive, exhibiting toxicological properties at high doses (Cohen and Ito 2002). Nilson and Wagner (1959) found no adverse effects on rats lifelong fed with kappa-/lambda-carrageenan from *C. crispus* or *G. mamillosa* at concentrations up to 25 %, while in two other unidentified strains of carrageenan, they found evidence of hepatic cirrhosis, but only at a concentration of 25 % and with no effect on mortality. Therefore, any **PS** from a novel source must be tested prior to be used in human applications.

7 Bioavailability and Metabolism

To our knowledge, there are no known bioavailability studies on the **PSs** from marine microalgae, the bioavailability of **PSs** from microalgae being yet to be studied on humans (Raposo et al. 2013). The **EPS** from *P. cruentum* is not

hydrolyzed in the gastrointestinal tract; therefore, its bioavailability is null or very reduced (Arad et al. 1993). However, some knowledge may be withdrawn from studies carried out with polysaccharides from macroalgae, plants, or microorganisms of structures similar to the **PSs** from microalgae.

Sulfated **PSs** from seaweeds, such as fucoidan from brown seaweeds (Senthilkumar et al. 2013) and carrageenans, alginates, and porphyrans from red seaweeds, are known to have biological effects that could be useful in the prevention or reversal of metabolic syndrome (Holdt and Kraan 2011).

As discussed previously in point 4.3, **PSs** could act as prebiotics. In fact, these **PSs** cannot be digested by human endogeneous enzymes, belonging, therefore, to the dietary fibers (Baird et al. 1977). They are able to modify gastrointestinal hormone secretion, glycemia regulation, and lipid metabolism, preventing, in this way, obesity (Parnell and Reimer 2012). One of the physicochemical properties of **PSs** is the ability to be fermented by the human colonic microbiota, resulting in beneficial health effects (Mišurcová et al. 2012). Also, in vitro and in vivo animal studies highlighted anti-hyperlipidemic and anti-hyperglycemic activities of **PSs** from microalgae (Raposo et al. 2013).

8 Clinical Trials

Reporting to what was already mentioned in Sect. 4 on bioactivity and applications, **PSs** constitute a good source for potential development of novel food ingredients and biomaterials. However, the exploitation of the biomedical potential of these **PSs** will be a long and challenging road, as the regulatory context of medical devices and, in this case, advanced therapy medicinal products are very demanding. Also, the lack of industrial-scale extraction and purification of many of these molecules is an obstacle for their application development. In fact, any clinical application will demand for the implementation and validation of industrial manufacturing methods. Besides this issue, the natural provenience of **PSs** imposes a strict control of their purity, stability, and safety, which imply extensive and, above all, expensive studies (Silva et al. 2012).

In spite of all these considerations, a large number of preclinical essays have already been conducted with microalgae. Recently, extensive studies have been performed to evaluate the therapeutic benefits of microalgae on several disease conditions including hypercholesterolemia, hyperglycerolemia, cardiovascular diseases, inflammatory diseases, cancer, and viral infections. Some studies reported the antioxidant and/or anti-inflammatory activities of *Spirulina* or its extracts, containing **PSs**, in vitro and in vivo, suggesting that *Spirulina* may provide a beneficial effect in managing cardiovascular conditions (Deng and Chow 2010). Some of the preclinical trials of polysaccharides from marine microalgae that have been carried out are listed in Table 6.

With respect to clinical essays and applications in the market, the area of cosmetics is one of the few known. An **EPS** from a cyanobacterium was reported to be effective in skin aging (Loing et al. 2011). There is a recent US patent defending the use of **sPSs**

Table 6 Preclinical trials of polysaccharides from marine microalgae (see also Table 4)

Microalgae species	Compound	Experimental model	Effects	References
Arthrospira sp.	Biomass	Several models	Hypolipidemic, antioxidant, and anti-inflammatory activities	Deng and Chow 2010
A. platensis	CaSp	Raw macrophages	Synthesis of TNF-α	Parages et al. 2012
Spirulina sp.	Spirulan-like substance	Several types of cell cultures	Antiviral activity against human cytomegalovirus (HCMV), herpes simplex virus (HSV-1), human herpes virus type 6 (HHV-6), human immunodeficiency virus type 1 (HIV-1), non-susceptibility of Epstein-Barr virus (EBV), and human influenza A virus (A/WSN/33)	Rechter et al. 2006
N. flagelliforme	Nostoflan	Vero, HEL, MDCK, and HeLa cells	Antiviral activity against HSV-1 (HF), HSV-2 (UW-268), HCMV (Towne), influenza (NWS)	Kenji et al. 2005
C. pyrenoidosa; *C. ellipsoidea*	Polysaccharide complex	Natural killer cells	Immunostimulating properties, inhibition of the proliferation of *Listeria monocytogenes* and *Candida albicans*	Barrow and Shahidi 2008
C. ellipsoidea	Chlorellan	Reticuloendothelial system in rats	Stimulate phagocytic activity of the reticuloendothelial system	Kojima et al. 1974
P. cruentum	(**EPSs**) degraded by Hermetic microwave and H2O2 under ultrasonic waves	Mouse tumor model, peritoneal macrophage activation, splenocyte proliferation assay	Antitumor and immunomodulatory activities of different molecular weight	Sun et al. 2012
Porphyridium sp.	Polysaccharide	NIH/3T3 cells	Antiviral activity against retrovirus MuSV-124 and MuSV/MuLV	Talyshinsky et al. 2002
P. aerugineum				
P. cruentum	**EPS**	Confluent cultures of human erythroleukemia cell line (HEL)	Antiviral activity against HSV type 1 (HSV-1; strain KOS), HSV type 1 (HSV-1; strain TK-KOS ACVr) and type 2 (HSV-2; strain G), vaccinia virus, and vesicular stomatitis virus	Raposo et al. 2014

in cosmetics. Indeed, microalgae extracts, mainly from *Arthrospira* and *Chlorella* (Stolz and Obermayer 2005; Spolaore et al. 2006), are incorporated in many face and skin care products (e.g., antiaging cream, refreshing or regenerating care products, emollient, and anti-irritant in peelers), sun protection, and hair care products (Martins et al. 2014). Alguard™ is a purified **sPS** from *P. cruentum* that is used in cosmetics worldwide as antiaging, anti-inflammatory, anti-irritant, skin maintenance, UVB damage prevention, soothing and healing creams, and lip balms. Also a mixture of **PSs** from microalgae makes part of the composition of a commercial product called Algenist™ antiaging skin care formulas in the form of alguronic acid.

There is a patent (WO 2007066340 A1, Arad and Atar 2007) defending the use of a material composed of algal polysaccharides for viscosupplementation in the treatment of degenerative joint disorders related to joint lubrication, preferably osteoarthritis, rheumatoid arthritis, gout, trauma, and age-related degeneration.

9 Conclusion

Microalgae are easy to grow organisms, and, in comparison with seaweeds, their culture conditions are easily controlled in closed systems, thus avoiding safety hazards. The chemical composition and structure and the rheological behavior of the polysaccharides they produce are relatively stable no matter the period/phase of harvest. However, the polysaccharides synthetized and/or released by marine microalgae can be so heterogeneous and structurally different that research on these compounds can be a very challenging task.

Polysaccharides may be regarded as key ingredients for the production of bio-based materials in life sciences (e.g., food, cosmetics, medical devices, pharmaceutics). The biological source and biodegradability of these biopolymers, coupled to the large variety of chemical functionalities they encompass, make them promising compounds. Despite having showed several interesting properties, including for human nutrition and health, their use in humans and clinical trials and bioapplications are yet to be explored, one of the reasons being the high molecular weights of the polymers. It would be an interesting issue to explore its use orally and therapeutically in human subjects, considering their anti-inflammatory, hypoglycemic, and anticoagulant/antithrombotic activities. However, the toxicity and bioavailability of such compounds are yet to be studied on humans. Although there are a large number of products on the market with biomass or extracts from microalgae, there are very few commercial products from polysaccharides isolated and purified on the market, but the outlook for such products is considered of major importance. Only a few patent applications on microalgae polysaccharides exist. However, the properties of polysaccharide-based products indicate great potential in the food and biomedical sectors.

Other areas of interest, due to their biochemical characteristics and rheological behavior, could be in engineering fields, such as naval (as drag-reducing agents), in food science/engineering, or in biomedical applications, as joint biolubricants and in arthritis treatment.

The extensive use of marine microalgae polysaccharides in these fields, however, would require a reliable supply of raw materials to guarantee the affordable price, sufficient purity, and constant high quality of these bioproducts. In addition, the possibility of obtaining polysaccharides in high quantities from modern microalgae biorefineries might be a competitive advantage with other sources.

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Pectic Polysaccharides and Their Functional Properties

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Beda M. Yapo and Dago Gnakri

Contents

Abstract

Since their first discovery in 1790–1825, pectins are still fascinating plant and food scientists who continue to carry out numerous structural as well as functional studies on them. This great interest of scientists for pectins is accounted for by their large spectrum of (bio)functionalities, starting from their natural location in plant cell walls as bioactive components for cell growth, defense, and protection via diverse manufactured food and nonfood products as techno-functional (gelling, emulsifying, film-forming, etc.) agents to terminate in human welfare as health-benefit (prebiotic, anticomplementary, antioxidant, anticancer, etc.) agents. The extraordinary functional versatility of pectins is thought to be intimately related to fine structure. Unfortunately, structurally, pectins are extremely diversified that establishment of structure-function relationship appeared so far a difficult task to go through. On the other hand, the extended structural variability of pectins presages for the finding of new functions hitherto unknown. Nevertheless, for some structurally well-known pectic cobiopolymers such as homogalacturonan, solid evidence for

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structure-related functions, especially gelling properties, has been provided, including new insights very recently.

After a brief introduction on the "pectin structural repertoire," the main sources of industrial pectins will be exposed, followed by a succinct structural description of the different pectic block cobiopolymers, commonly referred to as "pectic polysaccharides." Finally, some remarkable structure-related functions, namely, gelling, emulsifying/emulsion-stabilizing, and antitumor properties of pectins will be revisited in the light of the latest work.

Keywords
Pectins • Sources • Production • Structures • Properties

1 Introduction

Pectic substances (or merely pectins) are a group of extremely diversified polysaccharides from plant cell walls, mucilages, and exudates. No less than 17 different glycosyl residues (Vincken et al. 2003), such as α-D-galactopyranosyluronic acid (α-D-Gal*p*A), α-L-rhamnopyranose (α-L-Rha*p*), β-D-galactopyranose (β-D-Gal*p*), α-L-arabinofuranose (α-L-Ara*f*), β-D-xylopyranose (β-D-Xyl*p*), and β-D-apiofuranose (β-D-Api*f*), to name a few, are believed to be sugar constituents of pectins. Several structural studies using combinatory approaches including enzymatic and chemical fingerprints have revealed, in heterogeneously complex pectic macromolecules, that these glycosyl residues are not randomly distributed, but rather concentrated in different regions, giving rise to various block cobiopolymers. To date, the pectin group may encompass at least eight distinct block cobiopolymers (often referred to as "pectic polysaccharides"), namely, unbranched (or linear) homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), xylogalacturonan (XGA), apiogalacturonan (A_pGA), galacturonogalacturonan (GaGA), galactogalacturonan (GGA), and arabinogalacturonan (A_rGA) (Schols and Voragen 1996; Caffall and Mohnen 2009; Ridley et al. 2001; Yapo 2011a). The latter six are sometimes gathered under the generic terms of "substituted galacturonans." This striking structural diversity renders pectins functionally versatile with multiple biological (plant cell growth, defense, protection against harmful hosts, etc.), techno-functional (gelling, emulsifying/emulsion-stabilizing, thickening, film-forming, etc.), and health-benefit (immunological, prebiotic, anticomplementary, antioxidant, antitumor, etc.) properties. Pectin functionality is, indeed, intimately related to fine structure (Sørensen et al. 2009; Sila et al. 2009).

2 Marketable Pectins: Sources and Production

Most commercial (or industrial) pectins, mainly intended for the preparation of gelling food products, are produced from citrus (lemon, lime, orange, and grapefruit) peel and apple pomace (May 1990; Voragen et al. 1995; Sriamornsak 2003; Srivastava and Malviya 2011), two raw materials from the western juice industry,

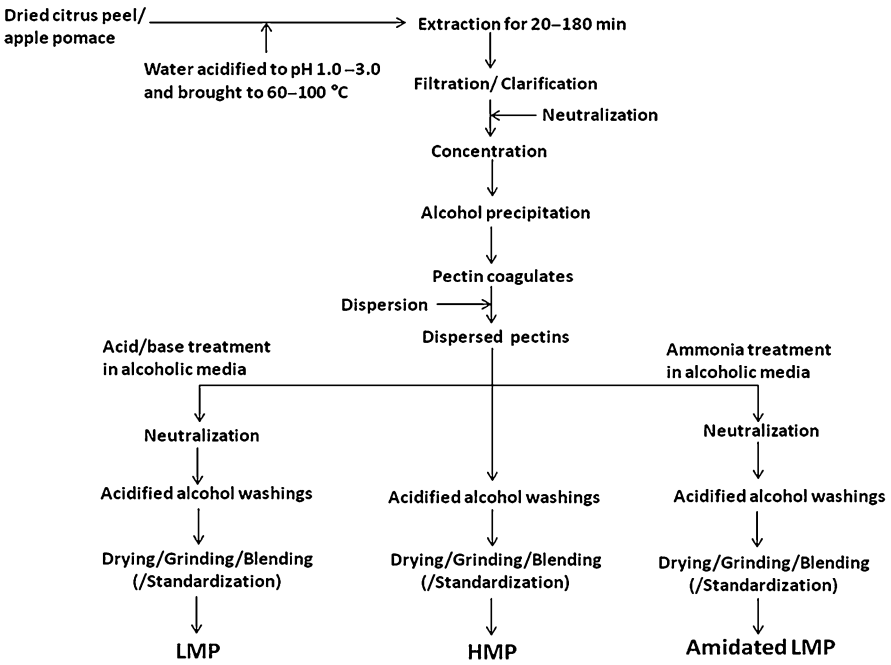

Fig. 1 Simplified scheme for the production of marketable HMP, LMP, and amidated LMP (LMP = low methoxy pectins; HMP = high methoxy pectins)

available in large quantities. The two industrial by-products are pectin-rich sources, with a pectin content of 15–30 % (for citrus peel) and 10–15 % (for apple pomace) on a dry weight basis. Moreover, acid-extracted citrus and apple pectins are usually high methoxy pectins (HMP) with high viscosity-average molecular weight ($\overline{Mv}$ ~100–300 kDa) and galacturonic acid content no less than 65 % on a dry weight basis as required by the international market. These features enable one to prepare good sugar-acid-mediated high methoxy pectin gels (HMP-SAG). In addition (amidated) low methoxy pectins (LMP) with good gelling properties can be produced from initially extracted citrus or apple HMP by partial de-esterification with acid (commonly)/base or with ammonia in alcoholic media (Fig. 1).

It is worth underlining that other commercially viable sources of pectins are sugar beet pulp (for producing pectin emulsifiers), sunflower head residues (for naturally (nonchemically tailored) LMP), and some tropical fruit by-products such as mango peel (for HMP), yellow passion fruit rind (for naturally HMP and LMP), and cashew apple pomace (for naturally LMP) (Schieber et al. 2001; Yapo and Koffi 2014). Passion fruit rind, for example, is used in some emerging countries such as Brazil for manufacturing marketable pectins.

Commercial pectins are usually extracted by acidified hot water under specified extraction conditions kept secret by producers but generally thought to be as follows: dry raw material to solvent ratio 1:35–1:15 (w/v), water acidified with

HNO_3 (or HCl) to pH 1.0–3.0, temperature 60–100 °C, and duration 20–180 min (Fig. 1). The pectin slurry is then filtered, concentrated, and precipitated with alcohol, usually isopropanol. The gelatinous pectin material is then extensively purified by acidified alcohol washings to remove various impurities including pigments and free saccharides of small sizes and salts, thereby converting pectin galacturonates into pectin galacturonic acid residues (the acid form of the pectin product). During this treatment, neutral sugar chains of solubilized pectin polymers are further hydrolyzed, which results in increase of the pectin galacturonic acid content. As pectins are extracted from natural plant materials in a practically unchanged form, they demonstrate different properties depending on the quality of the raw material (Herbstreith 2014a, b). Therefore, quality control and standardization of the pectins are very important criteria. The yield, sugar composition, esterification degree, and functional properties of pectins are influenced by extraction conditions, and therefore optimization is always a prerequisite for every new pectin source that is being investigated for marketing purposes.

To manufacture low-calorie gelling products, LMP which form Ca^{2+}-induced gels are needed. This compels producers to add a demethylesterification step to the production process yielding HMP. Usually, commercial LMP are produced by acid demethylation from primarily extracted citrus or apple HMP. In this case, initially methylesterified pectin GalA residues are randomly demethylated, which gives LMP products with randomly distributed unesterified GalA residues in the pectin chains. Ammonia treatment can also be applied, in which case methyl groups are partially replaced by amide groups, thereby yielding amidated LMP with randomly distributed unesterified GalA residues in the pectin chains. As required by the market, the pectin degree of amidation (DAm) should not exceed 25 %.

"Nonchemical" demethylation may also be performed by using demethylating enzymes, viz., fungal (f-PME) or plant (p-PME) pectin methylesterases. This produces LMP with non-blockwise (by f-PME) or blockwise (by p-PME) distribution of unesterified GalA residues within the pectin chains. More than acid demethylation, enzymatic demethylation is kinetically precisely controlled to produce LMP with the exactly desired degree of methylesterification (or degree of methoxylation, DM) without significant modification of the pectin molecular weight. However, this requires the use of highly purified PME preparations and will obviously add to the cost of production. For all these reasons, no commercial LMP have so far been enzymatically tailored.

Various extraction methods, which are relatively new, such as microwave-assisted extraction (MAE) and ultrasound-assisted (UAE) extraction, have been compared with the conventional acid extraction on a laboratory scale. Most studies showed that MAE or UAE gives higher amount of pectins with often higher viscosity-average molecular weight for considerably shorter extraction duration (Fishman et al. 2003; Wang et al. 2007; Bagherian et al. 2011). In addition, an environmentally friendly aspect of MAE and UAE methods using water solvent instead of dilute acid has also been put forward by some workers (Maran et al. 2013) with respect to generated effluents. On an industrial scale, however, the practicability of these "new methods" remains to be proven.

3 Structural Characteristics of the Different Pectic Cobiopolymers

3.1 Homogalacturonan

Homogalacturonan (HG) is generally the most abundant pectic polysaccharide, accounting for more than 50 % of complex pectins from various sources and up to 90 % of citrus pectins (Ridley et al. 2001; Voragen et al. 2009; Yapo 2011b). HG is an unbranched polymer of 1,4-linked α-D-Gal*p*A residues (Fig. 2), partially methylesterified at *C*-6 position and sometimes acetylesterified at *O*-2 and/or *O*-3 positions. The degree of methylesterification (DM) is the number of α-D-Gal*p*A residues, out of 100, esterified with methyl alcohol groups, and the degree of acetylesterification (DAc) corresponds to the number of α-D-Gal*p*A residues, out of 100, esterified with acetyl groups, assuming one acetylation per α-D-Gal*p*A residue. Depending on the value for DM of pectin HG, low methoxy pectins (LMP, DM <50 %) can be distinguished from high methoxy pectins (HMP, DM ≥50 %). Native HG may be (almost) fully methylesterified and only later partially de-esterified to about 70–80 % by plant PME. HG is renowned for its ability to form gels, a property widely utilized in the food industry and in all likelihood a property that determines some of the functions of (HG-containing complex) pectins in primary cell walls (Albersheim et al. 1996). Both DM and DAc of pectin HG influence its functional properties. The average degree of polymerization ($\overline{DP}$) of individual native HG is not known with certainty, but may be approximately of 50 in cell walls of commelinoid-related monocot species and a twofold higher in cell walls of dicotyledonous species at maturation stage (Voragen et al. 2009; Yapo 2009). The amount and $\overline{DP}$ of pectin HG also affect the pectin functional (especially gelling) properties (Yapo 2009).

3.2 Rhamnogalacturonan-I

Rhamnogalacturonan-I (RG-I) is generally quantitatively the second pectic cobiopolymer, which may represent 10–25 % of complex pectins and up to 45 % of sugar beet pulp, potato tuber, and tobacco leaf pectins (Ridley et al. 2001; Voragen et al. 2009; Yapo 2011a). RG-I is a structurally complex polysaccharide with a [→2)-α-L-Rha*p*-(1 → 4)-α-D-Gal*p*A-(1→]$_{n\geq 1}$ backbone partly branched, at *O*-4 (mainly) and/or *O*-3 positions of α-L-Rha*p* residues, with neutral sugar side chains of various types and sizes, viz., (1 → 5)-α-L-arabinan, (1 → 4)-β-D-galactan, arabinogalactan-I, and galactoarabinan (Fig. 3), and probably also by arabinogalactans-II.

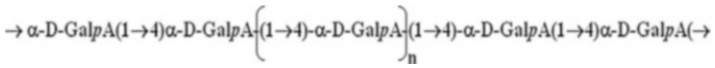

Fig. 2 Schematic representation of homogalacturonan

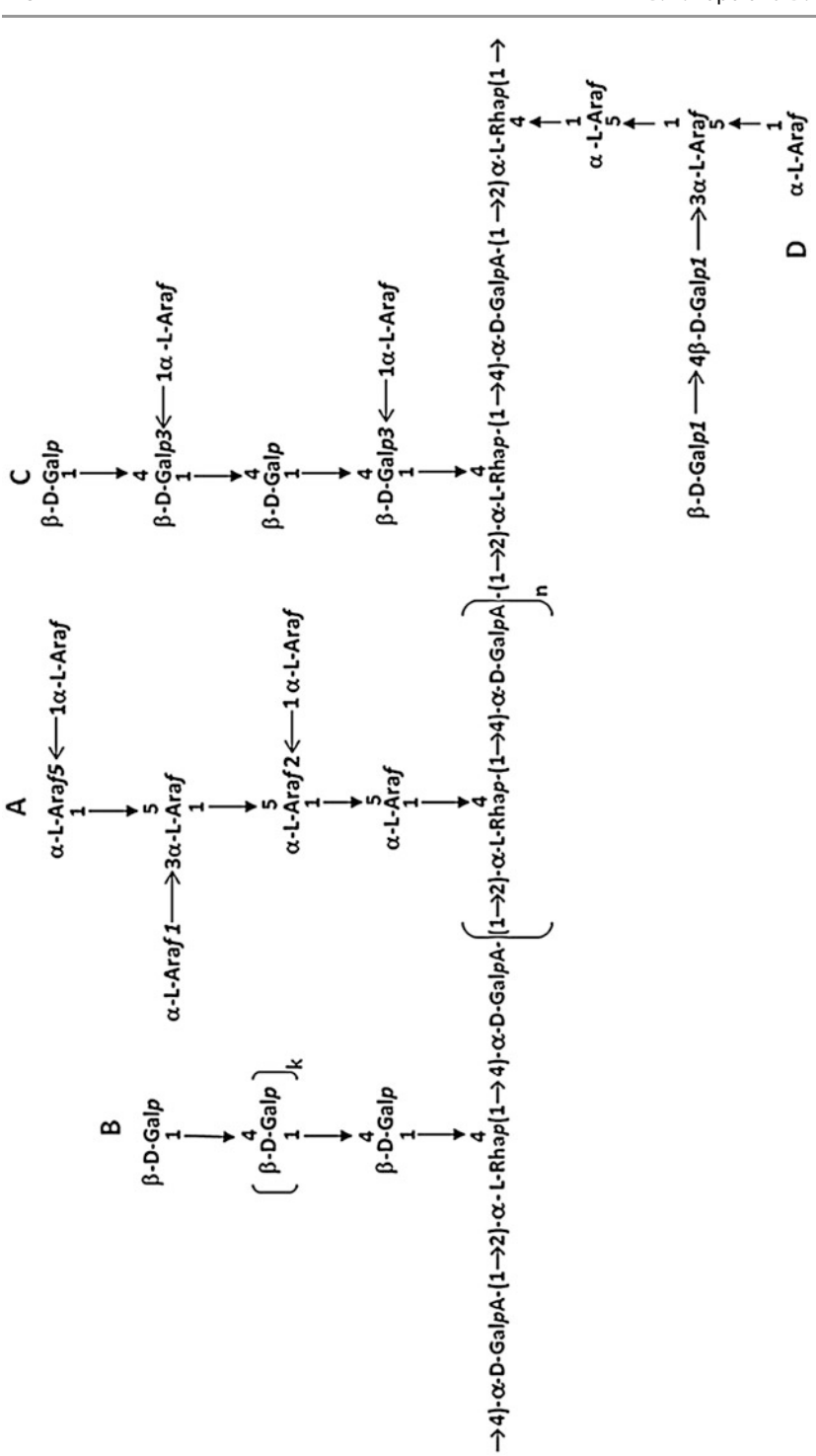

Fig. 3 Schematic representation of rhamnogalacturonan-I (With permission from Yapo (2011a). Copyright Taylor & Francis Group)

The $\overline{DP}$ of the backbone of individual native RG-I is not known with certainty and may be dependent on the polysaccharide origin; for example, ~30–40 diglycosyl repeats for citrus RG-I, 80 repeats for beet RG-I, and as many as 100–200 diglycosyl repeats for RG-I from suspension-cultured sycamore cells (Albersheim et al. 1996; Yapo 2011a). RG-I is structurally extremely varied, depending on plant source, organ, and even on location within the same cell tissue that it is nowadays viewed as a family of molecularly diversified polysaccharides with the same diglycosyl repeating backbone carrying side chains of different types and sizes.

3.3 Rhamnogalacturonan-II

Rhamnogalacturonan-II (RG-II) is undoubtedly the most ubiquitous of substituted galacturonans, being purified from primary cell walls of seedless plants such as ferns (e.g., *Ceratopteris thalictroides*), horsetails (e.g., *Equisetum hyemale*), and lycopods (e.g., *Lycopodium nummularifolium*) and all higher plants hitherto examined for. RG-II is, however, a quantitatively minor block cobiopolymer of complex pectins, accounting hardly for 0.1 % of the pectin-poor primary cell wall of commelinoid-related monocotyledonous species and for 0.5–8.0 % of the pectin-rich primary cell wall of non-commelinoid monocotyledonous and dicotyledonous species (O'Neill et al. 2004; Yapo 2011c). It is also remarkably structurally conserved throughout vascular plants and is believed to exist in the primary cell wall, predominantly as a borate diester cross-linked dimer (dRG-II) rather than as a monomer (mRG-II) (O'Neill et al. 2004). In vitro interconversion of dRG-II into mRG-II or vice versa is now well demonstrated. This pectic cobiopolymer, with an average molecular weight of 5.0–10.0 kDa, has an oligogalacturonan backbone ($\overline{DP}$ 11–15), which is branched with four well-defined side chains (A, B, C, and D) containing six unusual and specific (or diagnostic) sugars, namely, apiose (Api; 3-*C*-(hydroxymethyl)-*β*-D-erythrose), 2-*O*-methyl-fucose (2-*O*-Me-Fuc), 2-*O*-methyl-xylose (2-*O*-Me-Xyl), aceric acid (AceA; 3-*C*-carboxy-5-deoxy-L-xylose), 2-keto-3-deoxy-D-*manno*-octulosonic acid (KdoA), and 3-deoxy-D-*lyxo*-heptulosaric acid (DhaA) (Fig. 4). RG-II is, therefore, structurally different from RG-I in that rhamnose is not present in its backbone, but rather in three (A, B, and C) out of the four authenticated side chains and has only been named so for distinction sake.

3.4 Xylogalacturonan

Xylogalacturonan (XGA) is the second known substituted galacturonan. This pectic cobiopolymer has an (1 → 4)-linked α-D-galacturonan backbone, which is partially substituted at *O*-3 (mainly)/*O*-2 position by single nonreducing β-D-Xyl*p* residues and/or by relatively short side chains ($\overline{DP}$ 2–8) of 1 → 2/1 → 3/1 → 4/1 → 2,3/1 → 2,4/1 → 3,4-linked β-D-xylans (Fig. 5). The degree of substitution by β-D-Xyl*p* residues of the galacturonan backbone can range from approximately 20 % to

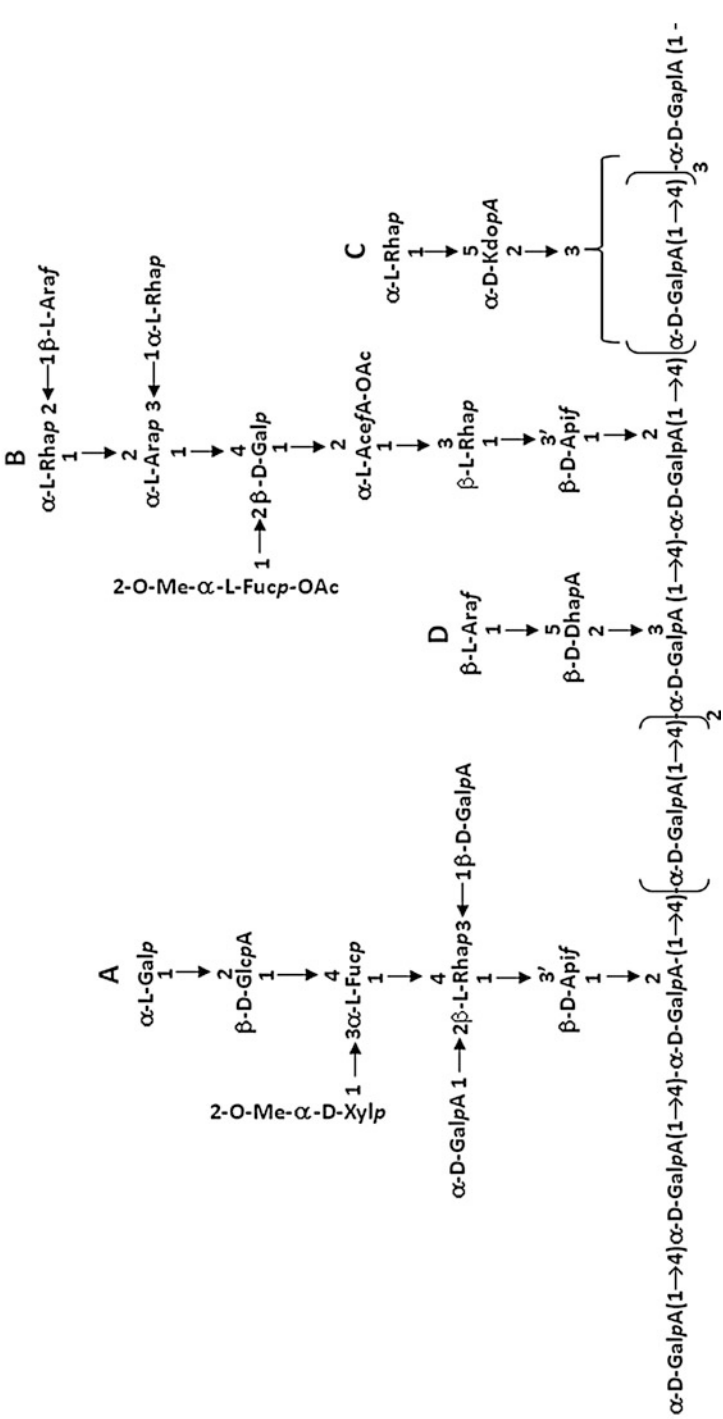

Fig. 4 Schematic representation of rhamnogalacturonan-II (With permission from Yapo (2011c). Copyright Hindawi Publishing Corporation)

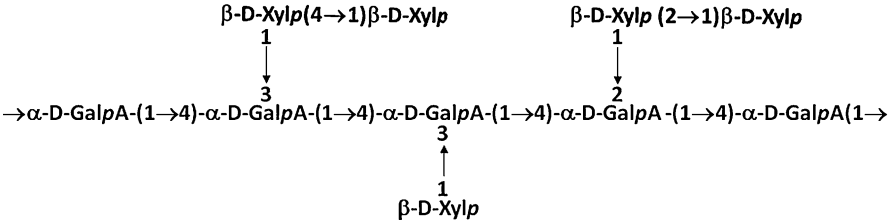

Fig. 5 Schematic representation of xylogalacturonan

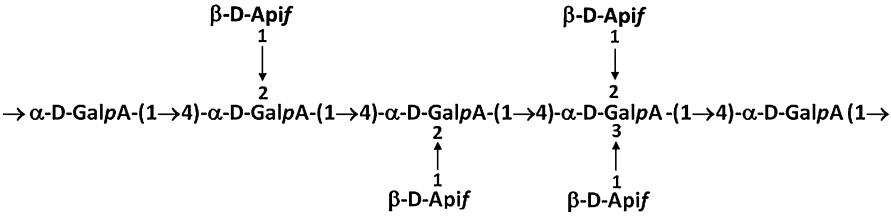

Fig. 6 Schematic representation of apiogalacturonan

100 %, depending on the polysaccharide origin. The $\overline{DP}$ of the galacturonan backbone of XGA is not known with certainty, but may be comparable to that of HG. Furthermore, the β-D-xylan side chains of XGA may be substituted with α-L-Ara*f*, α-L-Fuc*p*, and/or β-D-Gal*p* units. The DM of XGA is dependent on the polysaccharide source and may vary from about 40 to 90 %. In complex apple pectins, XGA is present in the vicinity of RG-I and is therefore an integral part of the so-called pectic hairy regions (Schols and Voragen 1996; Coenen et al. 2007).

3.5 Apiogalacturonan

Apiogalacturonan (A_pGA), an apiose-substituted galacturonan, is a relatively low molecular weight (20–25 kDa) pectic cobiopolymer from the primary cell wall of certain aquatic monocotyledonous species such as the duckweed *Lemna minor* (Lemnoideae) and eelgrasses of the Zosteraceae family (*Zostera marina*, *Z. pacifica*, and *Phyllospadix*). A_pGA has a (1 → 4)-linked α-D-galacturonan backbone, partly substituted at *O*-3 (mainly)/*O*-2 position by single nonreducing β-D-Api*f* (Fig. 6), (1 → 5)-linked β-D-Api*f* residues and/or by "apiobioside" (β-D-Api*f*-(1 → 3′)-β-D-Api*f*-(1→).

The degree of substitution of the galacturonan core may vary from about 5 to 90 %. Moreover, the galacturonan core of A_pGA is partially methylesterified to a usually low DM (15–30 %). The $\overline{DP}$ of the galacturonan backbone of A_pGA is not clearly known, but may be in the order of 65. A_pGA is likely an integral part of

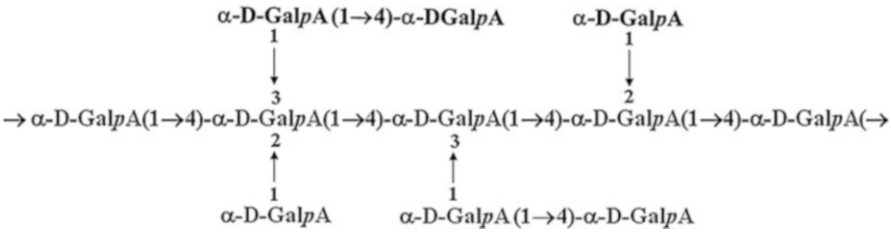

Fig. 7 Schematic representation of galacturonogalacturonan

complex pectins called lemnan and zosteran encompassing additionally blocks of HG and RG-I (Golovchenko et al. 2002). Unlike lemnan and zosteran, A_pGA is soluble in highly salted waters (containing up to 2 M NaCl). This property may be promoted by a high degree of apiosylation of the pectic polysaccharide and is likely related to the ability of these species (Lemnoideae and Zosteraceae) to grow and proliferate in aquatic and marine media. Contrary to zosteran, A_pGA is a rather poor-gelling agent for preparing sugar-acid-mediated gels. It is, however, not known if A_pGA may be a good gelling agent in calcium-mediated gels, considering its rather low DM.

3.6 Other Quantitatively Minor Substituted Galacturonans

Galacturonogalacturonan (GaGA), a galacturonic acid-substituted galacturonan, is a block cobiopolymer of complex pectins isolated from cell wall materials of plant species such as the marsh cinquefoil *Comarum palustre* and *Solanum licopersicum* (tomato) (Ovodova et al. 2006; Round et al. 2010). GaGA has a (1 → 4)-linked α-D-galacturonan backbone, partially substituted at *O*-2 and/or *O*-3 positions by single nonreducing α-D-Gal*p*A residues (Fig. 7) and/or by relatively long side chains of 1 → 3/1 → 4/1 → 3,4-linked α-D-Gal*p*A residues.

Galactogalacturonan (GGA), a galactose-substituted galacturonan, is a block cobiopolymer of various complex pectins such as gum karaya (from *Sterculia* species), zosteran (from *Z. marina*), panaxan (from *Panax ginseng* C. A. Meyer), and soya sauce acidic polysaccharide. GGA has a (1 → 4)-linked α-D-galacturonan backbone, partly substituted by single β-D-Gal*p* residues and/or 1 → 3/1 → 4/1 → 6/1 → 3,6-linked β-D-Gal*p* residues (Fig. 8). The galacturonan backbone of GGA can be highly methylesterified.

Arabinogalacturonan (A_rGA), an arabinose-substituted galacturonan, has a (1 → 4)-linked α-D-galacturonan core branched, via *O*-3 position and/or terminal reducing point, by single (nonreducing) or multiple side-stubs of α-L-Ara*f* residues (Fig. 9). This cobiopolymer has been isolated from complex pectins extracted from miscellaneous plant materials such as apple fruits, field-bean hulls, *Plantago psyllium* seeds, *Plantago major* leaves, *Amabilis* fir bark, alfalfa leaves and stems, and carnation (*Dianthus caryophyllus*) roots.

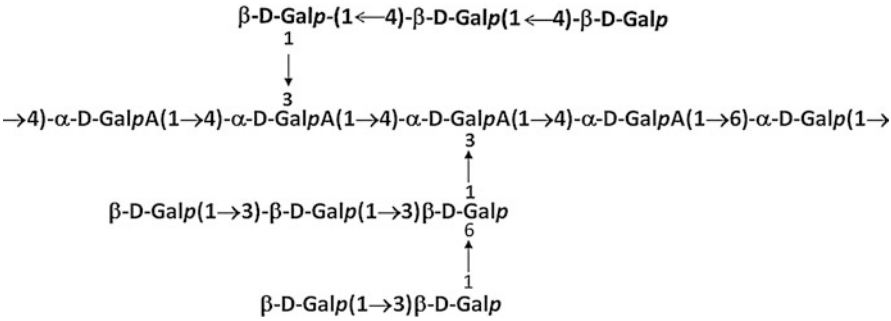

Fig. 8 Schematic representation of galactogalacturonan

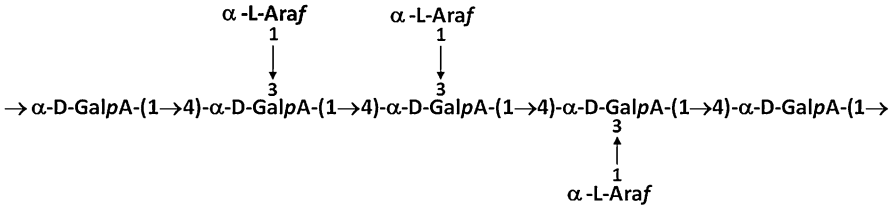

Fig. 9 Schematic representation of arabinogalacturonan

4 Structural Aspect of Complex Pectins

Native pectins in plant cell walls may generally be viewed as multi-polymer complexes which are formed by interconnections of various "pectic polysaccharide" elements, including block cobiopolymers of HG (predominantly), RG-I, and substituted galacturonans such as RG-II and to a lesser extent XGA (Albersheim et al. 1996; Schols and Voragen 1996; O'Neill and York 2003; Ovodov 2009; Voragen et al. 2009; Yapo 2011a). However, the type(s) of substituted galacturonans present in complex pectins may be dependent on the source. For example, in complex pectin fractions from cell wall materials of the marsh cinquefoil *Comarum palustre* and tomato, GaGA and/or RG-II appeared to be attached to HG and RG-I. In complex apple pectins, RG-II and XGA are associated to HG and RG-I. Zosteran and lemnan from *Z. marina* and *L. minor* (respectively) are composed of blocks of A_pGA, HG, and RG-I. In all cases, HG and RG-I elements appeared to be the most widespread in complex pectins, and therefore, most general schemes proposed for (complex) pectin structure included at least the two types of pectic polysaccharides, often referred to as pectin "smooth" (HG) and "hairy" (RG-I) regions. To date, three key hypothetical models exist in the literature. These are (i) the alternating "smooth" and "hairy" regions model (Schols and Voragen 1996; Fig. 10a), (ii) the "RG-I backbone" model (Vincken et al. 2003; Fig. 10b), and (iii) "the living thing-like" model (Yapo 2011b; Fig. 10c).

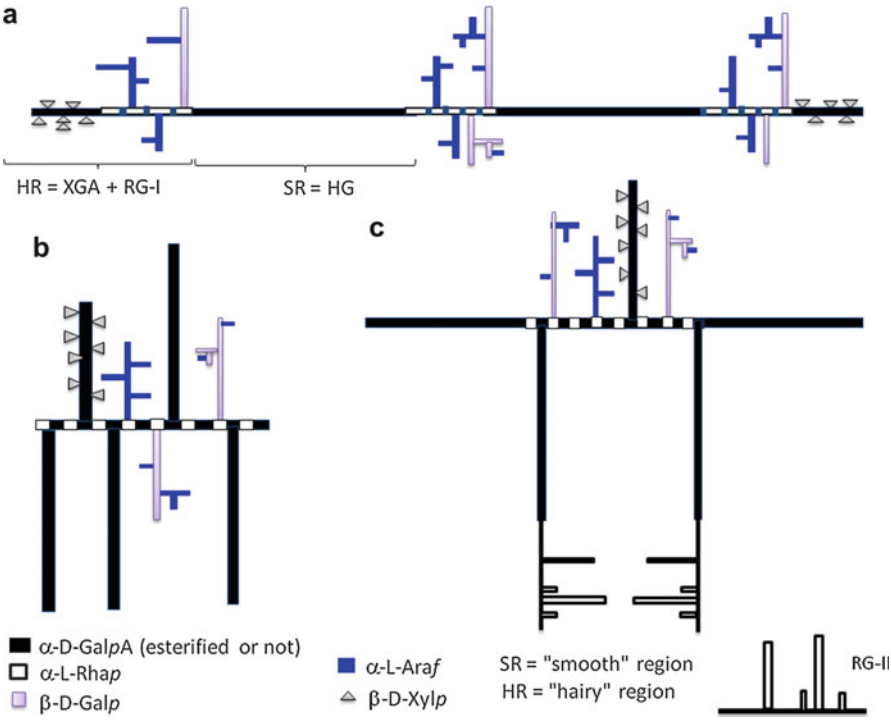

Fig. 10 The key hypothetical models proposed for pectins as multi-block cobiopolymer complexes of HG, RG-I, XGA, and/or RG-II (With permission from Yapo (2011b). Copyright www.Elsevier.com)

5 Functional Properties of Pectins

Pectins are undoubtedly the most functionally versatile biopolymers, with a large spectrum of biological (plant cell growth, defense, protection against harmful hosts, etc.), techno-functional (gelling, emulsifying/emulsion-stabilizing, thickening, film-forming, etc.), and health-benefit (biosorbent, immunomodulating, prebiotic, anticomplementary, antitumor, antioxidant, antimicrobial, etc.) properties. The extraordinary functional versatility of pectins may be related to their high structural variability. The impressive range of activities of pectins has remarkably been reviewed for years by several (groups of) workers (Voragen et al. 1995; Thakur et al. 1997; Sriamornsak 2003; Willats et al. 2006; Ovodov 2009; Sila et al. 2009; Yapo 2011a; Gullón et al. 2013; Espitia et al. 2014). However, new insights into the different mechanisms of action of pectin cobiopolymers are continuously provided for a better understanding of their structure-related properties. Will be dealt with here are the gelling, emulsifying/emulsion-stabilizing, and antitumor properties of pectin multi-block cobiopolymers.

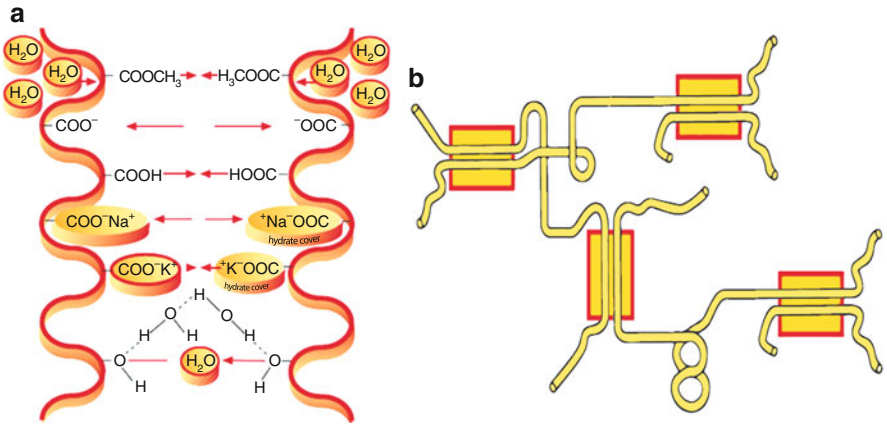

Fig. 11 Schematic representations: (**a**) Gelling mechanisms of high methylester pectins; (**b**) Bonding zones in the gel network. (With permission from Herbstreith and Fox Corporate Group. Copyright www.herbstreith-fox.de)

5.1 Gelling Properties of Pectins

As conveyed by the name per se, "pectin" (*in reference to the Greek word* "*pektikos*," *which means to* "*congeal*, *solidify or curdle*") is a naturally gelling polysaccharide. This oldest functionality of pectins is mainly exploited for manufacturing diverse gelling food products such as marmalades, jams, preserves (for HMP), and low-calorie jellies (for LMP). However, the mechanism of gelation of HMP is different from that of LMP.

HMP form sugar-acid-mediated gels (HMP-SAG). Gelation of HMP requires the presence of 55–65 % soluble sugar (generally sucrose) and acidic conditions (pH 2.0–3.5). Gelation occurs by formation of junction zones between the pectin HG chains, promoted by added cosolutes (sucrose). The soluble sugar added to the dispersed pectin system (intended for gelation), indeed, reduces the water activity and thus fosters pectin chain-to-chain interactions at the expense of pectin-solvent interactions. It may also function as a "coating agent" that aids in strengthening branch points, possibly through cooperative interactions and may influence self-association of pectin chains by "condensing" around them or binding to them (Fishman and Cooke 2009; O'Brien et al. 2009). Acid (low pH condition) prevents deprotonation of unesterified GalA residues of the pectin HG chains, thereby annealing (or at least minimizing) intermolecular electrostatic repulsions. The junction zones formed are then stabilized by interchain hydrogen bonds between protonated carboxyl groups and secondary alcohol groups of the pectin HG chains and by interchain hydrophobic interactions between pectin HG methylester groups (Fig. 11a). Gelation results in formation of vast tridimensional networks in which solvent and cosolutes are all immobilized (Fig. 11b). The gel formed is a thermo-irreversible cohesive system, resisting deformation and showing a stress/strain relationship for small deformation. Studies using atomic force microscopy (AFM)

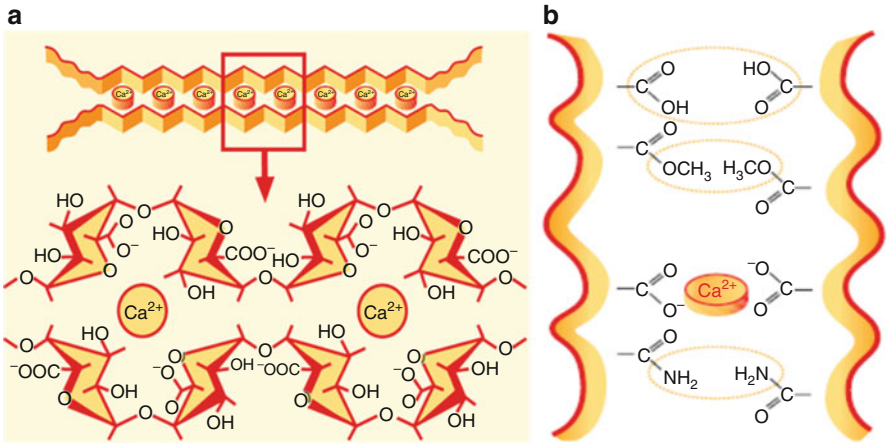

Fig. 12 Schematic representations: (**a**) Gelling mechanism after the "egg-box model" of (non-amidated) low methylester pectins; (**b**) amidated low methylester pectins. (With permission from Herbstreith and Fox KG. Copyright www.herbstreith-fox.de)

have, however, revealed that HMP-SAG comprise partially cross-linked networks, quite deformable in that they are held together by relatively easily disrupted secondary interactions such as hydrogen bonds and van der Waals forces, and this could actually be the basis of the unique ability of pectins to form spreadable gels (Fishman and Cooke 2009). Furthermore, the strength of the gels formed appeared to be correlated to the density of pectin strands and uniformity of pectin (chain) distribution within the gels (Fishman and Cooke 2009).

LMP solutions require, for gel formation, the presence of multivalent cations, especially Ca^{2+}, but not necessarily sugar (sucrose) and low pH conditions. Gelation can indeed occur within a larger pH range (3.0–7.0) whether soluble solids are added or not to the calcium-containing pectin system. This property allows LMP, unlike HMP, to be used in various food systems, such as low-calorie jellies, acidic milk products, yogurts, and desserts. Gelation is believed to result from formation of intermolecular junction zones, via Ca^{2+}-bridges, between unesterified GalA residues of the pectin HG chains, according to the so-called egg-box junction model (Fig. 12a), first posited for polyguluronate-rich alginates (Grant et al. 1973), since (1 → 4)-linked α-L-guluronate is a mirror image of (1 → 4)-linked α-D-galacturonate. The minimum length of sequences of unesterified GalA residues required for formation of stable egg-box junction structures may vary from approximately 6 to 20 residues, corresponding to a minimum array of approximately 3–10 site-bound Ca^{2+} between chains of twofold symmetry (O'Brien et al. 2009; Fraeye et al. 2010; Morris et al. 2011). Once formed, the junction zones are stabilized by van der Waals interactions and hydrogen bonds between protonated carboxyl groups and by electrostatic interactions. Cohesive LMP gels are thermo-reversible, with sometimes appearance of structural changes-related thermal hysteresis after a relatively long period of ageing.

It should be underlined that the ability of LMP to form egg-box-type junction zones does not only depend on its degree of demethylation (100 – DM) but also on intermolecular and intramolecular distribution patterns of unesterified GalA residues of the individual polyelectrolyte chains (Kohn and Luknar 1977; Taylor 1982; Voragen et al. 1995; Fraeye et al. 2010). Thus, the de-esterification pattern, according to the "blocky parameter" known as the absolute degree of blockiness, which represents the ratio of the amount of unesterified (mono, di-, and tri-) GalA residues, liberated by homogenous endopolygalacturonases, to the total amount of (esterified and unesterified) GalA residues within pectin chains, has been found to play a crucial role in the gelling behavior of LMP (Fraeye et al. 2010). In addition, the block size and the block size frequency may actually be the two key factors that govern calcium-mediated gelation of LMP, while stabilization of the gel formed requires junction-zone-terminating structures evolved from pectin RG-I block cobiopolymers (Yapo and Koffi 2013a).

The presence of amide groups improves, under low pH conditions, the gelling ability of LMP (Alonso-Mougan et al. 2002) and the elasticity and stability of the gel formed and decreases its tendency to syneresis by promoting additional hydrogen bonds (Fig. 12b). Thus, amidated LMP are able to form homogenous gels which are less sensitive to calcium ion concentration over a wide range. The presence of acetylester groups in polygalacturonate chains, by contrast, would hinder formation of junction zones, by decreasing the polyelectrolyte affinity for Ca^{2+} ions. This would mainly account for the rather poor gelling ability of amply acetylated pectins from, for example, cauliflower, chicory, cacao pod husk, endive, pumpkin, and sugar beet by-products. Nevertheless, good gelation has been reported for enzymatically deacetylated beet pectins (Oosterveld et al. 2000).

5.2 Emulsifying Properties of Acetylated Pectins

While high acetylation is likely to impair the gelling capability of certain HG-rich pectins, it may, due to the hydrophobic character, at least be partly accountable for the oil-in-water (O/W) emulsifying/emulsion-stabilizing activities of some amply acetylated pectins from various plant by-products such as sugar beet pulp, cacao pod husks, pumpkin pulp, and cauliflower leaves (Dea and Madden 1986; Yapo and Koffi 2013b). On the other hand (almost unacetylated) depolymerized pectins, from citrus, apple, or apricot by-products, containing 1–4 % proteinaceous compounds also appeared able to emulsify O/W systems (Akhtar et al. 2002; Baississe et al. 2010), suggesting that hydrophobic compounds other than acetyl groups might be involved in the pectin-emulsifying activity. Among acetylated pectins, much intention has been focused on (acid-extracted) sugar beet pectin (BP). It is posited that a hydrophobic protein moiety, relatively strongly associated to BP, is responsible for its emulsifying activity, while the hydrophilic polysaccharide part stabilizes the emulsion droplets formed by providing a protective thick layer, which bestows an effective steric stabilization during extended storage (Akhtar et al. 2002). AFM has enabled a group of workers (Kirby et al. 2008) to show

that BP is mainly composed of un-aggregated linear protein-polysaccharide complexes in which a single protein strand is attached to one end of the polysaccharide chains, thereby forming the so-called tadpoles that contribute to its unusually interesting emulsifying properties. However, other studies showed that the freak emulsifying activity of BP cannot only be explained by the presence of tadpole structures but could result from combination of several emulsion-promoting factors, viz., a high acetylation level, medium-sized polymer chains, and high amount neutral sugars that might constitute the interacting zones with protein which confers this activity without the need for pre-depolymerization (Yapo et al. 2007a). It has subsequently been found, in corroboration, that protein is predominantly bound to the neutral side chains of BP (Funami et al. 2011), which shows involvement of branched polysaccharide structures in the emulsifying/emulsion-stabilizing properties of BP. According to the same authors, contribution of BP ferulation might be little to null, in contrast to previous reports (Williams et al. 2005). Furthermore, BP has been found to exhibit, under acidic conditions (pH ~3), a higher emulsifying ability than gum arabic (the hydrocolloid emulsifier so far unequalled), at an approximately sevenfold lower polymer concentration (Nakauma et al. 2008). Because gum arabic is expensive to use in practice and that a rather high gum/oil ratio (~1:1) is required for producing fine stable emulsion droplets (Dickinson 2009), naturally acetylated pectins with demonstrated emulsifying properties may appear to be good alternative to gum arabic, the benchmark hydrocolloid emulsifier.

5.3 Anticancer Properties of Pectins

Among the miscellaneous potential health-benefit effects of pectic polysaccharides, special attention has recently been focused on their antitumor properties. In this connection, the so-called modified citrus pectin (MCP) has gained interest as the (pharmacological) product of choice for investigation. Most studies on the anticancer properties of pectic polysaccharides have indeed been carried out on MCP. Interestingly, the bulk of the studies (Platt and Raz 1992; Eliaz 2002; Nangia-Makker et al. 2002; Glinsky and Raz 2009) showed that MCP could be effective against any of the main four stages (cell adhesion, cell aggregation, angiogenesis, and metastasis) of development of galectin-3-meditated malign tumors (Fig. 13). Nevertheless, the molecular origin of MCP bioactivity has remained unknown hitherto, probably because MCP is in fact a heterogeneous mixture of pectic oligosaccharides (POS) and polysaccharides (PPS), produced by depolymerization of commercial citrus HMP ($\overline{Mv}$ ~100–300 kDa), based on modification of the reaction medium pH and temperature. In addition, preparation methods of MCP products varied widely from workers to workers to such a point that final products appeared considerably different from a molecular point of view.

Depending on the method of preparation, three molecularly different MCP products can be distinguished: polygalacturonan-rich MCP ($\overline{Mw}$ 10–20 kDa), commercially known as PectaSol (MCP_1); galactose-enriched RG-I-like MCP (MCP_2);

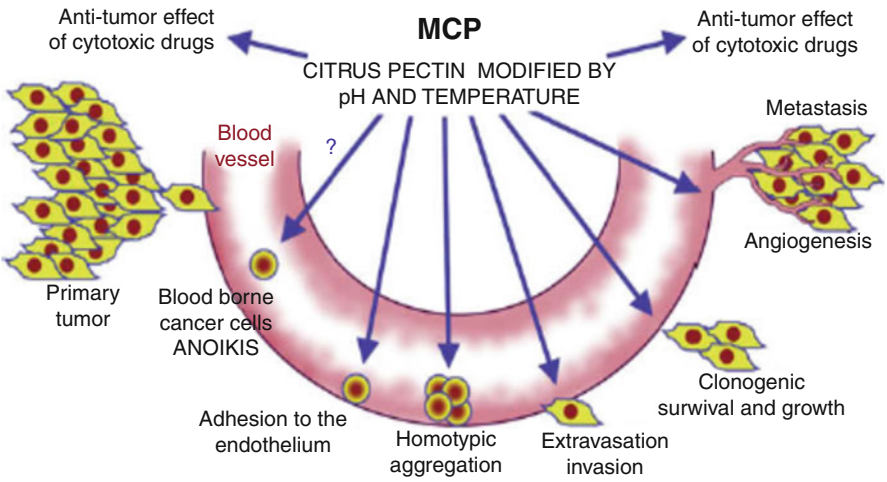

Fig. 13 Schematic representation of critical rate-limiting steps in cancer metastasis, which could be efficiently targeted by MCP. (With permission from Glinsky and Raz (2009). Copyright www.Elsevier.com)

and fractionated citrus pectin powder (MCP_3). The preparation method of PectaSol is not known, for obvious commercial purposes; however, according to the producer, this product belongs to a special class of polysaccharides known as polyuronides with low DM ($\leq$10) and $\overline{Mw}$ (~10–20 kDa). Therefore, MCP_1 is likely produced on the principle of difference in resistance of pectin glycosyl residue linkages to acid agents (Ara*f*-Ara*f* < Ara*f*-Gal*p* $\leq$ Gal*p*-Ara*f* < Gal*p*-Gal*p* < Rha*p*-Gal*p*A $\leq$ Gal*p*A-Rha*p* << Gal*p*A-Gal*p*A) and sounds like a relatively homogenous HG product, as judged from similar data in the literature (Yapo et al. 2007b; Yapo 2009). MCP_2 is obtained by hot alkaline treatment (β-elimination) of commercial citrus pectin followed by mild acid hydrolysis (Platt and Raz 1992; Nangia-Makker et al. 2002) and is classified as a nondigestible galactose-rich polysaccharide fiber of shorter size, readily soluble in water, and better absorbed and utilized by the human body than ordinary high molecular weight citrus HMP. MCP_3 is obtained by treating commercial citrus pectin under neutral pH and high-temperature conditions (Jackson et al. 2007), which causes β-elimination of the pectin HG regions, and is a mixed product of oligogalacturonans and galactose- and/or arabinose-rich-RG-I oligomers of various types. The discrepancy in the three methods adds to the complexity of the problem to solve, thereby reducing chances to unravel, in the short run, the molecular origin of the antitumor properties of MCP.

Furthermore, enzymatically purified okra pod RG-I, carrying short galactan side chains, also exhibited antiproliferative and proapoptotic activities (Vayssade et al. 2010), and hot water-extracted galactan-rich RG-I fractions from various plant materials such as yellow passion fruit rind, banana peel, and tobacco leaf showed efficacious antiproliferative activities in vitro

(Yapo 2011a; Silva et al. 2012). Moreover, specific binding of pectin galactans to galectin-3 has been reported (Gunning et al. 2009). All these recent reports strongly suggest that "modified pectins" may principally exert their antitumor activity, hitherto not clearly understood, at least by binding (via not yet identified linkages) to galectin-3 on the surface on malign cells. Elucidation of this extremely important "pharmacological" of pectins may lie in the use of enzymatically and/or chemically tailored model pectin products that are homogenous in size, in composition, and if possible in charge density.

6 Concluding Remarks

Since their first discovery in 1790–1825 and the correct prediction of Sir Henri Braconnot that pectins would have important functions in all plants and many applications in the art of the "confiseur," these naturally occurring polysaccharides continue to fascinate plant and food scientists who carry out numerous studies on them. However, It is a quite difficult task to deal with the impressive structural diversity and functional versatility of pectins, starting from their natural location within plant cell walls, as bioactive agents for growth, defense, protection of the cells, etc., via their technological properties as gelling, emulsifying/emulsion-stabilizing, film-forming, etc., agents in miscellaneous manufactured food and nonfood products to their physiological and pharmacological effects as health-benefit (biosorbent, immunomodulating, prebiotic, anticomplementary, antitumor, antioxidant, antimicrobial, etc.) agents for the human body. Pectins then represent an outstanding group of heterogeneously complex biopolymers with extraordinary functions, some of which remain to be understood in relation to their fine structure. This fully accounts for the particularly remarkable attention that multidisciplinary scientists should continue to pay to them by developing powerful techniques with the aim of unraveling the intriguing and so far puzzling structures of some pectic cobiopolymers (especially RG-I) and complex native pectins *in muro*, a result of which will surely be a great step toward a full understanding of structure-related functions and improvement of their known properties and finding of new area of applications.

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Recent Advances on the Development of Antibacterial Polysaccharide-Based Materials

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Chitosan, Cellulose, and Starch

Véronique Coma, Carmen S. R. Freire, and Armando J. D. Silvestre

Contents

Abstract

This chapter aims at presenting a comprehensive overview of the latest advances in the development of antibacterial materials based on chitosan, cellulose, and starch, which being the most abundant polysaccharides have a high potential to be explored in large variety of applications (e.g., food or biomedical fields).

Owing to its intrinsic antibacterial and film-forming properties, chitosan can be directly used to create antibacterial materials. Thus, the first part of this chapter focuses mainly on the factors affecting its inherent bioactivity and the strategies potentially used to enhance it, in order to broaden chitosan-based materials applicability. Then the strategies developed to impart antibacterial properties to cellulose and starch, mainly through chemical modification or combination with bioactive natural and synthetic components and polymers as

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well as with metal and metal oxide nanoparticles, were screened in detail, pointing out their antibacterial profile and prospective applications.

Keywords
Antibacterial activity • Cellulose • Starch • Chitosan • Biomedical products and devices • Food preservation

1 Introduction

Microbial contamination in food and medical fields can cause serious problems to human's health and safety.

In food preservation field, despite increased awareness of the importance of high-level hygiene in food supply chain, foodborne illnesses caused by microorganisms including *Salmonella* spp., *Staphylococcus aureus*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli* are still a large public health problem (De Moura et al. 2012; Coma 2008).

The European Food Safety Authority and the European Centre for Disease Prevention and Control reported, after analyzing information submitted by 27 European Union members, that campylobacteriosis was the most commonly reported zoonosis. The decreasing trend in confirmed salmonellosis and listeriosis cases in humans continued but not sufficiently (95,548 salmonellosis cases in 2011). One of the last reports available from the European Union (2006) showed that *Listeria* was the most severe pathogen on average. In addition, substantial increases have been observed, over the past few decades, in the incidence of diseases caused by pathogens *E. coli* 0157 (VTEC) and *Yersinia enterocolitica*, among others (EFSA 2011). It seems that the growing demand for prepared or minimally processed foods has contributed significantly to the increased number of outbreaks of foodborne illnesses (Lagaron et al. 2012).

In the biomedical field, despite the dramatic change in chemotherapeutic approaches, widespread antibiotic resistance and dissemination of communicable diseases remain as the leading causes of morbidity, mortality, and rising health costs. As specified by Harding and Reynolds (2014), infections arising from bacterial adhesion and colonization on medical device surfaces are a significant healthcare problem. The increasing use of invasive medical procedures for the implantation of devices leads to an increased risk for the development of device-associated infections. Estimates place the occurrence of bacterial-related infections for humans at 65 %, and are associated with the growth of bacterial biofilms on device surfaces (Harding and Reynolds 2014); once a suitable environment for attachment is established, bacterial species are capable of adhering to the surface and proliferating into microcolonies, eventually forming biofilms. Increased dependence on artificial implant replacement technology has contributed as new attachment opportunities to opportunistic, as well as to previously nonpathogenic resident flora. As mentioned by Ahmed (2012) as an example, *Staphylococcus epidermidis*

is primarily a human commensal bacterium of skin and mucosal surfaces with low pathogenic potential. With the introduction of implants, *S. epidermidis* became one of the most significant pathogens in biomaterial-associated infections (Schierholz and Beuth 2001). Moreover, there is a specific problem related to the use of nanofibers in medical applications. Nanofibers have shown to be particularly promising in fibrous scaffolds for tissue engineering, drug release, wound dressings, etc. Unfortunately, due to the high affinity for cellular attachment to such kind of scaffolds, many potential applications of nanofibers are limited by the possibility of bacterial adhesion and resulting infections (Coneski et al. 2014).

In this context, the development of antibacterial materials for food and biomedical applications, among others, can be one attractive solution. In addition, the generation of large amounts of wastes and the use of nonrenewable materials along with an increase in consumer environmental awareness led to the use of more eco-friendly materials. As mentioned by Cheng and Gross (2010), interest in biobased materials appears to be increasing proportionally to increased crude oil prices (Cheng and Gross 2010). Biobased materials available for films and coatings generally fall into the categories of polysaccharides, proteins, and lipids. Biopolymer-based materials may possess inherent antimicrobial activity, but usually, in order to exhibit specific functionality, bioactive compounds must be immobilized or incorporated into the polymer matrix. Among polysaccharides potentially used in active biomaterials, we can find:

- Chitosan, as an intrinsic antimicrobial aminopolysaccharide
- Cellulose, starch, hyaluronan, chondroitin sulfate, alginates, heparin, curdlan, pullulan, gellan as polysaccharides with induced bioactivity

The present chapter focuses on antibacterial polysaccharide-based materials, concerning antibacterial properties for active packaging for food preservation or biomedical applications (such as active medical device surfaces, active nanofibers, and wound dressing among other applications). The specific properties of chitosan and the easy accessibility and high production of cellulose and starch led to a very significant research interest and high scientific productions in terms of publications and industrial developments. As a result, in this chapter, we attempt to categorize the approaches that were commonly described from 2005 to date in the design of **antibacterial chitosan-**, **cellulose-**, and **starch**-based materials.

2 General Considerations on Antimicrobial Materials

2.1 Definitions

In food preservation and biomedical domains, different definitions associated with bioactive materials are found in the literature. To avoid misunderstandings, some terms frequently used in this chapter are defined in Table 1.

Table 1 Definition of some specific active materials adapted from Niu et al. (2014)

Bioactive materials	Materials which may include those that:
	Possess antimicrobial activities
	Exhibit immunoregulatory properties
	Promote tissue regeneration
	Possess cell-instructive and molecular signalling properties (i.e., regulating cell proliferation, migration, differentiation, protein expression, etc.)
	Are designed for biosensing
	Contain recognition sites for cleavage of enzymes involved in cell functions
	Incorporate bioactive molecules with specific properties such as antithrombotic, antihypertensive, antioxidative properties, etc.
Antibacterial materials	Materials capable of reducing the proliferation of bacteria
	Medical applications: antifouling, bactericidal and/or materials exhibiting a bacteriostatic effect
	Food packaging applications: bactericidal and/or materials exhibiting a bacteriostatic effect against bacterial strains encountered in foodstuffs
Antifouling materials	Materials able to decrease or avoid bacterial cells attachment (i.e., decrease the colonization of specific medical devices or food systems)
	Some of the low-adhesive, superhydrophobic and self- cleaning surfaces
Bactericidal materials	Materials that kill the live bacteria or some fraction therein
Materials exhibiting a bacteriostatic effect	Materials that hinder the growth of bacteria but do not imply whether or not bacteria are killed
	Inactivation action on the growth of bacterial strains

A few different approaches for imparting antibacterial functionalities have been attempted including the use of naturally antibacterial materials, the chemical modification of the polymer matrix itself, or the physical entrapment of bioactives.

2.2 Approaches for Imparting Antimicrobial Functionalities

Different classifications can be found for antimicrobial materials potentially used in biomedical or food applications. One of them is based on the antibacterial functional role that could be achieved through migratory or non-migratrory systems (Fig. 1). Briefly, materials can be distinguished, depending on whether the active agent is temporarily trapped within the biopolymer-based material or permanently attached to the chains (Lecomte et al. 2012).

The migratory systems can be used to deliver antibacterial agents to the food or the body target. This release can occur by different modalities such as simple

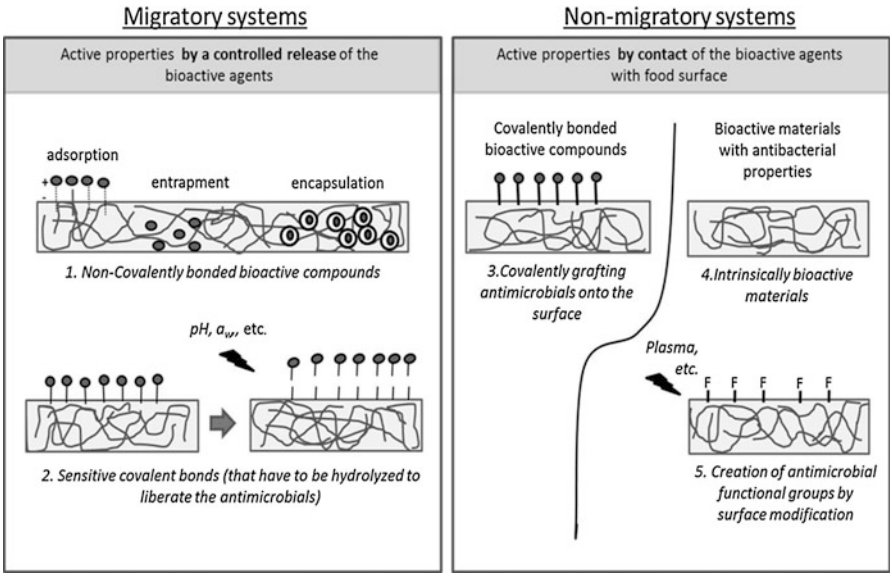

Fig. 1 The different approaches for imparting antimicrobial functionalities leading to migratory or non-migratory materials (Reproduced with permission from Coma and Olabarrieta 2014)

diffusion, degradation of loaded matrices, or hydrolysis of sensitive covalent bonds. In some cases, e.g., pH-sensitive bond, chemical bonds are created to allow an active agent slow-release system. In non-migratory systems the release of the antimicrobial agent is not required and even prohibited.

As another classification, we can propose three categories of antimicrobial materials. These are based on the following processes:

1. Direct utilization of active polymers exhibiting film-forming properties: active polymers created by chemical modification leading to bioactive properties or use of inherently antimicrobial polymers exhibiting film-forming properties
2. Incorporation of antimicrobial agents into the matrix
3. Coating of the matrix with a film-forming solution that acts as a carrier for the antimicrobial agents

An illustration of these categories is presented in Fig. 2.

Matrices with bioactive agents chemically bound to the polymer are included in the first category, and the immobilization of the antimicrobial onto the material can be used for drugs that have not yet obtained authorization for release. The other two categories can generally release the antimicrobials onto the surface where antimicrobial action is predominantly needed.

The potential use of chitosan, cellulose, and starch to develop antibacterial material following different strategies is then discussed below.

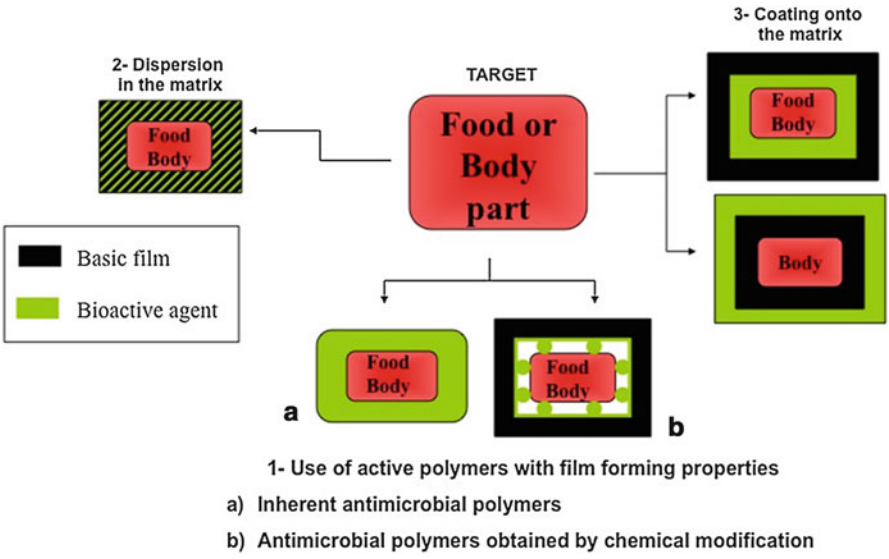

Fig. 2 The three categories of antimicrobial matrices (Adapted and reproduced with permission from Coma and Verestiuc 2012)

3 Polysaccharide-Based Materials with Antibacterial Properties

3.1 Antibacterial Chitosan-Based Materials: Use of an Inherently Active Polysaccharide

In the specific case of chitosan, a point of its antibacterial activity and factors affecting its ability is firstly proposed, due to its very particular properties in terms of bioactivity and to its film-forming ability. Because of the high scientific production regarding antibacterial activity of chitosan-based materials, the discussion was oriented to the strategies potentially used to enhance antibacterial activity in order to create highly active biobased materials.

3.1.1 General Considerations on Chitosan and Chitosan-Based Material Processing

Chitosan is a pseudonatural cationic polysaccharide, which is edible, nontoxic, biodegradable, and commercially available and that has been employed in a variety of applications. This linear copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose is the *N*-deacetylated form of chitin (Fig. 3). Chitin is widely distributed in nature, such as in crustaceans, currently the main source, insects, fungi, yeasts, and algae. In the case of fungal source, chitin is the characteristic component of the taxonomical groups zygo-,

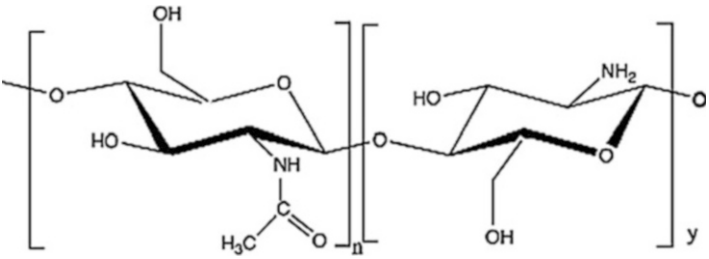

Fig. 3 Repeating unit of partially acetylated chitosan characterized by an average degree of acetylation

asco-, basidio-, and deuteromycetes. Yeasts and filamentous fungi contain glucan complexes with chitin or chitosan in their cell walls and septa (Muzzarelli et al. 2012). According to Shchipunov et al. (2009), as estimated, as much as 100 billion tons of chitin are produced annually in living nature. It constitutes a huge renewal resource of biomass that is still almost unutilized. Therefore, chitin and more precisely its derivative chitosan is believed to have a great potential for applications in many fields, being an extraordinary underexploited biopolymer of significant versatility (Muzzarelli 2010).

Most of the naturally occurring polysaccharides, e.g., cellulose, starch, alginate, dextran, pectin, agar, agarose, and carrageenans, are neutral or anionic in nature, whereas deacetylated chitin and chitosan are examples of cationic polysaccharides (Muzzarelli et al. 2012, Table 2). As already discussed above, since biocide-resistant bacteria and fungi, growing public health awareness of pathogenic micro-organism raised demands for safe, effective, and possibly biobased agents that were less prone to stimulating development of resistance (Kong et al. 2010). In this point of view, due to its broad antibacterial activity spectrum, effectiveness with high killing rate against Gram-positive and Gram-negative bacteria, and nontoxicity, chitosan, a GRAS polymer, is possibly one of the most studied as non-migratory antibacterial biomaterial (Anklam and Battaglia 2001; Kong et al. 2010; Coma and Verestiuc 2012; Coma 2012; Meng et al. 2014).

Chitosan can be used as an inherent bioactive material or as an antibacterial additive in synthetic or natural polymers (see the examples below with cellulose and starch) to prepare antibacterial materials.

Solutions of chitosan are prepared in acidic conditions and processed to the needed conformation (cast for a film, spun for fibers, freeze-dried for sponges) and sometimes immersed in a precipitation alkaline solution before washing and drying steps (Rinaudo 2013). Properties of chitosan materials depend on acid type and preparation procedure (Park et al. 2002; Lupei et al. 2013). With results in terms of basic film properties, acetic acid resulted in the toughest films followed by malic, lactic, and citric acid. Films prepared with citric acid had the highest elongation values (Park et al. 2002). Chitosan films prepared with malic acid showed the lowest oxygen permeability, followed by acetic, lactic, and citric acid. To summarize, the films elaborated with chitosan in lactic acid solution are more hygroscopic,

Table 2 Expected antibacterial properties of chitosan in food preservation or some biomedical applications according to Muzzarelli (2009) and Piozzi and Francolini (2013)

Chitosan in food preservation	
Packaging – coating	**Surfaces**
Decrease the risk of food spoilage and food-borne illnesses by reducing microbial contamination of food products during storage or distribution	Prevention of biofilm formation during food processing
	Biopesticide – Plant elicitor
	Controlling plant-diseases by displaying toxicity and inhibiting fungal growth. High interest for some mycotoxinogen fungal strains
	Eliciting activities of some coatings which lead to a variety of defense responses in host plants in response to microbial infections
Chitosan in biomedical applications (notably regenerative medicine)	
Wound healing	**Implantable medical devices**
Release of glucosamine and *N*-acetylglucosamine oligomers exhibiting antibacterial activities and stimulation of cellular activities	Decrease biofilm formation at the surface of medical devices, such as catheters, orthopedic implants, which is associated with the development of antibiotic-resistant, often life threatening, infections
Intrinsic antibacterial activity and controlled release of exogenous bioactive agents to prevent infection	
Tissue engineering	
Suitable for controlled release of antibiotics or other antibacterial agents	

permeable, and elastic and show a higher transparency. The films made from acetic acid solutions are generally less hygroscopic, show lower water vapor permeability, and are more resistant to traction but less elastic. As a result, chitosan film-forming solutions are more frequently elaborated using acetic acid. Because chitosan films are rigid and brittle in nature, plasticizers (i.e., glycerol, xylitol, and sorbitol) are generally used during solution casting to improve their flexibility and processability. As opposed to the usual casting–evaporation procedure, which requires the evaporation of a large quantity of solvent, plasticized chitosan with hierarchical structure, including multiple length scale structural units, can be prepared by a "melt"-based method. Chitosan was plasticized by thermomechanical mixing in the presence of concentrated lactic acid and glycerol (Meng et al. 2014).

3.1.2 Chitosan's Antibacterial Ability

Inherent Activity

Chitosan applications in advanced materials are mainly justified by their antibacterial activity against pathogenic and spoilage microorganisms. Chitosan has wide spectrum of activity and high killing rate against Gram-positive and Gram-negative bacterial strains.

The degree of deacetylation is one of the parameters, which allow to control some of the physical characteristics of films but also their antibacterial activity (Muzzarelli et al. 2012; Kong et al. 2010). Although the exact mechanism by which chitosan exerts its bioactivity is currently unclear, its polycationic nature is a crucial factor. Several mechanisms have been proposed such as:

- Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes which may cause:
  - The leaching out of intracellular constituents such as low molecular weight materials, nucleic acid, proteins, and so on (Raafat et al. 2008; Li et al. 2010)
  - The decreases of membrane permeability, preventing nutrient transport (Eaton et al. 2008)
- Binding of chitosan to DNA triggers inhibition of mRNA synthesis through penetration of the microbial nuclei by chitosan and interfering with the synthesis of mRNA and proteins.
- Chelation of metals, spore elements, and essential nutrients (Cuero et al. 1991).

Factors Influencing Antibacterial Activity

Antibacterial activity of chitosan and its derivatives relied on numerous intrinsic and extrinsic factors that include pH, temperature, salinity, microorganism species, presence or absence of metal cations, suspending medium, molecular weight (Mw), and degree of deacetylation (DD) (Kong et al. 2010). Because chitosans have been tested under widely varied conditions, it is hard to compare chitosan's antibacterial effect among results obtained by different researchers. However, some general factors influencing antibacterial activity have been identified and would be very useful to elaborate chitosan-based material with high efficiency.

Generally, the cytotoxic effect of chitosan and chitosan derivatives is dependent upon: (1) inherent properties of chitosan, (2) microbial factors, and (3) environmental factors, such as medium pH, temperature, and ionic strength. Only points 1 and 2 are discussed thereafter.

Factors Related to Inherent Properties of Chitosan and Chitosan-Based Matrices

One of the main factors influencing the antibacterial properties of chitosan materials is the deacetylation degree (DD) (Takahashia et al. 2008; Coma and Verestiuc 2012). As already mentioned, it is generally assumed that the cationic nature of chitosan, conveyed by the positively charged NH_3^+ groups of glucosamine, might be a fundamental factor contributing to its interaction with the negatively charged microbial cell surface, ultimately resulting in impairment of vital bacterial activities. Because the positive charge is associated with DD and pH values, a higher DD with more positive charge is especially successful in inhibiting the microbial growth. In other words, antibacterial activity of chitosan enhances with increasing DD.

The molecular weight (Mw) is the other parameter frequently identified as a factor affecting antibacterial properties. No et al. (2002) observed a minimum

inhibitory concentration (MIC) of chitosans ranged from 0.05 % to more than 0.1 % (w/v) depending on the bacteria and Mw of chitosan. They reported that chitosans (Mw > 28 kDa) showed higher antibacterial activities than chitosan oligomers (<22 kDa). However, numerous research works on bactericidal activity of chitosan have generated equivocal results concerning correlation between bactericidal activity and chitosan Mw. Some studies reported that increasing chitosan Mw leads to decreasing chitosan antibacterial activity. As an example, Gerasimenko et al. (2004) examined chitosan with Mw from 5 to 27 kDa and reported that antibacterial activity against *E. coli* increases as Mw decreased. The relationship between Mw and antibacterial is not cut and dry (Jung et al. 2014).

The physical state of chitosan seems to have also a decisive role in its antibacterial activity. As reported by Kong et al. (2010), soluble chitosan is generally more effective in inhibiting bacterial growth, due to the fact that the dissociating form in solution has an extending conformation, which enables a reaction with the counterparts to a sufficient degree. However, it tends to be difficult to know if the improvement of antibacterial activity is only due to an increase of water solubility or to the chemical modification conducted on the polymer structure. For example, reducing the Mw of chitosan markedly heightens solubility but not necessarily antibacterial activity. In the case of chitosan-based material, chitosan can be considered as a solid and generally leads to a lower antibacterial action than soluble chitosan (film-forming solution). Thus, because molecules of solid chitosan tightly contact with each other, the Mw of chitosan is of negligible concern. More generally, different physical states of chitosan (water solutions, beads, films, fibers, and hydrogels), as a crucial factor influencing antibacterial activity, are supposed to have been strongly considered but always being underestimated (Kong et al. 2010).

Antibacterial properties also depend upon the casting temperature of the films. Fernandez-Saiz et al. (2009) found that this capacity significantly decreased for higher temperatures. These authors have demonstrated that just-formed low Mw chitosonium acetate films present significant biocide properties against *S. aureus* and *Salmonella* spp. when cast at 37 °C or 80 °C, whereas this capacity is reduced when films are cast at 120 °C.

To conclude, the storage temperature could also be a key factor. Films should be stored at low temperature under dry conditions. Mayachiew et al. (2010) reported that ambient drying, low-temperature hot air-drying led to films with higher antibacterial activity and higher degree of swelling due to lower intermolecular interaction. If chitosan-based materials were maintained at high relative humidity conditions (i.e., 75 %) or relatively high temperatures (i.e., 37 °C), the samples presented a gradual loss of their antibacterial capacity, compared to lower temperatures and dry conditions (23 °C, 0 % RH) during the storage periods (Fernandez-Saiz et al. 2009). These results could be related to a substantial loss in the release of antibacterial protonated species as a result of cross-linking and/or other molecular entanglement process and/or chemical alterations of a more hydrophobic nature in the biomaterial.

Factors Related to Microorganisms

Chitosan is well known for its broad spectrum of activity. However, its antibacterial action is dependent on the bacterial genus, species, and Gram-stain group. The NH_3^+ groups of chitosan can alter bacterial surface morphology, which either increase membrane permeability in some bacteria or decrease membrane permeability in other bacteria (Tang et al. 2010; Wang et al. 2012). For example, Kumar et al. (2005) reported that chitooligomers caused pore formation and permeabilization of the cell wall of *Bacillus cereus*, whereas blockage of nutrient flow due to aggregation of chitooligomers was responsible for the growth inhibition and lysis of *E. coli*. According to Kong et al. (2010), based on the available evidences, bacteria appear to be generally less sensitive to the antibacterial action of chitosan than fungi. The bacterial effectiveness on Gram-positive or Gram-negative bacteria, due to different cell surface characteristics, is however somewhat controversial. However, as specified by Tang et al. (2010), the MICs reported to range from 100 to 10,000 mg L^{-1} against Gram-negative bacteria and from 100 to 1,250 mg L^{-1} against Gram-positive bacteria.

Finally, the age or the bacterial growth phase of the cell can also influence the antibacterial efficiency of chitosan (Jiang et al. 2013; Lahmer et al. 2014).

The phenomena involved in the antibacterial activity of both films and solutions were quite complex and depended not only on a series of factors but also upon the interactions between them (Jung et al. 2014). As a result, the sometimes conflicting results in literature, along with the differing effects of the various factors, highlight the need for additional research in order to elaborate chitosan-based material with optimum antibacterial ability.

3.1.3 Chitosan-Based Material: Strategies to Enhance the Antibacterial Ability of Chitosan-Based Materials

In the last decade many researchers attempted to modulate or increase this biopolymer activity, which will broaden its applications in active material field. This part of the chapter provides an overview of strategies used to enhance chitosan antibacterial activity, focusing on two specific topics: (i) strategies based on non-covalent grafting or simple combination with other compounds that also exhibit activity against bacteria and (ii) strategies based on permanent chemical grafting/modifications of chitosan.

Via Non-covalent Grafting Techniques or Simple Combination with Bioactives

Chitosan can be used as scaffold for controlled release of antibacterial agent. Chitosan films, porous chitosan, chitosan hydrogels, chitosan microspheres, and chitosan nanofibers were reported for the delivery of bioactives.

Positively charged groups make chitosan very sensitive to the presence of anionic substances in its solutions. This phenomenon can be useful to non-covalently immobilize antibacterial agents onto chitosan-based matrix. Moreover, a simple physical entrapment of antibacterial agent molecules in a chitosan matrix can also be envisaged to enhance antibacterial ability.

As a result, the antibacterial effect of chitosan can be improved by binding active **metal ions** such as Zn(II), Cu(II), Fe(II), Mn(II), or Ag(I) through different mechanisms such as ion exchange, chelation, or formation of ternary complexes. The minimum bactericidal concentration strongly decreased by one or two orders of magnitude for composite materials (metal-complexed chitosan) compared to chitosan or metal ions alone (Guibal et al. 2014). Hybrid materials with improved antibacterial ability can then be produced. Silver is the most widely used in biomedical fields due to its strong antibacterial activity at low concentrations. Ag^{+}-chitosan complex showed a wide antibacterial spectrum, with minimum inhibition concentrations 20 times lower than those of the initial chitosan. Material based on chitosan containing silver ions was found to decrease the amount of bacteria present by 98 % (No et al. 2002). Another example is the loading of silver nanoparticles in a chitosan matrix as recently studied by Ravindra et al. (2010). Vimala et al. (2010) fabricated a porous chitosan film impregnated with silver nanoparticles with potential applications in wound dressing. The antibacterial activity results revealed that these porous chitosan–silver nanocomposite films exhibited superior inhibition against bacterial strains such as *E. coli*, *Bacillus*, and *Klebsiella pneumoniae*.

Chitosan material may be also enriched by active molecules such as **protein**, **peptides**, or **antibiotics**. Enzymes or active peptides can be entrapped into the polysaccharide network and maintained by hydrogen bonds and/or electrostatic interactions. As mentioned by Macquarrie and Bacheva (2008), a simple process can be used to perform antibacterial protein– or peptide–chitosan interaction. The dissolution of chitosan in dilute acid and addition of an antibacterial protein-based solution before film formation can be sufficient. For pH-sensitive water-soluble antibacterials, this process could also be carried out at a pH closer to neutral by preparing chitosan acetate first of all from dilute acetic acid solutions of chitosan and then redissolving the salt in water. Arancibia et al. (2014) studied different blend coatings prepared by solubilizing chitosan in lactic acid and blending it with several shrimp active wastes including proteins. The incorporation of an astaxanthin-rich protein concentrate had increased the antibacterial capacity of the blend solution. Tin et al. (2009) combined ten **antibiotics** with different degrees of deacetylated chitosans and chitosan oligosaccharides. All the chitosans show synergistic activity with sulfamethoxazole, a sulfonamide antibacterial agent, against *Pseudomonas aeruginosa*. Another example is given by the study of Wu et al. (2014a). They prepared gentamicin sulfate-loaded carboxymethyl-chitosan hydrogel cross-linked by genipin and achieved a very high inhibition of *S. aureus* growth and biofilm formation. At the same time, Ordikhania et al. (2014) showed that the physical encapsulation of vancomycin in a chitosan hydrogel network allows the antibiotic controlled release. Antibacterial tests against *S. aureus* demonstrated that the infection risk of titanium foils used as orthopedic implant models was significantly reduced.

Antibacterial chitosan films and coatings have also been created by combination with a number of other active compounds, such as **essential oils**. The potential applications are generally for interactive food packaging. Due to the very large

amount of papers regarding this specific field, only some examples are given below. Azevedo et al. (2014) created efficient antibacterial edible coating using chitosan combined with *Lippia gracilis* extracts, which consists mainly in thymol and carvacrol. Sun et al. (2014) showed that chitosan coatings incorporated with certain essential oils (carvacrol, cinnamaldehyde, and *trans*-cinnamaldehyde) may be an effective alternative for fresh blueberry preservation. It has been observed that carvacrol at $\geq$0.1 % or *trans*-cinnamaldehyde at $\geq$0.2 % incorporated in chitosan coatings improved the inhibition of the *E. coli* growth. Iturriaga et al. (2014) prepared bioactive citrus extract–chitosan films through solvent casting–evaporation method, and the antilisterial activity of the material was clearly improved by the enrichment in essential oil.

Via Permanent Chemical Modifications of Chitosan

Because chitosan exhibits antibacterial functionalities based on the existence of amino groups, bioactive properties are related to a high degree of deacetylation. To potentially enhance bioactivity, efficient deacetylation methods have been proposed. In addition, chitosan being a multifunctional polymer containing both hydroxyl and amino groups, covalent or non-covalent graftings have been widely investigated to improve antibacterial properties.

Enhancement of the Deacetylation Degree

Conventional deacetylation of chitosan has been performed, by hydrolyzing acetamido groups by using highly concentrated aqueous alkaline solutions, such as caustic soda (Focher et al. 1990). Because the traditional method can cause degradation of chitosan backbones resulting in a lower molecular weight, Ishii et al. (2014) proposed a very interesting new method using 1-butyl-3-methylimidazolium acetate (BMIMOAc) as the reaction medium and catalyst. An imidazolium IL containing the acetate anion was used as a basic catalyst promoting the hydrolysis of remnant acetamido groups in chitosan. An enhancement from 77 % close to 87 % was attained by simple hydrothermal treatment (100 °C for 2 h), larger than the conventional method, which enhanced the DD to 79 %. The control of the amount of water added contributed to the improved homogeneity of the reaction, which is another key point for developing highly active deacetylated chitosans.

N and *O* Covalent Grafting

According to Kong et al. (2010), irrespective of their form or quantity, antibacterial agents typically require water for activity. The creation of water-soluble chitosan and its derivatives has been a central goal of investigations of antibacterial activity, which have included saccharization, alkylation, acylation, and quaternization. Furthermore, the grafting of antibacterials within the chitosan structure can also be a strategy to globally enhance bioactive properties. Various antibacterial substances, such as antibiotics, antiseptics, and enzymes, have been grafted on the surfaces of chitosan-based matrices (Yuan et al. 2013). The different strategies associated to a few examples are given below.

N-grafting: The easiest way to chemically modify chitosan is to produce *N*-chitosan derivatives.

For a polymeric quaternary ammonium biocide, the hydrophilic–lipophilic balance influences antibacterial properties by affecting the mode of interaction with the cytoplasmic membrane. Grafting **alkyl chains** on chitosan generally leads to higher antibacterial activity, and quaternization is one of the most studied chitosan modifications. Rationalization of the parabolic relationship between antibacterial properties and alkyl chain lengths has been debated. It has been attributed to (a) dual binding sites on the surface for which the relative binding affinities at each site differ for long and short alkyl substituents or (b) different aggregational behaviors for long and short hydrophobes (Kenawy et al. 2007). *N*-Alkyl chitosan derivatives were prepared by introducing alkyl groups into the amine groups of chitosan via a Schiff base intermediate. For quaternized derivatives, one direct effect is an enhancement of water solubility over a broad pH range (Mourya and Inamdar 2009). Quaternized chitosans generally lead to materials with stronger antibacterial activity, broader spectrum, and higher killing rates compared to unmodified chitosan materials (Belalia et al. 2008; Xu et al. 2011; Vallapa et al. 2011; Wiarachai et al. 2012).

Ma et al. (2008) synthesized an *N*-alkylated chitosan derivative by Michael addition reaction of chitosan and hydroxyethylacrylate in moderate conditions. Even if the chitosan derivatives exhibited an excellent solubility in distilled water, its antibacterial activity against *E. coli* was decreased compared with that of chitosan. But other bacterial strains have to be tested.

Peng et al. (2010) demonstrated that water-soluble quaternary ammonium salts, which are produced by the reaction of chitosan with glycidyl trimethylammonium chloride, provide chitosan derivatives with enhanced antibacterial ability. Hydroxypropyltrimethyl ammonium chloride chitosans with 18 % or 44 % substitution were significantly more effective than those with 0 % or 6 % substitution against *S. aureus*, methicillin-resistant *S. aureus*, and *Staphylococcus epidermidis*.

Water solubility of chitosans at neutral or basic pH was largely improved by specific attachment of **carbohydrates** to the 2-amino functions achieved by Maillard's reaction or further reductive alkylation of Schiff bases (Ying et al. 2011). These authors prepared chitosan derivatives with different degrees of substitution of various saccharides. The most potentially water-soluble chitosan was the Schiff base-typed chitosan–fructose derivative. No specific information about antibacterial activity was given but this derivative exhibited higher antioxidant activity. Unfortunately, even if the Maillard's reaction is promising and easy for commercial manufacturing of water-soluble chitosans, we could expect a decrease in terms of antibacterial activity due to the resulting non- or less-protonated chitosan derivative. However, Chung et al. (2011) showed high antibacterial activity of water-soluble chitosan derivatives prepared by Maillard's reactions against various Gram-positive and Gram-negative bacterial strains.

Another approach could be the functionalization of chitosan with **amino/peptide acid** moieties, containing potentially bioactive free amino groups. Many bioactive peptides are featured by their unique amino acid compositions

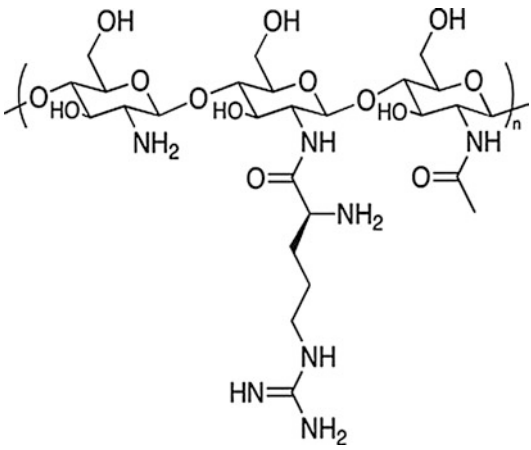

Fig. 4 Schematic representation of the chitosan-arginine derivative according to Lahmer et al. (2012)

such as arginine/lysine-rich biomolecules. Water-soluble arginine-functionalized chitosans (Fig. 4), obtained by the addition of a single arginine residue, via the formation of a stable peptide bond of the arginine carboxylic acid with the amine on the glucosamine unit, were studied by Tang et al. (2010) and Lahmer et al. (2012). The resulting cationic arginine-functionalized chitosans were soluble and polycationic at neutral pH, due to the higher p*K*a (12.48) of the guanidinium side chain relative to that of the amine of the chitosan. They strongly inhibited *P. fluorescens* and *E. coli* growth.

Another example included materials made from other basic amino acids, such as histidine-grafted chitosan. The imidazole ring present in the side chain of histidine has a pKa of approximately 6.0. At pH below 6, the imidazole ring is mostly protonated and carries two -NH bonds, with a positive charge. Even if no information was found about the antibacterial properties of histidine-grafted chitosan, histidine-containing peptides have demonstrated pH-dependent antibacterial activity (Tu et al. 2009), leading to a potential increase regarding the antibacterial ability. The functionalization of chitosan glucosamine moieties with histidine amino acid was found to enhance the buffering capacity over the parent chitosan polymer (Casettari et al. 2012).

Following the same idea, instead of amino acids, covalent fixation of active peptides, poly(peptides), or enzymes could be used to create new active chitosan-based matrices. Indeed, highly antibacterial active peptides can be potentially grafted onto the chitosan backbone; arginine- and tryptophan-rich peptides are very promising short peptides from this point of view. Tryptophan has a distinct preference for the interfacial region of lipid bilayers, while arginine residues endow the peptides with cationic charges and hydrogen-bonding properties necessary for interaction with the abundant anionic components of bacterial membranes (Chan et al. 2006). Poly(peptide)-grafted chitosan copolymer showed, in addition to being good candidates for developing a biomaterial with a better solubility in neutral conditions, a real potential to develop highly antibacterial biomaterials.

Poly(L-arginine)-grafted chitosan and poly(L-lysine)-grafted chitosan are generally good candidates notably in the biomedical applications (Zhou et al. 2013). Another approach is to covalently graft the enzyme on the biopolymer. Natural defense substances secreted by living organisms, such as hydrolytic enzymes, have recently emerged as a particularly attractive class of biocidal agents (Krajewska 2004). However, as mentioned by Yuan et al. (2013), only a few studies have been devoted to the immobilization of antibacterial enzymes on chitosan for improved antibacterial activity. Hen egg-white lysozyme, or 1,4-β-*N*-acetylmuramidase, a natural defensive and bacteriolytic enzyme, displays antibacterial activity especially for Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*. It can be conjugated to chitosan, via the 1,1′-carbonyldiimidazole (CDI) as a bifunctional linker, to enhance biocidal functionality (Yuan et al. 2013; Duan et al. 2007).

Covalent immobilization of **phenolic** compounds on chitosan could also be used to create new antibacterial materials. Because these compounds can possess antibacterial properties in addition to antioxidant activities, the grafting can result in derivatives with unique antibacterial ability (Liu et al. 2014). In this specific approach, the use of enzyme-catalyzed reaction is currently one of the most popular, naturally connected to green chemistry development. There are few enzymes known that might react with chitosan to build macromolecular structure and confer material function. Examples are the grafting of phenolic compounds using laccase or tyrosinase. Bozic et al. (2012) functionalized chitosan with quercetin or tannic acid using the laccase from *Trametes versicolor*, leading to a biobased product with synergistic antioxidant and antibacterial properties. Tyrosinase enzymes can also effectively graft phenolic compounds onto chitosan through their conversion into reactive *o*-quinone intermediates, which subsequently undergo nonenzymatic reactions with the nucleophilic amino groups of chitosan (Govar et al. 2003; Puskas et al. 2009). By using tyrosinase, Vartiainen et al. (2008) grafted octyl gallate and dodecyl gallate onto chitosan, resulting in coatings with strong antibacterial activity against *S. aureus* and *L. innocua*.

N,O-derivatization: To increase the water solubility and cationic charges at pH 7 and then maintaining or improving antibacterial properties, cationic moieties can be introduced onto both C_6–OH and C_2–NH_2 groups in the chitosan backbone. Even if the aim of the study was not to improve antibacterial activity but to synthesize a flocculant water-soluble cationic chitosan derivative, Jiang et al. (2011) prepared glycidyl methacrylate (GMA)-modified chitosan bearing double vinyl groups, through ring-opening reaction of GMA with the C_6–OH of chitosan (chitosan–GMA). Then, the flocculant was synthesized by means of radical copolymerization between chitosan–GMA and a cationic vinyl monomer. Strong antibacterial activity of this derivative can be expected, due to the presence of numerous cationic charges, but unfortunately they were not studied here.

In order to increase the number of free amino groups on chitosan chains, Xie et al. (2007) prepared an ethylamine hydroxyethyl chitosan (Fig. 5). A good water solubility of this derivative was observed in a relatively wide range of molecular weights, and antibacterial activity results ($M_n = 1.9 \times 10^5$) against *E. coli*, explored

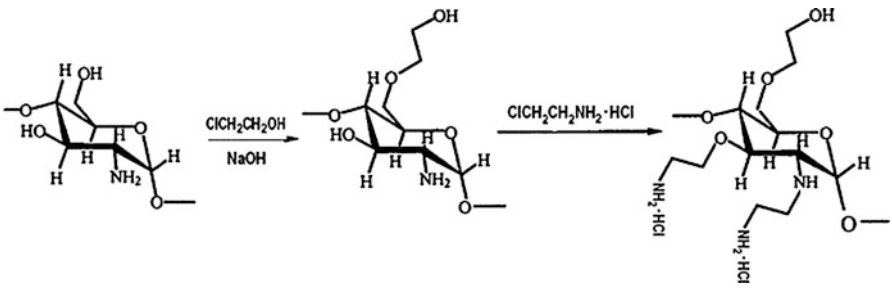

Fig. 5 Ethylamine hydroxyethyl chitosan synthesis according to Xie et al. (2007)

by using an optical density method, showed good inhibition effects but without a clear comparison with the unmodified chitosan.

O-Derivatization: Chemoselective substitution provides a way of developing novel chitosan derivative-based materials with really enhanced bioactive activity. *O*-Selectivity is still a challenge on chitosan because of the difference in reactivity between amino and hydroxyl groups of chitosan. An approach based on the selective protection of the amino group followed by a deprotection step can be useful. Interestingly, Ifuke et al. (2011) proposed a highly chemoselective *N*-phthaloylation of chitosan by performing a reaction in aqueous acetic acid media. The degree of substitution values of phthaloyl groups was approximately 1. These authors mentioned that phthaloyl group is most useful for amino group protection from the point of easy deprotection and chemoselectivity in mild conditions. With recent interest in green chemistry, the use of water as solvent for protection process on chitosan could be a key point in the future. *N*-Phthaloyl chitosan could act as a precursor to allow further modifications of hydroxyl groups at C_3 and C_6 positions. That could be a solution to prepare new antibacterial chitosan derivatives with free amino groups.

Another approach to proceed to the selective *O*-modification of chitosan is to conduct derivatization in the presence of H_2SO_4 as a catalyst. Even if the protecting effect of H_2SO_4 on the amino group was not yet clear, it seems that it can be related to the salt formation of the primary amino group. A water-soluble chitosan derivative, *O*-fumaryl-chitosan (Fig. 6), was prepared by Feng and Xia (2011), using the selective partial acylation of chitosan and fumaric acid in the presence of H_2SO_4. Fumaric acid is a food-grade acidulant with strong bactericidal activity. The prepared derivatives had good solubility over a wide pH range, and the antibacterial activity, investigated against *E. coli* and *S. aureus*, was much stronger than that of chitosan. At pH 7.0, chitosan was not antibacterially active since it was not soluble and therefore the amino groups were not positively charged. Interestingly, the *O*-fumaryl chitosan was completely soluble at pH 7.0 and was very effective in decreasing the viable cell population of both strains. Bioactivity increased with increasing DS and the *O*-fumaryl chitosan with a DS of 0.48 appeared as the best one to be used as a food preservative. Unfortunately, the authors did not specify if

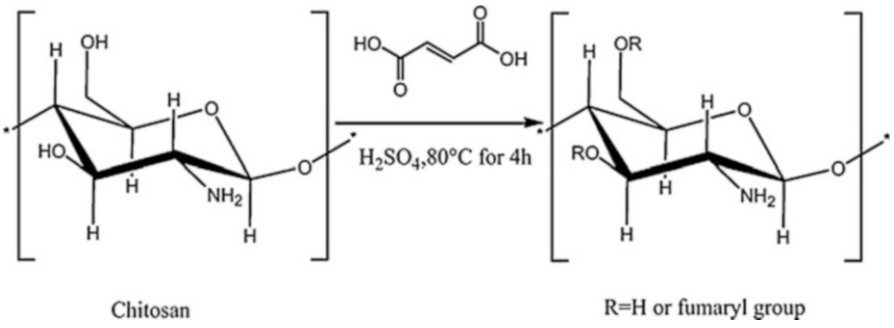

Fig. 6 *O*-fumaryl chitosan according to Feng and Xia (2011)

the chitosan derivative has maintained its polymerization degree and, in other words, its film-forming properties to be used directly as a bioactive material.

3.2 Antibacterial Cellulose- and Starch-Based Materials

3.2.1 Cellulose

Cellulose, the main component of plant cell walls, is by far the most abundant natural polymer and has been explored for a long time in the paper and textile industries. The production of a wide range of cellulose derivatives, as well as the emerging of nanocellulose forms (as nanofibrillated cellulose (NFC), obtained by mechanical disintegration of plant fibers, and bacterial cellulose (BC) produced by several nonpathogenic bacteria), opened the chance of using this biopolymer in other technological fields, including packaging and coatings, films, nanocomposites, and biomedical materials and devices, among many others.

In most of these areas and singularly in food packaging, textiles, and biomedical products, the control and/or prevention of contaminations by microorganisms constitutes a key issue because of their straight relation with human's health and life quality. Therefore, antibacterial materials and in particular antibacterial cellulose-based materials have attracted a great deal of attention in the latter decades as a sustainable alternative for the traditional materials used in many fields. Antibacterial cellulose-based materials are obtained primarily by the simple combination of cellulose (or cellulose derivatives) with distinct biocides (including metal and metal oxide nanoparticles and salts, polymers, and organic molecules) or by chemical grafting/modification with antibacterial moieties or groups.

Antibacterial Cellulose/Metal (Metal Oxide) Nanoparticle Composites

In the last decade, the combination of different forms of cellulose, like plant fibers (Csóka et al. 2012; Raghavendra et al. 2013; Simonc et al. 2008; Teli and Sheikh. 2012; Wang et al. 2011, 2013b; Zhu et al. 2009), cotton fabrics (Kim et al. 2010; Park et al. 2012), filter paper (Imani et al. 2011; Tang et al. 2009; Tankhiwale and Bajpai 2009), microcrystalline cellulose (Dong et al. 2014; Huang et al. 2013;

Jankauskaitė et al. 2014; Li et al. 2011, 2012a), bacterial cellulose (Barud et al. 2011; Dobre and Stoica-Guzun 2013; Jung et al. 2009; Liu et al. 2012a; Maneerung et al. 2008; Maria et al. 2010; Sureshkumar et al. 2010; Wu et al. 2014b; Yang et al. 2012a, b, 2013; Zhang et al. 2013), nanofibrillated cellulose (Díez et al. 2011; Martins et al. 2012; Xiong et al. 2013), as well as cellulose derivatives (e.g., cellulose acetate (Hyuk Jang et al. 2014; Perera et al. 2014; Scheeren et al. 2011; Son et al. 2006), hydroxypropyl cellulose (Angelova et al. 2012), and cellulose acetate phthalate (Necula et al. 2010)), with silver nanoparticles (Ag NPs), following diverse methods, is definitely the most extensively studied strategy for the design of antibacterial cellulose-based materials because of the recognized strong activity of Ag NPs.

For example, Wang et al. (2013b) reported the preparation of cellulose fiber/TiO_2 nanobelt-Ag NP hierarchically structured paper with photocatalytic and antibacterial properties. The characterization results showed that the Ag/TiO_2 nanobelt cellulose composite paper holds a typical hierarchical structure; specifically the TiO_2 nanobelt-based nanomaterials adsorb on the cellulose fiber surface forming an organic/inorganic hybrid paper, whereas Ag NPs are assembled on TiO_2 nanobelt surface creating metal oxide composite nanobelts. The photocatalytic activity and cycle stability of the hybrid paper in degrading methyl orange solution were enhanced by increasing the fraction of TiO_2 nanobelts. The papers with 40 wt% of TiO_2 nanobelt exhibit high photocatalytic activity even after three photocatalysis cycles. Paper with Ag-loaded TiO_2 nanobelts displayed also good antibacterial effect against *E. coli* due to the release of silver ion from the paper. In a distinct line, Tankhiwale and Bajpai (Tankhiwale and Bajpai 2009) described a cerium ammonium nitrate-initiated graft copolymerization of acrylamide onto cellulose filter paper followed by entrapment of Ag NPs (12–42 nm) by in situ reduction of Ag^+ ions with sodium citrate. It was suggested that the cross-linked polyacrylamide network formed onto the cellulose fibers stabilizes the Ag NPs avoiding their agglomeration, as observed when using plain paper filter. This material demonstrated fair biocidal activity against *E. coli* and it was proposed as a potential candidate for use as antibacterial food packaging material. In a different work, antibacterial active metal–cellulose hybrids were produced by covalent assembly of Ag or Pd NPs on cellulose fabric via impregnation of thiol-modified cellulose in colloidal Ag or Pd nanoparticle solutions (Park et al. 2012). The robust linkage between the NPs and the cellulose fabric, as confirmed by X-ray photoelectron spectroscopy, leads to the suppression of the release of metal NPs from the fabric. Therefore, these hybrids may avoid cell damage caused by diffusion and fixation of metal NPs into the cells but also act as a bioactive textile.

In another study, a simple microwave-assisted approach to produce microcrystalline cellulose/Ag NP composites by reducing silver nitrate in ethylene glycol was developed (Li et al. 2011). Ethylene glycol acts as the solvent, reduction agent, and microwave observer in the system. Thermogravimetric analysis showed that the temperature and heating time in the preparation stage had minor effect on the thermal stability of the ensuing nanocomposite. In addition, these materials exhibited strong antibacterial activity against *E. coli* and *S. aureus*. A comparable

methodology was also used to prepare cellulose/AgCl nanocomposites (Li et al. 2012a); however, in this case the formation of the nanocomposites was based on the simultaneous formation of AgCl nanoparticles and cellulose precipitation. More recently, antibacterial cellulose/Ag/AgCl hybrids against *E. coli* and *S. aureus* were also fabricated by means of an environmentally friendly and rapid methodology using a cellulose solution in NaOH/urea, $AgNO_3$, and $AlCl_3$ with ultrasound agitation (Dong et al. 2014). In a distinct manner, new antibacterial nanocomposite films against *E. coli* and *S. aureus* were also prepared by employing amino acids (phenylalanine or tryptophan) functionalized TEMPO-oxidized microcrystalline cellulose with Ag NPs prepared by in situ reduction via homogeneous (in an ionic liquid solution) and heterogeneous (in a aqueous dispersion) methods (Huang et al. 2013).

Bacterial cellulose has received particular attention on the design of novel hybrid materials due to its tridimensional porous structures that allow the entrapment of several molecules and nanoentities, such as Ag NPs. For instance, Sureshskumar et al. (2010) developed innovative magnetic antibacterial nanocomposites based on BC and Ag NPs (Fig. 7). Precisely, the nanofibrous network of BC was homogenized with a ferric and ferrous mixture, and magnetite nanoparticles were incorporated into BC structure by adjusting the alkaline pH. The magnetic BC membrane was then coated with a polydopamine layer, and finally Ag NPs were incorporated by soaking with a silver nitrate solution that reduced spontaneously. The obtained BC–Ag nanocomposite membranes own superparamagnetic properties and antibacterial action against both *E. coli* and *B. subtilis* bacteria and were found to be effective on the sterilization of fermentation culture mediua. More recently, Yang et al. (2012a) described a hydrothermal synthesis of a BC–Ag NP composite using BC as both reducing and stabilizing agent. Under optimum conditions, small-size Ag NPs (17.1 + −5 nm) were formed on the BC

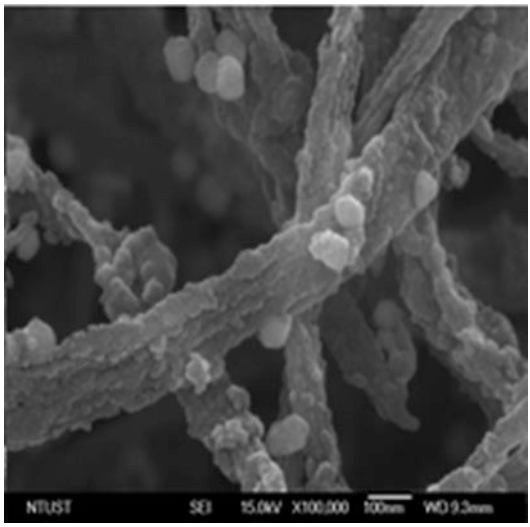

Fig. 7 Scanning electron microscopy (SEM) image of magnetic antibacterial nanocomposites based on BC and Ag NPs (Adapted and reproduced with permission from Sureshkumar et al. 2010)

matrix, with an Ag content of 1.78 % (%w/w) and an MIC (*S. aureus*) value of 1.30×10^{-4} mug/CFU. In another study, *Gluconacetobacter xylinus* bacteria were utilized as versatile factories that produce bacterial cellulose and induced the formation of Ag/AgCl nanoparticles, yielding BC/Ag–AgCl nanocomposites with Ag contents of 0.05 % (Liu et al. 2012a). The obtained nanocomposites showed required activity against *S. aureus* and *E. coli* demonstrating enormous potentialities as antibacterial wound dressing materials.

Nanofibrillated cellulose has also been explored for the preparation of cellulose/Ag nanocomposites materials. For instance, the functionalization of nanofibrillated cellulose using silver nanoclusters as a way to prepare novel functional nanocellulose/nanocluster composites was recently reported by Diez et al. (2011). This functionalization was mediated by poly(methacrylic acid) that simultaneously protected the silver nanoclusters and allowed hydrogen bonding with cellulose, as tentatively suggested by the authors. The ensuing composites retained the attractive properties of silver nanoclusters, namely, fluorescence, and antibacterial activity against *E. coli*. Xiong et al. (2013) developed novel catalytic and antibacterial (against *S. aureus*) Fe_3O_4/Ag@NFC nanocomposites which enable tunability from highly porous aerogels to stiff films. In a quite different strain, antibacterial composites of nanofibrillated cellulose and Ag NPs were prepared by means of electrostatic assembly, using distinct electrolytes as macromolecular linkers, of Ag NPs onto NFC (Martins et al. 2012). These NFC/Ag nanocomposites were further used as fillers in starch-based paper sheet coating formulations for the development of antibacterial paper products against *S. aureus* and *K. pneumoniae*.

Cellulose derivatives, e.g., cellulose acetate (Hyuk Jang et al. 2014; Perera et al. 2014; Scheeren et al. 2011; Son et al. 2006), hydroxypropyl cellulose (Angelova et al. 2012), and cellulose acetate phthalate (Necula et al. 2010), due to their film-forming ability and other specific properties have been also used for the design of innovative antibacterial films by combination with Ag NPs. For instance, metal (Ag, Au, and Pt) NPs containing membrane cellulose films were obtained by combining NPs of monomodal size distributions in the ionic liquid 1-*n*-butyl-3-methylimidazolium bis(trifluoromethane sulfonyl)imide with a syrup of cellulose acetate in acetone (Scheeren et al. 2011). The addition of the ionic liquid to the membrane increased its elasticity and toughness. High antibacterial activity against *E. coli* and *S. aureus* was perceived in membranes with metal NP concentrations as low as 0.2 mg/g of cellulose acetate.

In addition to the well-documented Ag NPs, other metal (e.g., Cu NPs; Grace et al. 2009; Jia et al. 2012; Mary et al. 2009; Oporto et al. 2013; Pinto et al. 2013) and metal oxide (e.g., ZnO NPs; Chaurasia et al. 2010; Martins et al. 2013; Wang et al. 2014) NPs have also been suggested as antibacterial agents to fabricate bioactive cellulose-based materials.

For instance, Pinto et al. described novel nanocomposites containing copper NPs and nanowires in both plant and bacterial cellulose matrices prepared by in situ and ex situ approaches (Pinto et al. 2013) (Fig. 8). The results showed that the chemical and morphological nature of the copper nanostructures and of the cellulose substrates have a great influence on the antibacterial activity of the nanocomposites

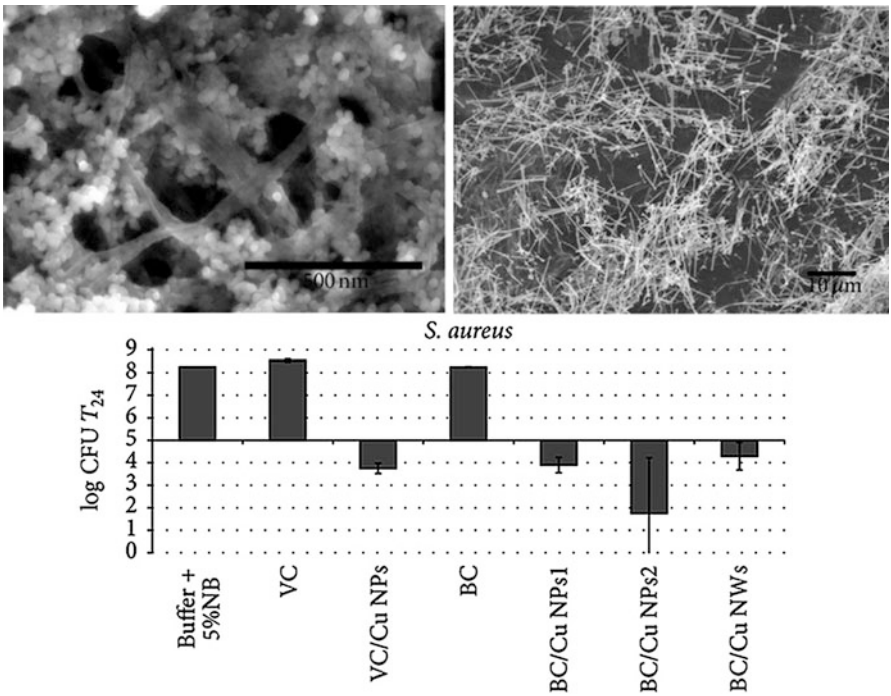

Fig. 8 SEM images of BC-Cu NPs and BC-Cu nanowires and antibacterial activity of cellulose/Cu nanocomposites with variable Cu content against *S. aureus* (Adapted and reproduced with permission from Pinto et al. 2013)

against *S. aureus* and *K. pneumoniae*, with plant fibers and Cu NPs being the most effective substrate and nanofiller, respectively. Zong et al. reported a simple method to prepare cellulose-based nanocomposites with antibacterial properties by introducing Cu NPs (10–20 nm) on carboxymethyl cellulose using sodium borohydride as the copper-reducing agent (Oporto et al. 2013). In a different study, regenerated cellulose films coated with Cu NPs were fabricated from cellulose–cuprammonium solution through coagulation in aqueous NaOH followed by reduction with sodium borohydride (Jia et al. 2012). Cu NPs were found to be definitely embedded on the surface of the cellulose films that showed effective antibacterial activity against *S. aureus* and *E. coli*. Indeed, the reduction of viable bacteria could be observed within 0.5 h of exposure, and all bacteria were killed within 1 h.

Antibacterial composites of NFC and ZnO NPs have been prepared by electrostatic assembly in aqueous medium and using polyelectrolytes as macrolinkers (Martins et al. 2013). These ZnO/NFC systems were further tested as fillers in starch coating formulations for paper sheets. Antibacterial paper sheets (against Gram-positive and Gram-negative bacteria and at both light exposure and dark conditions) with low ZnO content (<0.03 %) and slight enhancements in air

permeability and mechanical performance were obtained. The results obtained seem to suggest that the mechanism for the ZnO antibacterial activity is not mediated merely by the photoactivity of this semiconductor but likewise by the presence of oxidized species formed at the NP surface. Recently, Wang et al. (2014) reported the development of monolithic, flexible, and porous ZnO biocomposite foams (with 70 % ZnO load and a BET (Brunauer–Emmett–Teller) surface area of 92 $m^2\ g^{-1}$) with a hierarchical architecture assembled by controlled hydrolysis and solvothermal crystallization using a BC aerogel as a pattern in ethanol. This ZnO/BC nanofoam revealed outstanding antibacterial activity toward *E. coli* under both daylight and UV light that could be a result of the efficient integration of the porous structures and small ZnO nanocrystals and which give them prospective as bioactive wound healing dressings and water sterilization materials.

Finally, the use of metal salts (e.g., AgCl (Kim et al. 2009), $AgNO_3$ (Ng and Jumaat 2014), and silver sulfadiazine (Luan et al. 2012)) as antibacterial agents, particularly explored in the past, was only marginally reported in the last decade mainly due to the emerging of the nanotechnology domain associated with this field, as described in the latter paragraphs. Though gallium has emerged as a new therapeutic agent, in a quite recent study (Valappil et al. 2013), a novel antibacterial gallium exchanged carboxymethyl cellulose was developed and tested for the vulnerability on *P. aeruginosa*. The results indicate that an increase in average molecular weight (from 90,250 to 700 kDa of Ga–carboxymethyl cellulose) triggered a decrease in antibacterial activity. The sample with MW 250 kDa and DS 0.9 showed the most promising activity against planktonic *P. aeruginosa* (0.85 log (10) CFU reduction compared to sodium-carboxymethyl cellulose after 24 h).

Antibacterial Cellulose-Based Materials with Bioactive Polymers and Organic Molecules

The development of antibacterial cellulose-based materials through the combination with bioactive polymers and organic molecules has also been extensively investigated in the latter years, with particular emphasis for the use of biopolymers as chitosan, proteins, and peptides.

Chitosan, the deacetylation product of chitin, is well documented by its film-forming ability and antibacterial properties and due to its polysaccharide-based structure has been extensively explored for the development of bioactive cellulose-based materials (Abou-Zeid et al. 2011; Bu et al. 2012; Fras et al. 2012; Liu et al. 2012c, 2013; Ren et al. 2012; Zemljic et al. 2012) for several applications.

For example, water-soluble *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride, prepared by reaction of chitosan with glycidyltrimethyl ammonium chloride, was used to modify cotton fabrics for improving aqueous pigment-based inkjet printing and antibacterial properties (Bu et al. 2012). Scanning electron microscopy images showed that water-soluble chitosan derivative was obeyed onto the cotton fabrics surface and formed a film structure. Modified cotton samples showed good antibacterial activity, with an inhibitory rate for both *S. aureus* and *E. coli* higher than 95 % when the concentration of the chitosan derivative used in the pretreatment solution was 0.8 %. Moreover, the antibacterial effect was found to be

long lasting for 20 laundering cycles. However, the hand feeling of modified cotton fabrics presented a slight decrease. Mututuvari et al. developed a green methodology to prepare multifunctional composites for bone tissue regeneration containing cellulose (to impart mechanical strength), chitosan (to induce antibacterial activity), and hydroxyapatite (to impart osteoconductivity). Chitosan and cellulose were co-dissolved in an ionic liquid and then regenerated from water. Hydroxyapatite was then formed in situ by alternately soaking chitosan/cellulose composites in aqueous solutions of $CaCl_2$ and Na_2HPO_4. These hybrid composites retained the desirable properties of their constituents. For instance, the tensile strength of the composites was improved 1.9 times by increasing cellulose content from 20 % to 80 %. Integrating chitosan in the composite materials resulted in antibacterial activity against Gram-positive (methicillin-resistant *S. aureus*, *S. aureus*, and vancomycin-resistant *Enterococci*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria. In another study, aiming to enhance the antibacterial and mechanical properties of agar films (Liu et al. 2013), a chitosan–methylisothiazolinone complex was first prepared, by an ionic gelation method, adsorbed onto microfibrillated cellulose (MFC), and then the obtained composite was incorporated into the agar films. In comparison with the pure agar films, the tensile strength of the agar composite films was increased by about 19 % for 10 wt% of chitosan–MFC composite. The antibacterial tests revealed that the agar composite films showed notable antibacterial activities against both *E. coli* and *S. aureus* and therefore are suitable to be used as multifunctional agar films in the medical domain. In a recent study, He et al. (2014) reported the development of functional chitosan-coated oxidized regenerated cellulose gauze for biomedical applications. Oxidized regenerated cellulose gauze, prepared by oxidation of regenerated cellulose using NO_2/CCl_4, was treated with dissolved chitosan in aqueous acetic acid and finally neutralized with NaOH/ethanol. The results of the hemostatic test on rabbit liver and ear-artery injuries showed that the adsorption of high molecular weight chitosan to the surface of regenerated cellulose gauze significantly improved the hemostatic effect of the material without compromising its antibacterial activity against *E. coli* and *S. aureus* and degradability.

Quaternary ammonium salts or polymers containing quaternary ammonium groups are also well recognized for their high antibacterial activity. Zhao and Sun (2007) employed three quaternary ammonium salts, namely, 4-aminolaurylpyridinium chloride, 4-benzoylamino-1-dodecylpyridinium bromide, and 4-(1-naphthoyl)amino-1-dodecylpyridinium bromide, in the antibacterial finishing of undyed and dyed cotton cellulose fabrics. These quaternary ammonium salts could establish ionic interactions with sulfonate groups on the dyed cotton fibers, which contributed to higher exhaustion uptakes of the salts and better antibacterial activities against *E. coli*. In another study, antibacterial BC films for potential application as functional wound dressing for acute trauma treatment were produced by immersion of freeze-dried BC membranes in a benzalkonium chloride solution (a cationic surfactant type antibacterial agent) followed by an additional freeze-drying stage. Some physical and antibacterial properties of the ensuing BC films were inspected, and the results indicated that the loading capacity of the BC dry film was about 0.116 mg/cm^2 when soaked in 0.102 % benzalkonium chloride

solution. With respect to the antibacterial activity, a stable and prolonged antibacterial effect for at least 24 h was achieved against *S. aureus* and *B. subtilis*, Gram-positive bacteria commonly found on the contaminated wounds. Chen et al. (2013) reported a simple method to produce durable antibacterial activity on cellulose by deposition of a CO_2-philic silicone with pendant quaternary ammonium groups, synthesized through hydrosilylation reaction of poly(methylhydrosiloxane) (PMHS) and 2-(dimethylamino)ethyl acrylate in the presence of platinum-based catalyst and subsequent quaternization with 1-bromohexane, onto cellulose by adsorption from supercritical CO_2. Offered data revealed that the antibacterial layer, against *S. aureus* and *E. coli*, was very stable toward washing and UV irradiation due to the low surface tension and relatively high bond energy of the backbone of silicone.

Westman et al. (2009) developed a polyelectrolyte multilayer film with antibacterial properties prepared by the layer-by-layer assembly of hydrophobically modified polyvinylamine and polyacrylic acid on porous cellulose membranes. The methodology included the preliminary introduction of negative charges onto cellulose membranes by NaBr and TEMPO-catalyzed oxidation which generated anchoring sites for the positively charged polyvinylamine. A substantial decrease in bacterial (*E. coli*) growth, ascribed to the modified polyvinylamine, was observed with increasing number of polymeric bilayers (between three and six layers of cationic polymer) on the cellulose membranes.

The use of carboxymethyl cellulose to form polyelectrolyte polymer complexes with cationic antibacterial-wet-strength polymers with guanidine residues in an effort to further increase wet strength and dry strength of paper was also investigated (Qian et al. 2008a) as well as its antibacterial efficiency (Qian et al. 2013). In a different work, Liu et al. (2012b) prepared a series of antibacterial nanofibrous membranes against *E. coli* from cellulose acetate and polyester urethane and using polyhexamethylene biguanide as the antibacterial agent by coelectrospinning or blend electrospinning. The presence of cellulose acetate in the nanofiber membrane improved its hydrophilicity and permeability to air and moisture. Drug release dynamics of the membranes was controlled by its structure and component ratios. The controlled-diffusion membranes exerted long-term antibacterial effect for wound healing.

Antibacterial proteins, specifically lysozyme and lactoferrin, were also incorporated into a matrix of cellulose and carboxymethyl cellulose fibers during the preparation of conventional paper sheets (Barbiroli et al. 2012). More than 60 % of the proteins added (alone or in combination) to the slurry used in the paper preparation were released in buffered saline solutions. The released proteins retained their structural and functional features, indicating that the papermaking process did not affect their structure. The antibacterial activity on common food contaminants was also preserved in the released protein, and a synergic effect between the two proteins was clear in tests carried out with paper sheets containing both proteins against *L. innocua*. Additional tests on thin meat slices placed on these paper sheets indicated that lysozyme was most active in preventing growth of this particular microbiota. In another study (Gemili et al. 2009), antibacterial

packaging materials were obtained by incorporating lysozyme into cellulose acetate films. The maximum release rate, soluble lysozyme activity, and antibacterial activity were achieved with the film prepared with 5 % cellulose acetate solution and 1.5 % lysozyme. Increasing cellulose content decreased the porosity of the films and therefore the release rate and the antibacterial activities of the films. In general, the incorporation of lysozyme did not cause significant reductions in tensile strength and elongation at break values of the films. In a quite distinct vein, Basmaji et al. (2014) reported novel antibacterial bacterial cellulose/polyhexanide biguanide composites produced by symbioses culture between polyhexanide biguanide and green tea culture medium resulting in a 3-D structure consisting of an ultrafine network of biocellulose/PHMB nanofibers matrix (2–8 nm), highly hydrated (99 % in weight), with higher molecular weight, and biocompatible.

In a different study, cellulose fibers were rendered antibacterial against *E. coli* by using cationic beta-cyclodextrin polymer host–guest complexes with antibiotics (butylparaben and triclosan) (Qian et al. 2008b) (Fig. 9). Results of inhibition zones and shaking flask methods of antibacterial-modified cellulose fibers showed that both antibiotics/cationic β-cyclodextrin complexes had exceptional antibacterial activities when spread over on the cellulose fibers; however, triclosan appeared to be more effective. Morphology investigations by atomic force microscopy (AFM) suggested that these complexes inhibited bacteria through disturbing their

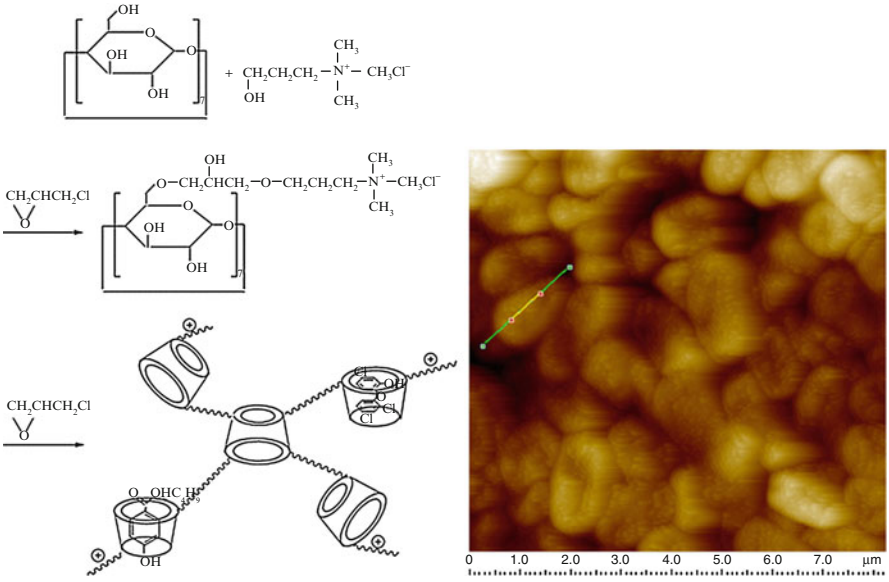

Fig. 9 Schematic representation of the synthesis of cationic β-cyclodextrin polymers and its inclusion with antibiotics. And morphology of *E. coli* treated by butylparaben/ cationic β-cyclodextrin polymer complex (Adapted and reproduced with permission from Qian et al. 2008b)

metabolism instead of damaging the cell membrane (Fig. 9). In a more recent study, Peila et al. (2013) compared two different production ways of obtaining antibacterial cotton fabrics against *E. coli* and *S. aureus* by triclosan inclusion into a beta-cyclodextrin cavity, namely, before or after grafting of beta-cyclodextrin on the cotton fabric. It has been revealed that the stability of the antibacterial finishing depends on the production method, attaining a more durable antibacterial action in case of previous triclosan inclusion followed by grafting. This result proposes that the immobilization onto the fiber has affected the cyclodextrin cavity accessibility. Imra et al. (2010) described novel bioactive composite coatings based on hydroxypropyl methylcellulose and the broad-spectrum food preservative nisin. Glycerol was used as plasticizer. The tensile strength of composite films decreased and the elongation at break increased significantly. Moreover, the transparency and water permeability of the hydroxypropyl methylcellulose films were undesirably affected by the additives. Finally, film bioactivity revealed efficacy against *Listeria* > *Enterococcus* > *Staphylococcus* > *Bacillus* species.

Several other organic biocides, including triazine derivatives (Hou et al. 2009), oregano and sage essential oils (Royo et al. 2010), potassium sorbate (Jipa et al. 2012a; Sayanjali et al. 2011), phenolic compounds (cinnamaldehyde (Nutcha et al. 2012; Rosa et al. 2013), eugenol (Nutcha et al. 2012), thymol (Rodríguez et al. 2014), carvacrol (Rosa et al. 2013; Tunç and Duman 2011), anthraquinones (Zhuo and Sun 2013), and benzoic acid (Dobre et al. 2012)), sorbic acid (Jipa et al. 2012a), dyes (Decraene et al. 2006; Farouk and Gaffer 2013), and streptomycin sulfate (Rajan et al. 2014), have also been used to impart antibacterial properties to cellulose-based materials.

For instance, biodegradable bacterial cellulose-based films, incorporating sorbic acid as antibacterial agent, have been obtained (Jipa et al. 2012b). Monolayer films, prepared using powdered BC and poly(vinyl) alcohol, were coated with BC membranes to obtain multilayer films. Results indicated that both sorbic acid and powdered BC concentration influenced the water sensitivity, release rate, and antibacterial ability of mono- and multilayer films. The release of sorbic acid was faster when powdered BC concentration was higher but significantly slower, as a consequence of formed crystal dissolution, when sorbic acid concentration was increased. The antibacterial activity was tested against *E. coli*, and the results achieved indicated that it was mainly influenced by the sorbic acid concentration and water solubility of the films. These biocomposite films could be used as antibacterial food packaging materials. In a recent study (Zhuo and Sun 2013), two anthraquinone derivatives were incorporated onto cotton fabrics by a vat dyeing process. The dyed cotton fabrics revealed durable light-induced biocidal activity against washing and long-term light exposure but dependent on the anthraquinone compounds used. In a very recent report (Rajan et al. 2014), antibacterial electrospun nanofibrous scaffolds (diameters 400–700 nm) were prepared by physically blending polyurethane, cellulose acetate, and zein. To prevent common clinical infections, streptomycin sulfate, an established antibacterial agent, was integrated into the electrospun fibers. These novel composite nanoscaffolds showed

enhanced blood clotting ability in comparison with original polyurethane nanofibers. The existence of cellulose acetate and zein in the nanofiber composite membrane enhanced its hydrophilicity and bioactivity (against *E. coli*, *S. typhimurium*, *V. vulnificus*, *S. aureus*, and *B. subtilis*) and created a moist environment for the wound, which can accelerate wound recovery.

Antibacterial Cellulose-Based Materials Obtained by Chemical Modification

The development of antibacterial cellulose-based materials by simple blending with antibacterial agents constitutes an up-front strategy; however, it is normally accompanying by the leaching of the antibacterial agents during usage and consequent decrease of the bioactivity. The introduction of permanent antibacterial activity is normally accomplished by chemical grafting of different antibacterial moieties onto the cellulose backbone, though this field is still less explored.

One of the first studies on this domain reported the covalent bond of a reactive melamine derivative, 2-amino-4-chloro-6-hydroxy-*s*-triazine, onto cotton cellulose through nucleophilic substitution (Sun et al. 2005). Upon chlorine bleaching treatment, the covalently bound melamine derivative moieties could be transformed into chloromelamine derivatives, affording strong, long-lasting, and rechargeable antibacterial activity against 10^6–10^7 CFU/mL of drug-resistant Gram-negative (*S. aureus* spp.) and Gram-positive (*E. coli* spp.) bacteria under both waterborne and airborne conditions. However, the inclusion of chlorine bleaching in the process is not very motivating considering its negative environmental impact. In a very attractive work, microfibrillated cellulose was grafted with a quaternary ammonium compound (octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride) by an adsorption-curing process (Andresen et al. 2007). Films obtained from the modified MFC showed substantial antibacterial capacity against *S. aureus*, *E. coli*, and *P. aeruginosa* even at very low concentrations of antibacterial agent immobilized on the surface (0.14 % of atomic nitrogen from the quaternary ammonium compound). The zone of inhibition test demonstrated that no quaternary ammonium compound diffused into the surrounds, corroborating that the films were definitely of non-leaching type. These films could be suitable for application as antibacterial separation filters.

More recently, a similar approach was applied to bacterial cellulose aiming to design functional nanostructured membranes for biomedical applications (Fernandes et al. 2013). Precisely, aiming to mimic the natural antibacterial activity of chitosan macromolecules, antibacterial nanostructured bacterial cellulose membranes were obtained by chemical grafting of aminoalkyl groups onto the surface of its nanofibrillar network (Fig. 10). Interestingly, these novel grafted bacterial cellulose membranes are simultaneously lethal against *S. aureus* and *E. coli* and nontoxic to human adipose-derived mesenchymal stem cells (ADSCs). In addition to these motivating biological properties, the bioactive nanostructured membranes also showed enhanced mechanical and thermal properties. In a more complex way, Rouabhia et al. (2014) reported novel gentamicin-activated BC membranes obtained by chemically grafting RGDC peptides (R, arginine; G, glycine; D, aspartic acid; C, cysteine) using the coupling agent 3-aminopropyltriethoxysilane

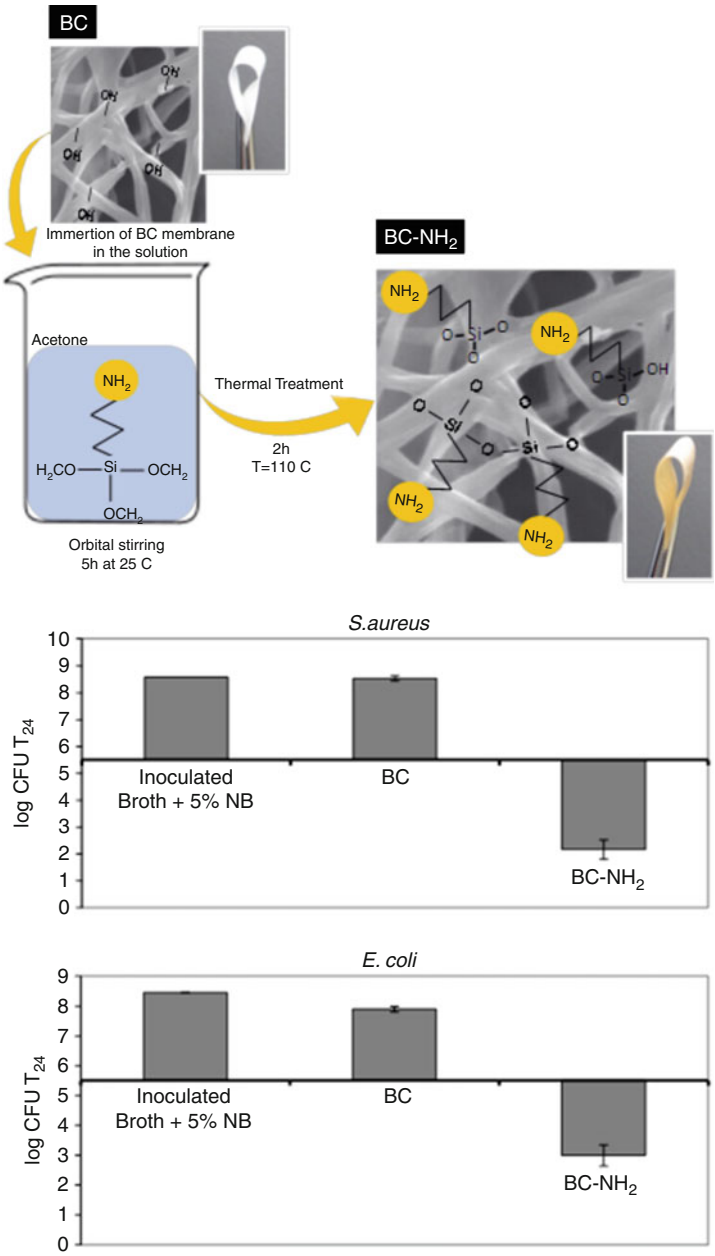

Fig. 10 Mechanism of silane-bacterial cellulose chemical grafting using APS as active reagent to prepare BC membranes with antibacterial properties. And antibacterial activity of blank BC and BC-NH2 mats respect to *S. aureus* and *E. coli* of. The obtained results are compared with reference sample (unmodified BC mat) as well as inoculated broth added with 5% of Nutrient Broth (NB) (Adapted and reproduced with permission from Fernandes et al. 2013)

followed by covalent linkage of gentamicin onto the surface of the BC. As well as in the preceding example, the gentamicin–RGDC-grafted BC membranes are bactericidal against *S. mutans* but nontoxic to human dermal fibroblasts and thus may be advantageous for multiple applications such as functional wound healing and drug delivery systems. In a previous study (Lochman et al. 2010), the effect of topically applied gentamicin attached to a new biodegradable carrier formed by microdispersed oxidized cellulose in microfiber and nanofiber form for treatment of acute wound infection was also investigated. However, in this case gentamicin was not chemically attached to cellulose.

In another pioneering work, 2-(dimethylamino)ethyl methacrylate (DMAEMA) was polymerized from cellulosic filter paper via reversible addition–fragmentation chain transfer (RAFT) polymerization (Roy et al. 2008). The tertiary amino groups of the grafted polymeric chains were subsequently quaternized with alkyl bromides of different chain lengths (C8–C16) to afford a large concentration of quaternary ammonium groups on the cellulose surface. The antibacterial activity of the obtained materials against *E. coli* was found to be reliant on the alkyl chain length and on the degree of quaternization, with the PDMAEMA-grafted cellulose fiber with the highest degree of quaternization and quaternized with the shortest alkyl chains showing the highest activity. In a different line, Elegir et al. (2008) developed antibacterial cellulose packaging via laccase-mediated grafting of phenolic compounds (*p*-hydroxybenzoic acid, caffeic acid, gallic acid, dopamine, eugenol, isoeugenol, and thymol). The handsheet papers obtained by laccase antibacterial surface treatment exhibited a greater efficiency against Gram-positive (*B. subtilis*, *E. hirae*, *S. epidermidis*, *S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, and *K. pneumonia*) bacteria than those treated only with monomeric phenolic compounds. Antibacterial effect was dependent on the grafted structure, time of the reaction, and concentration of phenolic derivatives. For example, the laccase antibacterial surface obtained in the presence of caffeic acid or *p*-hydroxybenzoic acid produced paper handsheets with strong bactericidal effect on *S. aureus* even at low phenol monomer concentration (4 mM). Feese et al. (2011) and carpenter et al. (2012) described the synthesis and characterization of groundbreaking cellulose nanocrystals surface-modified with a cationic porphyrin. The porphyrin moiety was appended onto the cellulose surface via the Cu(I)-catalyzed Huisgen–Meldal–Sharpless 1,3-dipolar cycloaddition. The obtained materials showed outstanding efficiency toward the photodynamic inactivation of *M. smegmatis* and *S. aureus*, although only slight activity against *E. coli*, and therefore could have potential utilization in the health care and food preparation industries. In a different study, the antibacterial properties of oxidized cellulose (commercial oxidized cellulose sample for biomedical applications with different carboxylic acid %, up to 20.6 %) and its salts (with Ag^{+}, Mg^{2+}, Na^{+} or Zn^{2+}) in linters, microsphere, and textile form were tested by a dilution method against a range of several microbial strains, namely, *E. coli*, *P. aeruginosa*, *S. epidermidis*, *B. licheniformis*, *A. niger*, *P. chrysogenum*, *R. oryzae*, *S. brevicaulis*, *C. albicans*, and *C. tropicalis* (Vytrasova et al. 2008). The results of this study showed that the acidic form of the oxidized cellulose with zinc has a pronounced potential to be

explored in biomedical applications, e.g., as dressing material, against bacteria as well as against fungal *Candida* species.

Chitosan cross-linked cellulose fibers were obtained by means of a benign process aiming to confer antibacterial properties to cellulose fibers (Alonso et al. 2009). Citric acid was used as the cross-linker agent and NaH_2PO_4 as catalyst in formerly UV-irradiated cellulose fibers. Further heat dried-cure process and washing with detergent, water, and acetic acid (0.1 M) yielded a maximum incorporation of 27 mg of chitosan/gram of functionalized cellulose. The material with the highest chitosan content displayed augmented thermal stability compared to the initial cellulose and chitosan samples. The colony-forming units/mL for *E. coli* and the biomass and spore germination fraction of *P. chrysogenum* decreased significantly on the cross-linked materials as compared to native cellulose fibers. For illustration, the sample with the highest chitosan content incorporated inhibited the fungal growth with 66 % less biomass amount than that obtained with raw cellulose. In a different study, the flocculation and antibacterial properties of water-soluble quaternized cellulose derivatives synthesized by reaction with 3-chloro-2-hydroxypropyltrimethylammonium chloride in NaOH/urea aqueous solutions (degree of substitution (DS) of 0.38, 0.50, and 0.74) were investigated (Song et al. 2010). The results showed that the obtained quaternized cellulose derivatives were effective flocculating agents for montmorillonite over a wide range of pH values and that powerfully inhibit the growth of *E. coli* and *S. aureus*, being potentially applicable as an original wastewater treatment agent with high flocculation efficacy as well as effective antibacterial effect. Bieser et al. (2011) reported the synthesis of contact-active antibacterial coating that only degraded in the presence of cellulase. Specifically, 2′,3′-dideoxyadenosine (DDA) function was grafted to a cellulose backbone via a poly(2-ethyloxazoline) spacer. It was found that all coatings were effective active against *S. aureus* and those with high DS and long polymeric spacers degraded in water. This constitutes an interesting strategy for the development of new antifouling paints useful for marine fouling prevention. In another study (Salimpour Abkenar and Mohammad Ali Malek 2012), two generations of poly(propylene imine) dendrimers with amino-terminated groups were successfully grafted on cotton fabric using citric or glutaric acids as cross-linking agents, as confirmed by FTIR analysis. Moreover, nitrogen content determination revealed the presence of the dendrimers on the cotton fabric even after five washing cycles. The dendrimer grafted cotton fabric showed a 99 % reduction in microbial counts against *S. aureus*, *E. coli*, and *C. albicans*. Kang et al. (2012) synthesized a novel quaternized *N*-halamine precursor (3-chloro-2-hydroxypropyl)-(5, 5-dimethylhydantoinyl-1-ylmethyl)-dimethylammonium chloride by a two-step reaction that was then efficiently grafted onto the surface of cellulose by a dehydrochlorination reaction. The quaternized precursor grafted on cellulose was finally converted to an *N*-halamine structure by a chlorination reaction with a diluted NaClO solution. The antibacterial experiments revealed that the chlorinated grafted cellulose fabric was capable of 5-log inactivation of *S. aureus* and *E. coli* within 5 min. This methodology could be used in the antibacterial finishing

of textiles. In another recent study, a "clickable" quaternary ammonium compound, specifically *N*-(2-ethoxy-2-oxoethyl)-*N*,*N*-dimethylprop-2yn-1-aminium bromide, was synthesized through an artless reaction and covalently bonded to a cellulose derivative containing an azido group (3-*O*-azidopropoxypoly(ethylene glycol)-2,6-di-*O*-thexyldimethylsilyl cellulose, DS 0.54 at C3) to form non-leaching antibacterial cellulose-based material via the simple Cu(I)-catalyzed alkyne-azide [2 + 3] cycloaddition reaction. Noteworthy antibacterial activity of the obtained cellulose derivative (DS = 0.30 at C3) was established by testing against *E. coli* (about 50 % of the bacteria were killed when 0.4 mg of the compound were added to a solution containing 2,700,000 CFU of live cells). This investigation offers a simple and robust route toward the development of permanent antibacterial materials and biomedical devices.

3.2.2 Starch

In recent decades starch has attracted an enormous interest as a source of new renewable materials, due to its low cost, biocompatibility, nontoxicity, and simplicity to convert into a thermoplastic material, referred to as thermoplastic starch (TPS), through the breaking of the hydrogen bond interactions between polymeric chains by a plasticizer at specific temperature and/or mechanical energy conditions (Carvalho 2008; Chivrac et al. 2009). The preparation of TPS-based materials is often preceded by the treatment of starch in water at specific temperatures leading to the dissolution of starch granules due to the disruption of the hydrogen bonds between the polysaccharide chains, leading to the so-called gelatinized starch solutions, that can be used to coat materials (e.g., food products and nanoparticles, among others) or to produce TPS-based films or membranes by casting. However, starch-based materials present several limitations such as poor mechanical performance and water resistance and, particularly relevant in the present context, the intrinsic lack of biological activity. To overcome such limitations, the most common approach involves the combination of starch with other chemicals or polymeric materials in gelatinized solutions used to produce coatings or blended/composite films where the specific target properties are imparted by those additives (bioactive polymers, natural compounds, or metal nanoparticles among others), while preserving most of the connotations associated with the starch matrix, such as its biodegradability, biocompatibility, renewable character, and in a large number of cases edibility.

Here an overview of the most common strategies, reported in the last decade, to impart antibacterial activity to starch containing materials will be comprehensively explored. The major strategy used to produce starch-based materials with antibacterial properties (mainly edible coatings, films, and membranes, aimed at reducing fresh food contamination and extending shelf life) involved its combination with bioactive plant extracts (with special emphasis in essential oils and phenolic extracts, or corresponding pure compounds), followed by the blending with chitosan and several other bioactive materials. Another approach pursued was the combination with metal nanoparticles (especially silver); however, in most of these studies, starch was used to coat/stabilize the metal nanoparticles which might

find uses in active packaging and biomedical applications rather than to produce edible films and coatings.

Antibacterial Starch-Based Materials with Plant Extracts

One of the simplest examples regarding the preparation of starch-based antibacterial materials involves the use of gelatinized starch solutions with bioactive plant extracts and their direct use in food coatings. As an illustrative example, Goswami et al. (2009) spread pre-gelatinized pea starch with 0.5 % thyme essential oil over chicken breast meat after inoculation with several bacteria (from the genera *Salmonella*, *Listeria*, *Campylobacter*, and *Pseudomonas*), followed by packing in plastic bags and storage at 4 °C. It was shown that the starch/thyme oil coating effectively reduced the viability of *Salmonella*, as well as the growth of *Listeria* and *Pseudomonas*, and eliminated *Campylobacter* during storage.

Edible coatings combining tapioca starch with decolorized hsian-tsao leaf gum matrix and glycerol and with various green tea extracts, obtained under different extraction conditions, were also evaluated for their antibacterial activity (Chiu and Lai 2010). All the green tea extracts showed a pronounced inhibition of Gram-positive bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*, increasing with the extract content, but not of Gram-negative bacteria, such as *Escherichia coli* and *Salmonella enterica*. When the various green tea extracts were added to the edible coating formulations, a strong antibacterial activity on Gram-positive bacteria was also observed as evaluated by using cylinder diffusion and antibacterial migration tests. It is proposed that the green tea extract active components leave the coating matrix and migrate to the surface of the substrate to increase the nongrowth area. These edible coatings with green tea extracts were sprayed over several food components (fruit-based salads, romaine hearts, and pork slices), demonstrating a pronounced antibacterial activity against Gram-positive bacteria, during refrigerated storage of romaine hearts and pork slices for 48 h. These coatings also showed that they could successfully reduce the aerobic counting and growth of yeasts/molds in fruit-based salads, as compared to the control samples. These two studies (Chiu and Lai 2010; Goswami et al. 2009) clearly demonstrate the potential of the addition of bioactive extracts to starch-based edible coating formulations to prevent food products spoilage and contamination and therefore improve their safety and shelf life.

Distinct plant extracts and selected bioactive compounds were also included in starch films leading to antibacterial materials. One of the first approaches comprises the use of phenolic-rich plant extracts as will be highlighted by the following examples (Corrales et al. 2009; Kuorwel et al. 2014; Li et al. 2013; Nouri and Mohammadi Nafchi 2014; Pyla et al. 2010).

As an illustrative example, Corrales et al. (2009) used grape seed extracts, with high total phenolic content and antioxidant activity (mostly attributed to their flavonoid and phenolic acid composition), to prepare antibacterial starch-based films. The extracts were tested against several bacteria showing that they inhibited the growth of Gram-positive foodborne pathogens but not Gram-negative ones. These extracts were incorporated into pea gelatinized starch solutions, followed by

solvent casting. The films containing 1 % of grape seed extracts revealed an increment in thickness and decrease in the puncture and tensile strength compared to control ones. Furthermore, the in vitro tests of the films containing the grape seed extracts with pork loins infected with *Brochothrix thermosphacta* showed a bacterial growth reduction in 1.3 log CFU mL^{-1} after 4 days of incubation at 4 °C, demonstrating that these films can be used to inhibit the growth of pathogens in meat products, improving meat quality and shelf life.

Tannic acid (a commercially available complex mixture of polyphenolic structures which can be obtained from several plants), known as a food additive with antibacterial activity against common food pathogens, was also used as such or after an hydrothermal treatment, to produce starch-based films by casting (Pyla et al. 2010). The resulting films were then tested against *E. coli* and *L. monocytogenes*. It was demonstrated that the bacterial inactivation curves correlated well with the kinetics of release of tannic acid from the starch films.

Li et al. (2013) studied the antibacterial activity against *E. coli* of cornstarch films incorporating extracts of several Chinese medicinal herbs, evaluating also the mechanical properties and moisture resistance of the resulting films. The authors highlighted the effect of the addition of *Scutellaria baicalensis* and *Schisandra chinensis* extracts, which, despite some decrease in mechanical properties (e.g., extensibility) and water resistance, induced strong antibacterial properties on the films.

Nouri et al. (2014) studied the biological activity of sago starch films incorporating different contents of betel leaf ethanolic extract, showing a decrease in tensile strength with increasing elongation with growing extracts content, whereas the water and oxygen permeability increased. These films were claimed to show a good inhibitory activity against nonspecified Gram-positive and Gram-negative bacteria with the exception of *P. aeruginosa*.

The incorporation of essential oils or some pure monoterpenic compounds into starch edible films has also been considered in several studies (Ehivet et al. 2011; Kuorwel et al. 2011, 2013, 2014). For example, Ehivet et al. (2011) prepared sweet potato starch-based edible films by casting, containing origanum (*Thymus capitatus*) oil and different plasticizers (propylene glycol, sucrose, sorbitol, and glycerol), and evaluated their antibacterial properties against *S. enteritidis*, *E. coli*, and *L. monocytogenes* as well as their mechanical properties and water vapor permeability. The films produced with 2.5 % (w/v) starch and 40 % (w/v) sorbitol demonstrated the best mechanical and barrier performance, and their antibacterial activity was shown to be directly related with the content of origanum oil, in the range of 0.5–2 % (v/v).

In a study with pure essential oil components, namely, linalool, carvacrol, and thymol incorporated in starch-based films, Kuorwel et al. (2011) demonstrated their antibacterial activity against *S. aureus* both in vitro and inoculated in cheddar cheese. In vitro, using the agar diffusion method, it was demonstrated that the antibacterial activity increased significantly with the concentrations of the antibacterial agents. Furthermore, all the starch films containing the bioactive agents inhibited the bacterial growth in inoculated cheddar cheese, with the highest

activity being reported for thymol, followed by carvacrol and linalool. However, as in most studies mentioned above, it was shown (Kuorwel et al. 2014) that the increasing amounts of the antibacterial agents decreased the tensile strength of the films and increased the elongation at break. However, at low contents these three components did not drastically affect the transparency or the water vapor permeability and the morphology of the films.

In addition to the straightforward combination with bioactive extracts (again mainly phenolic-rich extracts and essential oils) with starch, several studies additionally involved the blending with other polymeric materials to improve the properties of the ensuing starch-based materials (Chiu and Lai 2010; Jagannath et al. 2006; Maizura et al. 2007; Wu et al. 2010).

Jagannath et al. (2006) prepared starch–casein-based edible films by casting, followed by heat pressing, with the incorporation of neem (*Melia azardirachta*) water extract. The films showed suitable water vapor and oxygen permeability, as well as thermal and mechanical properties, which were in general enhanced with the hot pressing operation. The addition of the neem extract did not affected these properties and imparted the films with antibacterial properties against *E. coli*, *S. aureus*, *B. cereus*, *L. monocytogenes*, *Pseudomonas* spp., and *S. typhimurium*.

Wu et al. (2010) studied the production of starch–polyvinyl alcohol films impregnated with catechin-rich green tea extracts. Although the mechanical properties (tensile strength and elongation) decreased with increasing amounts of incorporated extract, the inclusion of 1000 ppm of catechins in the films resulted in a considerable antioxidant capacity and antibacterial activity against *E. coli*, as well as some yeasts in raw beef at refrigeration temperature, although a slightly negative effect over meat color was observed.

The combination of tapioca starch with decolorized hsian-tsao leaf gum used above to produce antibacterial coatings (Chiu and Lai 2010) has been also used in the preparation of films incorporating potassium sorbate and an ethanolic thyme extract, which have been tested against *L. monocytogenes*. The inclusion of the thyme extract contributed to a smoother microstructure of the films, as evidenced by SEM analysis, but in general their mechanical performance (tensile strength and modulus) decreased with the increasing content of the antibacterial extract. The antibacterial properties of the films were lower than those of the isolated extracts which was attributed to limited migration of the extract from the film. These films were also tested in fresh beef demonstrating to inhibit the growth of the tested bacteria.

Edible films of partially hydrolyzed sago starch (*Metroxylon sagu*) and alginate, with glycerol as plasticizer and lemongrass oil as bioactive component, were tested for their antibacterial activity against *E. coli* based on the zone inhibition assay and proved to be active in all lemongrass oil concentration levels (Maizura et al. 2007). It was also noticed that the antibacterial activity of these films against *E. coli* was substantially enhanced by the addition of the plasticizer (20 % glycerol). Furthermore, although the addition of lemongrass oil did not have any interaction with the functional groups of film components, as measured by FTIR, the elongation at break and water vapor permeation of the plasticized materials were found to

increase significantly with increasing lemongrass oil content. In another study, starch and alginate films incorporating sodium dehydroacetate or a rosemary extract have been studied for their antibacterial activity against *E. coli* (Yan et al. 2013). Both additives reduced starch crystallinity, as well as the mechanical properties of the films (tensile strength and elongation at break), and increased both the surface roughness and the water vapor permeability, as well as the color of the films. Films with either sodium dehydroacetate or rosemary extract showed a strong anti-*E. coli* effect.

In a distinct application Arfa et al. (2007) used octenyl-succinate-modified starch (and soy protein isolates for comparative purposes) as paper coatings and inclusion matrices for antibacterial cinnamaldehyde and carvacrol and tested for their antibacterial activity against *E. coli*. Both modified starch and soy protein isolates demonstrated good retention ability of the bioactive compounds during paper coating and drying. The ability of coated papers to release these compounds over storage time was assessed and demonstrated that both compounds are gradually released from the coating; furthermore, the favorable antibacterial properties are related with the total release of the bioactive agents.

Antibacterial Starch-Based Materials with Chitosan

The combination of starch and chitosan to produce edible films and membranes has also been investigated with some detail. Here chitosan brings both its filmogenic and antibacterial properties to the resulting materials arising from the presence of amino groups of the glucosamine unit and its ability (in the cationic form) to disrupt bacterial membranes.

Most starch/chitosan films were prepared using gelatinized starch and chitosan (and cationized water-soluble chitosan; Tome et al. 2012) solutions with a plasticizer (most often glycerol) by casting (Durango et al. 2006; Liu et al. 2009; Mei et al. 2013; Tomé et al. 2013; Vásconez et al. 2009; Yoksan and Chirachanchai 2010; Zhong et al. 2011) or by extrusion (Bie et al. 2013; Pelissari et al. 2009; Tome et al. 2012).

In general, the obtained starch/chitosan edible films/membranes are thermally stable with a good flexibility (Durango et al. 2006) and with elongation at break and water vapor permeability considerably improved when compared to the single components films, mainly due to the interaction between the hydroxyl groups of starch and the amino groups of chitosan (Liu et al. 2009); additionally, some additives, such as oregano essential oil (Pelissari et al. 2009) or potassium sorbate (Shen et al. 2010), also improved film properties such as elongation and water vapor permeability. Furthermore, it was also demonstrated that the mechanical properties and water vapor permeability of starch/chitosan films might also be affected by the nature of the acid used to dissolve chitosan, with the best results in terms of mechanical properties being obtained with acetic and lactic acid, respectively (Zhong et al. 2011).

Apart from the combination of starch/chitosan with other bioactive additives, the combination with polylactic acid (Bie et al. 2013) and bacterial cellulose (Tomé et al. 2013) has also been reported. In the case of the combination

with polylactic acid (Bie et al. 2013), the resulting material acted as a slow chitosan release system whose rate depended on the hydrophilicity of the blend, which in turn was mainly determined by its starch content. The combination of starch/chitosan with bacterial cellulose (Tomé et al. 2013) was aimed at improving the mechanical properties of the materials, without affecting its transparency, taking advantage of the unique features of the bacterial cellulose nanofibrillar structure.

Another distinct approach involved the preparation of antibacterial starch/chitosan nanocomposite films loaded with spherically shaped (20–25 nm) silver nanoparticles by casting (Yoksan and Chirachanchai 2010). The incorporation of Ag NPs leads to a slight improvement in tensile strength and oxygen barrier properties and to a decrease in water barrier properties.

The starch/chitosan films showed excellent antibacterial properties against *S. enteritidis* (Durango et al. 2006; Pelissari et al. 2009), *E. coli* (Bie et al. 2013; Liu et al. 2009; Mei et al. 2013; Pelissari et al. 2009; Shen et al. 2010; Yoksan and Chirachanchai 2010; Zhong et al. 2011), *S. aureus* (Bie et al. 2013; Mei et al. 2013; Pelissari et al. 2009; Shen et al. 2010; Tome et al. 2012, 2013; Yoksan and Chirachanchai 2010; Zhong et al. 2011), *B. cereus* (Pelissari et al. 2009; Yoksan and Chirachanchai 2010), and *L. monocytogenes* (Mei et al. 2013) but not against *Lactobacillus* spp. (Vásconez et al. 2009). In specific cases, it was suggested that the antibacterial activity of the films was not only due to chitosan but also to the addition of other bioactive agents such as oregano essential oil (Pelissari et al. 2009), potassium sorbate (Shen et al. 2010), *Cornus officinalis* fruit extract, and pine needle essential oil and nisin (both isolated or combined with each other) (Mei et al. 2013). The antibacterial properties of the starch/chitosan films are also affected by the acid used to dissolve chitosan, with the best results against *S. aureus* and *E. coli* being reported with malic acid (Zhong et al. 2011).

Antibacterial Starch-Based Materials with Metal Nanoparticles

The use of metal nanoparticles as antibacterial agents found a tremendous boost in recent years, with particular emphasis in silver nanoparticles. In this context, there are also a considerable number of studies dealing with metal nanoparticles (again mainly silver) and starch; however, as will be highlighted in the key examples put forward below, instead of being used to produce edible coatings or films, starch is being used as a stabilizing agent for silver nanoparticles (Ag NPs). Nevertheless in most cases these stabilized Ag NPs have been successfully tested for their antibacterial activity.

Ag NPs stabilized with starch are normally produced from $AgNO_3$ aqueous solutions in the presence of solubilized starch, followed by reduction either by γ-irradiation (Kassaee et al. 2008), by hydrothermal treatment (Mohanty et al. 2012), or by reduction with sodium borohydride (Raji et al. 2012), glucose (Gao Xianghua et al. 2013), and other monosaccharides (Kahrilas et al. 2014) or even by starch itself (Ayala Valencia et al. 2013; Taheri et al. 2014) or starch dialdehyde (Wang et al. 2013a) acting as the reducing agent, resulting in starch coated Ag NPs.

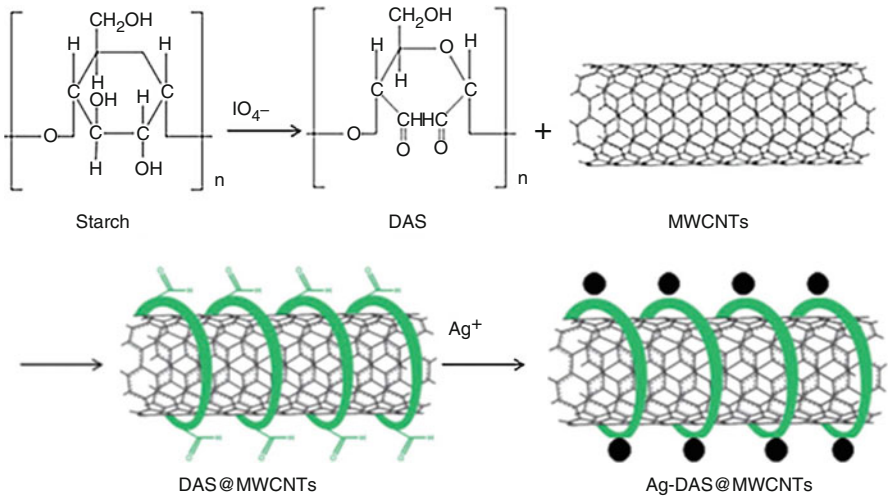

Fig. 11 Schematic description of the process of production of multiwalled carbon nanotubes decorated with silver nanoparticles using starch dialdehyde (Reproduced with permission from Wang et al. 2013a)

Ag NPs coated with starch are most commonly spherical with average size distributions in the range of 10–30 nm (Ayala Valencia et al. 2013; Gao Xianghua et al. 2013; Kahrilas et al. 2014; Mohanty et al. 2012; Raji et al. 2012), although cubic shape NPs with average sizes of 14 nm have also been reported (Kassaee et al. 2008),

In general, it was demonstrated that Ag NPs are embedded in starch macromolecules (Kassaee et al. 2008) and tested as such against distinct bacterial strains. In a more sophisticated approach, Wang et al. (2013a) have bound starch dialdehyde (obtained by partial oxidation of starch with sodium periodate) to multiwalled carbon nanotubes. In a second step the starch dialdehyde was used as the reducing as well as a complexing agent to deposit Ag NPs onto the surface of starch modified multiwalled carbon nanotubes (Fig. 11), which demonstrated to have superior antibacterial activity against several bacteria as enumerated below.

Ag NPs have also been prepared in situ and incorporated into starch base nanocapsules by a miniemulsion/polyaddition polymerization of 2,4-toluene diisocyanate using polyglycerin-polyricinoleate as surfactant (Taheri et al. 2014). The resulting round-shaped nanocapsules were then carboxymethylated with monochloroacetic acid under alkaline conditions. The nanocapsules' shell thickness could be tuned from 13 to 29 nm by varying the amount of cross-linker.

Starch materials with Ag NPs were shown to have a broad range of antibacterial activity against Gram-positive and Gram-negative pathogens, and at the same time to be nontoxic to macrophages at the bactericidal concentrations, and can even augment their intracellular killing potential (Mohanty et al. 2012). In detail, the antibacterial activity of Ag NPs has been demonstrated against *E. coli*

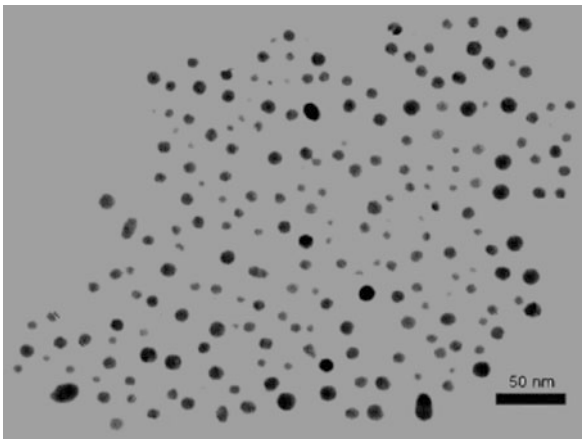

Fig. 12 TEM images of starch capped copper nanoparticles (Reproduced with permission from Valodkar et al. 2012)

(Gao Xianghua et al. 2013; Kahrilas et al. 2014; Kassaee et al. 2008; Raji et al. 2012; Taheri et al. 2014; Wang et al. 2013a), *S. typhi* (Mohanty et al. 2012), *P. aeruginosa* (Kahrilas et al. 2014; Mohanty et al. 2012; Raji et al. 2012), *S. aureus* (Ayala Valencia et al. 2013; Gao Xianghua et al. 2013; Kahrilas et al. 2014; Mohanty et al. 2012; Raji et al. 2012), *S. flexneri* (Mohanty et al. 2012), *S. epidermidis* (Raji et al. 2012; Taheri et al. 2014), *M. smegmatis* (Mohanty et al. 2012), *Bacillus megaterium* (Wang et al. 2013a), *Bacillus subtilis* (Kahrilas et al. 2014; Wang et al. 2013a), *Klebsiella pneumonia* (Kahrilas et al. 2014), and *Janthinobacterium lividum* (Kahrilas et al. 2014).

Apart from Ag NPs coated with starch, a few other examples of conjugations of starch with NPs include copper nanoparticles (Valodkar et al. 2012) and zinc oxide nanorods (Nafchi et al. 2012).

As in the case of Ag NPs, monodisperse copper NPs, with average size of 10 nm (Fig. 12), were prepared by microwave irradiation using starch as green capping agent; the ensuing solutions were shown to exhibit excellent bactericidal action against *S. aureus*, *E. coli*, and *S. typhi* (Valodkar et al. 2012). It was also demonstrated that copper nanoparticles capped with starch are not cytotoxic against 3T3L1 cells at the concentrations required for antibacterial activity, opening the perspective of application of these NPs in biomedical field.

Finally, zinc oxide nanorods were incorporated into sago starch solutions at different concentrations and nanocomposite films were obtained by casting (Nafchi et al. 2012). These films showed excellent antibacterial properties against *S. aureus* directly related with the nanorod content. Additionally, the inclusion of the zinc oxide nanorods substantially decreased the water permeability and increased the hydrophobicity of the film surface and low light transmittance in the UV-Vis.

Antibacterial Starch-Based Materials with Miscellaneous Components

Apart from bioactive plant extracts and chitosan, several other components have been used to prepare antibacterial starch-based material, namely, lysozyme (Basch et al. 2012; Dawson et al. 2005; Li et al. 2012b), nisin (Basch et al. 2012;

Dawson et al. 2005), and the combination with other antibacterial cationic polymers as polyhexamethylene guanidine (Guan et al. 2008a, b; Ziaee et al. 2010), among others discussed below.

Pea starch-based materials with lysozyme were prepared by Nam et al. (2007) under different extrusion conditions aiming to obtain extrudates with strong mechanical properties able to be used in biodegradable rigid containers, with antibacterial activity against *B. thermosphacta* due to the gradual release of lysozyme. The release of lysozyme from microgels of oxidized starch was also evaluated (Li et al. 2012b). Here starch was oxidized with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and chemically cross-linked with sodium trimetaphosphate (STMP), yielding a gel that could be converted into a finely powdered material. The dry gel particles were then suspended in water to which a solution of lysozyme was added. The microgel is negatively charged and interacts with positively charged lysozyme by electrostatic attraction. Application of the lysozyme-containing starch particles to environments contaminated with amylase producing microbes may lead to the hydrolysis of starch. As a result, lysozyme is released and will inhibit the growth of lysozyme-sensitive bacteria. The potential application of this material was demonstrated against *B. licheniformis* and *B. subtilis*. Due to the frequent presence of amylase-producing bacteria in food products, this material might find application in food protection systems. Finally, lysozyme was also incorporated in pea protein-/corn starch-based films, and its release into a food simulant (agar gel) was evaluated (Fabra et al. 2014) showing that the protein diffuses from the film at increasing rate with increasing temperature and concentrated mainly on the surface of the simulant where most of its antibacterial activity against *L. monocytogenes* was observed, being therefore able to preserve the surface of food products from microorganisms.

Another bioactive proteic material, the antibacterial peptide nisin has been incorporated in starch powders and films (Basch et al. 2012; Dawson et al. 2005). Nisin has been adsorbed into cornstarch powder (among other food-grade powders), by placing them in agitated nisin solutions, followed by dehydration and testing against *L. plantarum* or *L. monocytogenes* (Dawson et al. 2005). Nisin adsorbed into starch preserved 54 % of its original activity, and between 45 % and 61 % of the peptide was released over time (depending on the initial load). The starch-based powder showed an intense antibacterial activity against *L. monocytogenes*. Nisin and potassium sorbate have also been incorporated into tapioca starch edible films plasticized with glycerol (Basch et al. 2012), which demonstrated that the combination of both bioactive agents resulted in an enhanced activity against *L. innocua* as well as some yeast, when compared with films with a single compound. The addition of the bioactive agents led to some reduction in the mechanical properties of the films, when compared to pure starch, but they were still suitable for packaging applications.

In another approach, antibacterial starch materials were prepared by coupling starch with polyhexamethylene guanidine via a reaction with a diepoxide (Guan et al. 2008a), and their adsorption onto cellulose fibers was studied in detail (Guan et al. 2008b). These modified starch materials showed a high antibacterial activity

against both *E. coli* (Guan et al. 2008a; Ziaee et al. 2010) and *S. aureus* (Guan et al. 2008a), due to the disruption of cell membranes, which as in the case of chitosan is attributed to the cationic nature of the material, both when adsorbed onto cellulose fibers in paper sheets (Guan et al. 2008a) and recycled fibers (Ziaee et al. 2010). In adequate doses, these modified starches promoted a 100 % growth inhibition of the studied bacteria.

In another study implying the cationization of starch-based materials, quaternary phosphonium starch has been grafted with acrylamide and allyl triphenyl phosphonium bromide at different ratios by simultaneous gamma irradiation, and the resulting copolymers were tested against *S. aureus* (Song et al. 2013). The key factor influencing the antibacterial activity of the material was the degree of cationization, which demonstrated that the grafted starch derivatives with higher cationic degree were able to kill >99.75 % of the bacteria within 30–60 min.

Bursali et al. studied the preparation and antibacterial activity of casted films based on boron/starch/polyvinyl alcohol hydrogels by blending gelatinized starch with polyvinyl alcohol and boric acid with or without the addition of glutaraldehyde as cross-linking agent (Bursali et al. 2011). The best film formulations, incorporating boron complexes and glutaraldehyde, showed an antibacterial activity against *E. coli* comparable to that of the standard antibiotic erythromycin, whereas cross-linked films without boron complexes were not active. Starch/poly(vinyl alcohol) blends prepared by using citric acid as plasticizer and glutaraldehyde as the cross-linker have also been used to prepare composite films with cellulose as reinforcing agent (Priya et al. 2014). The good adhesion of the starch/poly(vinyl alcohol) blends to the fibers resulted in a composite with improved mechanical properties when compared to isolated starch/poly(vinyl alcohol) films. The composite films showed a good antibacterial activity against *S. aureus* and *E. coli*.

In another study, Mehyar et al. (2007) investigated the use of pea starch and calcium alginate incorporating trisodium phosphate and sodium chlorite as antibacterial coatings on chicken skin and demonstrated its effectiveness against *Salmonella* spp. It was verified that the antibacterial activity of the films was significantly higher than that of trisodium phosphate and sodium chlorite applied alone, which might be related with the high adhesion of the starch-based formulation, when compared to the isolated compounds that rapidly dripped from the skin.

4 Conclusion: Future Trends

The possibility of incorporating antibacterial agents either in surface coatings or within material matrices has attracted considerable global attention in recent years. For example, in the food preservation fields, antibacterial materials have been identified as one of the most promising forms of active packaging technologies. Also, in a context of fatal nosocomial and community-acquired infections, the development of antibacterial materials has opened novel opportunities to inhibit microbial adhesion and limit transmission. In addition, it would be highly desirable to really develop biodegradable (or even edible in case of food preservation fields)

and sustainable matrices capable of safe and long shelf-life integration of bioactive substances.

In this perspective, it is clear that biobased materials will act as an "added value" argument for the upward use of sustainable biomass-derived material components. The present appraisal clearly demonstrates that abundant polysaccharides can be used to effectively prepare antibacterial materials either by improving/modulating the properties of naturally antibacterial chitosan or by de novo conferring these properties to cellulose or starch. The combination of antibacterial activity with other well-known properties of these polysaccharides opens wide perspectives for the future development of innovative functional materials. In this context, nanomaterials and nanotechnology in general are emerging as powerful tools for the design of new materials with unique functional properties among which antibacterial activity will certainly be targeted with increased efficiency and specificity (Lagaron et al. 2012).

However, most of the research studies mentioned in this chapter have been carried out often in vitro and at a laboratory scale. Because antibacterial material efficiency may be influenced by interfering substances that inhibit the antibacterial action, it is thus necessary to conduct in vivo studies more frequently. Furthermore, the scale-up of the production of those materials that prove to be successful in vivo is also a big challenge to address in the coming years to allow them to be turned into marketable products.

To conclude, the development of bioactive materials based on a release-on-demand of antibacterials is a real new challenge. The release of antibacterial agents from materials in response to an increase in microbial population is still in the research phase, especially in the case of food preservation, and far from a final optimized commercial process. That is to say, the development of stimuli-responsive materials could be a very good opportunity for new preservation trends.

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Modified Polysaccharides for Drug Delivery 57

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Contents

Abstract

In recent decades, polysaccharides have come to play an important role in pharmaceutical science, among them in the domain of nanotechnology. As a result, there is an increased interest in their isolation, synthesis, modification, characterization, and application in this relevant new topic in nanotechnology. This has led to the use of modified polysaccharides in changing nanocomposite morphology and properties. Delivery of hydrophobic molecules, drugs, and proteins is difficult due to poor bioavailability following administration. Thus, modifications to natural polymeric carrier systems are being investigated to

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improve drug solubility, stability, and induced toxicity. Due to problems of toxicity and immunogenicity, natural modified polysaccharides are being explored as substitutes for synthetic polymers in the development of new drug-delivery systems, such as coating or copolymer material. By conjugating different entities to the polysaccharide backbone, resultant materials can be used for preparing self-assembled micelles, coating polymeric microspheres, and self-reorganized nanostructures, improving drug release in tumoral areas.

Keywords

Drug delivery • Cytotoxicity • Polysaccharides • In-vitro and in vivo toxicity • Natural products • Bioactivity • Mechanism of action

1 Introduction

Many polysaccharides possess inherent bioactivity that can facilitate mucoadhesion, enhanced targeting of specific tissues, and reduced inflammatory response. Furthermore, the hydrophilic nature of a great number of polysaccharides can be exploited to enhance circulatory stability. This chapter describes the advantages of polysaccharide use in the development of drug-delivery systems and provides an overview of the polysaccharide-based micelles developed to date. Smart pH and thermonanocontainers modified by succinate cellulose (Metaxa et al. 2012) and dextran xerogel (Angelopoulou et al. 2012) were reported by Metaxa et al. and Angelopoulou et al., respectively. Despite these properties and advantages, some problems can occur. For example, chitosan is soluble in acidic media and therefore cannot be used as an insoluble sorbent under these conditions, except after physical and chemical modifications (Shahidi et al. 1999). To avoid this problem, different polysaccharide derivatives have been developed. Cyclodextrins, water-soluble in their native form, are often modified to prepare new insoluble derivatives. The hydrophilic nature of starch is also known to be a major constraint that seriously limits the development of starch-based materials. Chemical derivatization has been proposed as a way to solve this problem and to produce water-resistant materials. However, the strategy for modification depends on the proposed final purpose for the material. If a water-soluble starch derivative is desired for application in wastewater flocculation or as an additive in paper manufacturing, then a random conversion of hydroxyl groups to aminopropyl (Gonera et al. 2002), hydroxyalkyl (Wesslén and Wesslén 2002), or betaine (Auzély-Velty and Rinaudo 2003) is necessary. The use of nanotechnology not only changes the structures of polysaccharides but also changes functionality of the materials and their properties.

Many polysaccharide properties are yet to be discovered, and the application of nanotechnology may open up new horizons that can noticeably change every aspect of human life, and this includes use for polysaccharides found in the exoskeleton of crab, shrimp, prawn, lobster, squid pen, and other marine products. Despite the abundant availability in nature of polysaccharides, they have not yet been

completely accepted by the scientific community. This chapter deals with various polysaccharides and discusses their physical, chemical, and biological properties; characterization; synthesis of polysaccharide-based nanoparticles, nanospheres, and nanogels; and biomedical applications.

The idea of targeting drugs in the pathogen area is quite old and was first proposed by Paul Ehrlich (1854–1915) in the early twentieth century (Torchilin 2010; Park et al. 2010). Ehrlich was awarded the Nobel Prize in Medicine (1908) for his work in the field of immunization and his ideas for specific targeting of drugs, the inspiration for the 1940 film script "Dr. Ehrlich's Magic Bullet" (Arruebo et al. 2007). There are now several different approaches to specific targeting of therapeutic and diagnostic agents that permit direct drug transfer to the affected area (organ or tissue) (Torchilin 2010).

A drug delivery system allows the introduction of a therapeutic substance or diagnostic agent to the body and improves drug efficacy and safety, controlling pace, timing, and mode of delivery. Such systems are used to transfer diagnostic and therapeutic agents either on the experimental or clinical level (Torchilin 2009, 2006). In recent years, research has focused primarily on developing drug delivery systems with sizes in the range of nanometers (10^{-9} m) (Arruebo et al. 2007). Such systems are called nanoparticles and solid, colloidal, bodies, which are composed of macromolecules and range in size from 10 to 1,000 nm (Singh and Lillard 2009). The diameter of human cells ranges between 10 and 20 microns, while various structural units of the cell range from a few to several hundred nanometers. Therefore, nanoparticles carrying the drug may interact with biomolecules on the surface and inside the cell without causing irreversible damage to that (Kim 2005). Nanoparticles are able to offer a variety of useful properties:

1. Long life to the body, high stability of the systemic circulatory system, and improve drug solubility and biocompatibility
2. Targeting of pathogenic tissue and organs
3. Sensitivity to external stimuli (e.g., external heating effect of magnetic field or ultrasound), or to the pathogen stimuli area (e.g., changes in temperature, redox environment, or pH)
4. Transfer of a reporter that provides information on metabolism and with the ability to specifically target a system (Torchilin 2009, 2006; Singh and Lillard 2009; Torchilin 2007a, b)

Nanotechnology therefore may provide a solution to problems faced by conventional therapy, offering an effective alternative treatment and better patient quality of life (Kim 2005).

Nanoparticles for transport of drugs consist of a variety of materials. Natural and synthetic polymers have been used extensively (polymeric nanoparticles, micelles, dendrimers), inorganic materials such as oxides of gold, iron, and silicon (inorganic nanoparticles), lipids (liposomes), organometallic compounds (nanotubes), and even viruses (viral nanoparticles). The material that makes up nanospheres, can be synthetic or natural polymers, which must meet certain criteria. Initially, they

should be biodegradable or biocompatible to be excreted from the body in a short time and without the risk of uncontrolled accumulation. Furthermore, these polymers, and products of their metabolism, should not be toxic and or cause an immune response (Vauthier and Bouchemal 2009).

Among others, polymers primarily used for nanoparticle synthesis in drug delivery are (Edlund and Albertsson 2002):

1. Physical origin:
   - Polysaccharides
   - Proteins (e.g., collagen, gelatin, albumin)
2. Synthetic co-polymers (Vauthier and Bouchemal 2009)
   Aliphatic polyesters:
   - Polyglycolic acid poly(glycolide), PGA
   - Polylactide poly(lactide), PLA
   - Polylactide-polyglycolide copolymer oxeosPoly (glycolideco-lactide), PLGA
   - Polycaprolactone poly(e-caprolactone), PCL
   - Polyanhydride
   - Polyaminoacids

The pharmaceutical compound is dissolved and then trapped either within the sphere, in the polymer network, or linked to the polymer by chemical modification. Drug entrapment – ideally by a nanoparticulate drug system – must have high drug-retention capacity, which depends on a drug's material composition. Drug entrapment can be done in two ways: either by incorporation during nanosphere production (incorporation method), or adsorption during incubation with isotonic solution of the pharmaceutical compound (adsorption/absorption technique).

Drug encapsulation depends upon polymer network solubility, polymer molecular weight, and interactions between polymer and drug. For small molecules, investigations show a significant role in maximizing the amount that can be trapped is played by ionic interactions between the drug and the network hardware (Mohanraj and Chen 2006).

2 Bioactivity

Polysaccharides have several advantages over synthetic biopolymers for use as a basis for synthesis of new drug-delivery systems. They are nontoxic, have good biocompatibility, and low production costs (Doshi and Mitragotri 2009). It is a fact that a combination of polysaccharides with synthetic water-soluble polymers leads to production of materials with improved biochemical and mechanical properties (Nurkeeva et al. 2003). They can extend lifespan in the body, which increases absorption of the entrapped drug. For all the above reasons, polysaccharides

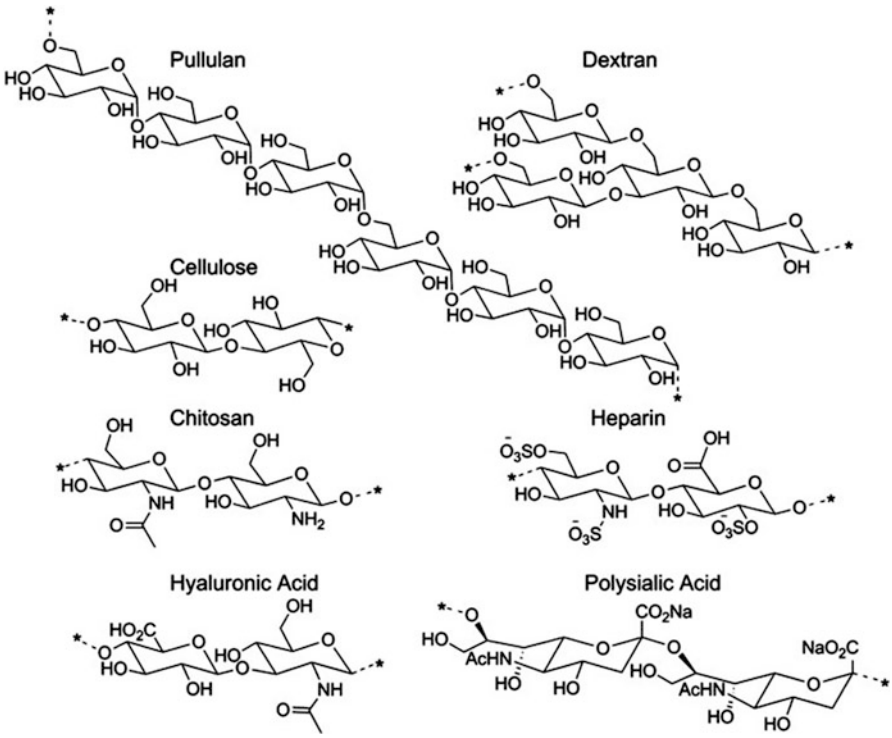

Fig. 1 Structures of polysaccharides used in the development of drug-delivery systems and the possible modified site (Zhang et al. 2013)

are ideal raw materials for synthesis of promising biomaterials in the field of drug-delivery systems (Fig. 1).

3 Bioactivity Induced by Polysaccharides' Modification

The purpose of chemical modification is to improve mechanical and chemical properties, biocompatibility, improved solubility, and control of biodegradability. In particular, modifications to polysaccharides have followed different approaches:

- Combination or chemical ligation with synthetic biopolymers
- Surface coating of micro- or nanosphere polysaccharides with biocompatible synthetic polymers
- Crosslinking with different types of reagents
- Increased hydrophobicity via alkylation reactions
- Configuring of glucuronic/mannuronic ratio, or of deacetylation degree, respectively (d'Ayala et al. 2008).

4 Cellulose

In 1838, the French chemist Anselme Payen isolated for the first time a solid plant tissue by treatment with acids and ammonia and the extraction thereof using water, alcohol, and ether. This solid residue was named *keloulozi* (cellulose) in 1839. The name means sugar (ose) combined with cell (from the English language) (Klemm et al. 2005; Pérez and Samain 2010a). Cellulose is a linear polysaccharide homopolymer of β-D-glucose (anhydrous D-glucopyranose). Structural cellulose units link with beta-1, 4 glycosidic bonds, forming long chains. Each cellulose molecule has three hydroxyl groups per glucose moiety, apart from the end edges (Williamson et al. 2002; Kamel 2008). Cellulose is the most common organic polymer in nature, with ~1.5 × 1,012 t of biomass being the structural component of the cell wall of green plants, certain species of algae, and some oomycetes (Pérez and Samain 2010b). In tall plants forming cellulose, microfiber has a diameter from 2 to 10 nm. Hydrogen bonds and forces, such as van der Waals force, stabilize microfibers and make them insoluble, chemically resistant, and mechanically robust (Williamson et al. 2002). Cellulose is located also in prokaryotes (*Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Rhizobium* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Sarcina ventriculi*) and eukaryotes.

Cellulose is insoluble in water and most common solvents. The poor solubility comes from the strong intermolecular and intramolecular hydrogen bonds developed between the independent polymeric chains. Although it is almost insoluble, it is used in many applications and is an almost inexhaustible source of raw material for the increasing demand for eco- and biocompatible products. Unlike carbohydrates with a low molar mass, reactions of cellulose are characterized by intermolecular interactions, cross-linking polymer, chain elongation, and effects of side-chain functional groups. Based on the literature, several chemical modifications of cellulose (cellulosics) have been reported (Kamel 2008). Cellulose derivatives are widely used in industrial-scale coatings, by optical films, for building materials, and in food, pharmaceutical, and cosmetic formulations. Also, because of its chirality, cellulose is for immobilization of proteins, antibodies, and heparin (HP), as well as for separating enantiomeric molecules and in the development of new synthetic materials with synthetic and natural polymers.

Cellulose is insoluble in water because of its high crystallinity. However, it may be converted to water-soluble polymer with various chemical modifications. Thanks to its structure and reactivity, cellulose can be designed and synthesized into a variety of new water-soluble polymers. Such derivatives are methylcellulose (MC), hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC), and hydroxypropyl methylcellulose (HPMC), all of which are commercially available (Nurkeeva et al. 2003). Chemical modification of cellulose is performed to develop derivatives of cellulose (cellulosics) that are broadly reactive, low cost, reproducible, recyclable, and biocompatible; therefore, it may be used for pharmaceutical applications. Cellulose derivatives are most commonly used to modify the release of drugs in tablet and capsule formulations; as a control agent for tablet binding,

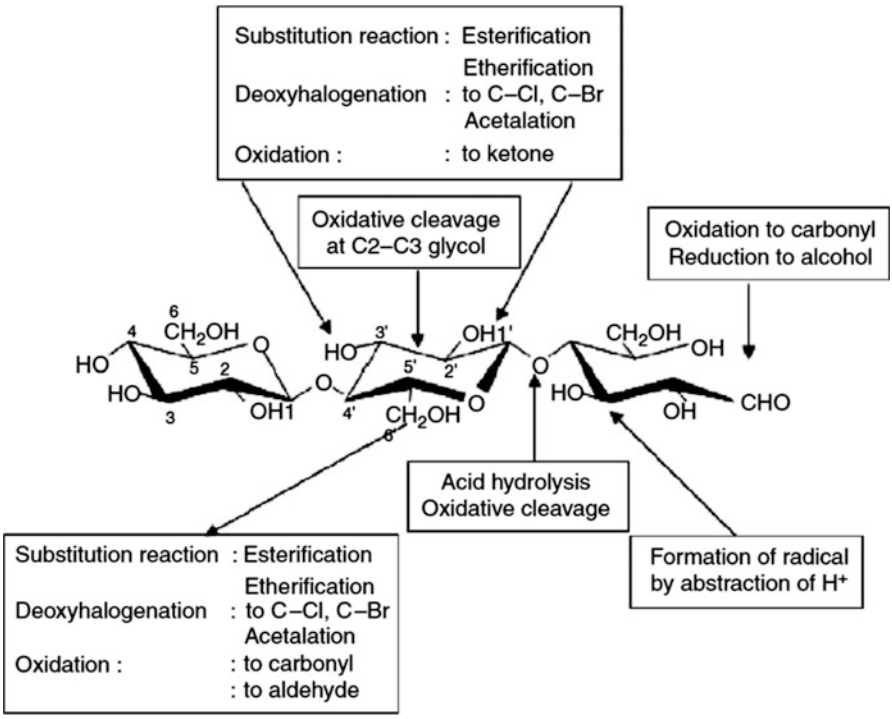

Fig. 2 Sites of cellulose modification (Pérez and Samain 2010b)

thickening, and rheology; for film formation, water retention, improving adhesive strength, and suspending and emulsifying (Kamel 2008; Fig. 2).

Hydroxypropyl cellulose (HPC) is a nonionic, biocompatible, and pH-sensitive derivative of cellulose (Kamel 2008). It is soluble in organic solvents and water but is sensitive to changes in temperature, with a minimum critical solubility temperature (LCST) in water of 41 °C (Cai et al. 2003). Hydroxypropyl cellulose used in ophthalmic formulations, mainly as a component of artificial tears.

Cellulose esters play a vital role in the development of modern drug-delivery technology. Their properties are well adapted to the needs of pharmaceutical applications and additionally permit the construction of drug-delivery systems that meet the basic needs of patients.

5 Chitin and Chitosan

Chitosan is a naturally occurring linear polysaccharide produced by alkaline deacetylation (Felt et al. 1998) of chitin, and in nature it is present only in some species of fungi (Mucoraceae) (Agnihotri et al. 2004). Chitin is the major component of the protective cuticle of crustaceans such as crab, shrimp, lobster, etc. (Felt

et al. 1998), and is a cell-wall component in certain fungi (Sinha et al. 2004; Dash et al. 2011). Nonetheless applications are minimal compared with cellulose and chitosan due to the absence of chemical activity (Agnihotri et al. 2004). Chitosan and chitin are linear polysaccharides structurally similar to cellulose, with replacement of the hydroxyl and amino groups with C6 acetyl-amido, respectively (Felt et al. 1998).

Chitin is a homopolymer of beta-(1,4)-*N*-acetyl-glucosamine, whereas chitosan is a copolymer of glucosamine and *N*-acetyl glucosamine linked by beta-1, 4 glycosidic bonds (Park et al. 2010). Generally, chitosan is provided in the form of dry flakes in solution or powder form. It has a molecular weight range between 3,800 and 20,000 Da and may have undergone deacetylation by 66–95 % (Agnihotri et al. 2004). Because amino groups can be charged positively, they can interact with negatively charged surfaces or polymers and metal ions, such as cobalt; these properties become useful in pharmaceutical applications (Agnihotri et al. 2004).

Chitosan is a cationic polysaccharide with both neutral and acidic pH. It has a weak base, is insoluble in water and organic solvents, but dissolves in a slightly acidic environment (pH <6.5). At low pH the amine is protonated and charged positively improving the chitosan solubility in aq. solutions. When the pH rises above 6.5, the amine is deprotonated, the polymer is discharged, and it becomes insoluble (Sinha et al. 2004). Therefore, chitosan precipitated in alkaline solution or polyanion solutions forms a gel at low pH. The degree of deacetylation and the molecular chitosan weight also affects the polymer solubility. After processing, chitosan becomes a stiff, crystalline structure due to the intramolecular and intermolecular hydrogen bonds.

Chitosan is a biocompatible and biodegradable polymer. It is not toxic, and has LD_{50} 16 g/kg body weight in experimental animals, and a price similar to sugar and salt. It is compatible with biological tissue and does not cause allergic reactions. Cleaved from enzymes into harmless products (amino sugars), they are absorbed fully from the human body without causing side effects (Kean and Thanou 2010). Chitosan has pharmacological properties, and its activity enhances wound healing, has an antiacid effect, and helps prevent stomach ulcers (Felt et al. 1998). It also has antimicrobial action and absorbs toxic metals, such as mercury, cadmium, and lead. Chitosan is used in dressings for protecting the injured eye, while membranes of chitosan films are used as artificial liver (Sinha et al. 2004). These properties make it ideally suited for medical and pharmaceutical purposes. For example, the properties of chitosan allow rapid blood clotting, and for this reason, it recently gained approval in the United States and Europe for use in bandages and hemostatic agents (Li et al. 2008).

A very important property of chitosan is its antimicrobial activity, which was effectively demonstrated against many bacteria, filamentous fungi, and yeasts. Chitosan has a wide spectrum of activity and high levels of Gram-positive and Gram-negative bacteria death, but exhibits low toxicity against mammalian cells. Ever since the broad-spectrum antibacterial activity of chitosan was first proposed by Allen (Allan and Hardwiger 1979), together with the significant commercial interest, the antimicrobial properties of chitosan and its derivatives have attracted great attention from researchers (Kong et al. 2010).

Although chitosan was discovered by the early nineteenth century, only in the last two decades has it been used in biological applications and drug-delivery systems, such as nanoparticle microspheres. Chitosan can be synthesized using various techniques and used to entrap drugs and release them in a controlled manner (Janes et al. 2001a). Spheres of chitosan can be synthesized by cross-linking with glutaraldehyde, formaldehyde (Jameela and Jayakrishnan 1995), and citric acid (Varshosaz and Alinagari 2005), and by the physical agent genipin extracted from the fruit of gardenia (Mi et al. 2002). Apart from the method of cross-linking, chitosan spheres are synthesized by techniques, such as multiple emulsion, solvent evaporation, coating deposition, successive bark chitosan, etc. (Lu et al. 2011; Fig. 3).

Chemical modification of chitosan is particularly interesting, as attached groups do not change the fundamental skeleton, thus maintaining the original physico-chemical and biochemical properties; while they may on the other hand lead to new and improved properties. Many modifications are reported in the literature, such as oligomerization, alkylation, acylation, hydroxy alkylation, carboxy alkylation, sulfation, phosphorylation, enzymatic modifications, copolymerizations, and many others. Chemical modification provides a wide range of derivatives with modified properties for specific applications in areas such as pharmaceutics, biomedicine, and biotechnology. A variety of modifications, including hybrids with chitosan, sugar, cyclodextrin, dendrimers, and crown ethers, have also emerged as interesting multifunctional macromolecules (Mourya and Inamdar 2008; Fig. 4).

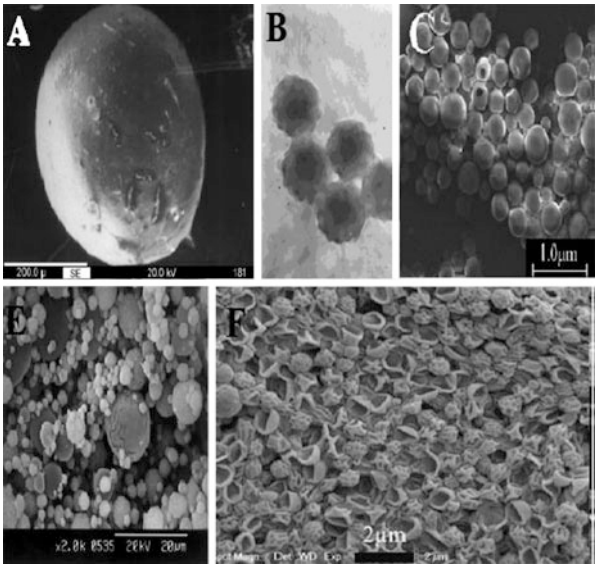

Fig. 3 SEM and TEM images of chitosan spheres: (**a**) with the cross-linking process in emulsion (Agnihotri et al. 2004), (**b**) with the sequential deposition bark technique, (Lu et al. 2011), (**e**) with the spray-drying method (Mi et al. 2002). (**f**) Coated spheres of polystyrene (Li et al. 2008)

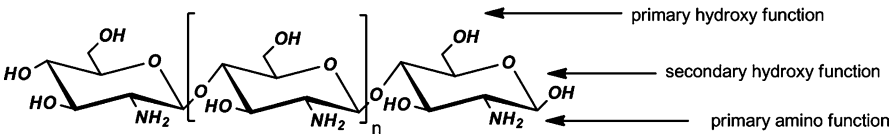

Fig. 4 Functional groups in chitosan

6 Glycosaminoglycans - Heparin

Glycosaminoglycans are a family of polysaccharides that normally cover the surface of all eukaryotic cells. Glycosaminoglycans (GAGs) are negatively charged polysaccharides composed of repeating disaccharide units. HP and heparan sulphate (HS) are related species of GAGs.

HP coupled with nanomaterials has recently been investigated for chemical and biological properties. HP improves the biocompatibility of nanoparticles and their performance in various biological applications. HP has coupled surface magnetic and metallic nanoparticles and biodegradable synthetic biopolymers. HP has also been entrapped in nanoparticles. These changes lead to the use of heparin in new applications ranging from improved anticoagulation, cancer treatment, tissue engineering, and biosensors (Kemp and Linhardt 2010).

7 Cross-Linked Polysaccharides

Polysaccharides and their derivatives can be covalently crosslinked to prepare nanoparticles for use as drug carriers. The cross-linking process involves formation of covalent bonds between function groups and functional cross-linking agents.

The free amino groups in chitosan contribute to its solubility in acidic media and in reactivity with physical and chemical cross-linkers. Glutaraldehyde cross-linked chitosan microspheres are very promising for controlled drug delivery for very long time release (Jameela and Jayakrishnan 1995), but its toxicity on cell viability limits its utility in the field of pharmaceutics (Liu et al. 2008).

Recently, other cross-linkers, such as genipin, glyoxal, and other polymers, have been examined for their possible applications in the biomedical field, but few studies are reported. Microspheres prepared with glyoxal were more hydrophobic, nonspherical, and smaller in size due to the high degree of cross-linking. These microspheres increased the controlled release period for centchroman (Ormeloxifene) in comparison with chitosan and glutaraldehyde cross-linked chitosan microspheres (Gupta and Jabrail 2006).

Chitosan has been successfully modified by condensation reaction using natural di- and tricarboxylic acids (succinic acid, malic acid, tartaric acid, and citric acid) and as cross-linking agents for intramolecular cross-linking of the chitosan linear chains, to form pH-sensitive nanoparticles (Bodnar et al. 2005). A novel

biocompatible chitosan derivative, *N*-succinyl-chitosan (NSCS), with a well-designed structure, has been successfully synthesized by Aiping et al. NSCS can self-assemble regular nanosphere morphology in distilled water, are nontoxic, and have cell-compatible properties (Aiping et al. 2006).

8 Polysaccharide Modification with Small Molecules

Generally, small molecules such as ligands on the surface of nanoparticles can be easily fitted and low cost. Their small size allows for the attachment of several molecules on a nanoparticle. Thus, while the online pharmaceutical compound with a ligand was limited to only a few ligand-binding properties, in contrast, connection of nanoparticles with linkers allows the introduction of thousands of such small molecules.

In the study of Zhang et al. 2007, chitosan was modified with oleoyl to prepare self-assembled nanoparticles with a diameter of ~250 nm and an almost spherical shape as carriers for hydrophobic antitumor agents. The antitumor drug doxorubicin was efficiently loaded into nanoparticles, and in vitro studies were subsequently performed (Aiping et al. 2006).

Choochottiros et al. studied the modification of the chitosan amine with phthalic anhydride and primary hydroxyl with polyethylene glycol and carbolytic acid (PEG-COOH) in order to synthesize amphiphilic-chitosan-monodispersed nanospheres (range 100–300 nm) with pH sensitivity. Incorporation of the model drug lidocaine into modified chitosan nanospheres was successful (Choochottiros et al. 2009).

Thiolated chitosan was synthesized by covalent attachment of glutathione (GSH) to chitosan by forming amide bonds between amine groups of chitosan and carboxylic acid of GSH. Poly(methyl methacrylate) nanoparticles coated with chitosan–glutathione, and the insoluble anticancer drug paclitaxel (PTX), was encapsulated in the nanoparticles (Akhlaghi et al. 2010).

In this context, molecules such as folic acid (Torchilin 2006, 2007b; Alexis et al. 2008) and various sugars are used extensively. The vitamin folic acid is essential for cell survival of these cells; for this reason, folate receptors are overexpressed in many types of cancer cells. The folate receptor (FR) interacts with folic acid on the surface of nanoparticles, resulting in endocytosis. This produces some problems for the potential clinical use of folic acid. Generally though, folic acid ligands are inexpensive, do not generate an immune response, are nontoxic, and easy to attach to the nanoparticles and is very stable in the bloodstream (Byrne et al. 2008). Nevertheless, immunochemical studies show that there is overexpression of folate receptors in normal tissue, such as liver and placenta (Sudimack and Lee 2000).

Mansouri et al. synthesized and characterized nanoparticles folate (FA)-chitosan-DNA and evaluated their cytotoxicity in vitro. Chitosan-DNA nanoparticles and FA-chitosan-DNA are prepared using reductive amidation and a complex process of aggregation. The authors concluded that FA-chitosan nanoparticles are promising candidates as nonviral gene vectors (Mansouri et al. 2006).

Some long-chain fatty acids, such as hexanoic acid, decanoic acid, linoleic acid, linolenic acid, palmitic acid, stearic acid, and oleic acid, have been used for modifying polysaccharides (Liu et al. 2008).

9 Graft Copolymerization

The ability of grafting synthetic polymers to polysaccharides has attracted attention in recent years, and useful derivatives have been developed. Graft copolymerization reactions introduce side chains and lead to the formation of novel types of hybrid materials composed of natural and synthetic polymers. HPC has been modified by attaching acrylates or degradable moieties as side groups. Through free-radical polymerization of side chains in aqueous solutions at temperatures above their LCST, the modified HPC network is cross-linked (Cai et al. 2003).

10 Composites and Hydrogels

Hydrogels are hydrophilic network structures that can absorb from 20 % to 1,000 times their weight in water. The ability of the network to maintain a large volume of water is considered one reason BC (BC) implants do not cause an immune response. BC hydrogels have great potential for biological application (e.g., cardiovascular implants, trauma, burns, and tissue-regeneration scaffolds). According to the study by Taxima et al., films of BC composite are produced by the addition of carboxymethyl (CM) cellulose, methyl cellulose, or polyethylene glycol to BC culture.

The cross-linking process of two cationic hydroxyethyl celluloses of different hydroxyethyl- and ammonium-group contents polyquaternium-4 (PQ-4) and PQ-10) with ethylene glycol diglycidyl ether (EGDE) was characterized and optimized. Hydrogels presented a significant loading capacity of diclofenac, with which they interrelate through ionic and hydrophobic interactions (Rodríguez et al. 2003).

11 Shelf Life and Stability of Nanoparticles in Systemic Circulation: Biocompatibility

Life within the organization plays an important role in drug-delivery systems. For the immune system, drug-delivery systems are "foreign," which become opsonizational before completing their function. For this and other reasons, properties of each nanoparticle are long life, stability, and – of course – biocompatibility. The longer the lifetime, the more stable and slower is drug release in the pathogen area. As well as long life in the circulation, leading to better drug targeting either passively or when using specific ligands that target receptors in the pathogen area, systems have more time available to act on the target (Torchilin 2006).

Prolongation of life nanoparticles into the organism occurs by modifying the surface with synthetic polymers, such as PEG. Coating nanoparticles with PEG results

in the formation of a polymeric layer over particle surfaces, which is impermeable to other solutes, even at relatively low polymer concentrations. "Steric stabilization" of PEG nanoparticles prevents interaction with blood components, thus preventing nanoparticle opsonization, phagocytosis, and their ability to capture reticuloendothelial system (RES). The term spatial (steric) stabilization was used to describe the protective capacity of PEG nanoparticles. As a protective polymer, PEG has a powerful combination of properties, such as excellent solubility in aqueous medium, flexible polymer chains, low toxicity, low accumulation in cells, and minimal impact on the pharmaceutical activity of the transported drug (Torchilin 2006).

Despite the fact that PEG is the most frequently used polymer for ensuring long life of drug-delivery systems, other biocompatible, soluble, and hydrophilic polymers are used to protect the nanoparticles. Other hydrophilic polymers used to protect and stabilize drug-delivery systems is the modified poly(acryl amide), poly (vinyl pyrrolidone) (PVP), poly(acryloyl morpholine) (PAM), poly(2-methyl-2-oxazoline), poly(2-ethyl-2-oxazoline), phosphatidyl polyglycerol, and polyvinyl alcohol (PVA).

A thermosensitive hydrogel was synthesized by block copolymerization of monomethoxy poly(ethylene glycol) macromere (PEG) onto a chitosan backbone using potassium persulfate as a free radical initiator. These block copolymers are promising and attractive materials for biomedical applications (Ganji and Abdekhodaie 2008).

11.1 Polysaccharides as Coating Materials

To the potential of nanoparticles in therapeutic applications, surface modification is most commonly used. Nanoparticles should ideally present a more hydrophilic surface in order to prevent macrophage capture (Cho et al. 2012), since the body recognizes hydrophobic moieties as foreign matter and quickly triggers the monocyte phagocytosis system (MPS). Therefore, covalent modification of nanoparticle surfaces with hydrophilic polymers avoids, or at least delays, the phagocytosis process (Lemarchand et al. 2004), thus improving circulation time, which increases the probability that nanoparticles will reach their target (Kumari et al. 2010). Using PEG or PEG-containing copolymers is the most common strategy to attain that objective. PEG is a hydrophilic nonionic polymer that presents high biocompatibility, biodegradability, and nontoxicity. PEGylation lowers the positive surface charge and decreases zeta potential, which increases nanoparticle physical stability and biocompatibility (Janes et al. 2001a). This methodology creates a hydrophilic layer around the nanostructure, thus avoiding protein absorption due to steric repulsive forces. However, the most important part of nanoparticle coating with PEG is increased circulation time, inducing the rate of sustained drug release.

Polysaccharide-coated nanoparticles are attractive candidates for biomedical applications due to their biocompatibility, biodegradability (Ladaviere et al. 2007), and application in drug-controlled delivery. Recognition of polysaccharide-coated nanoparticles is reduced by the MPS. As they are hydrophilic polymers,

polysaccharide coating induces stability in the blood circulation system (Ma et al. 2008). Other significant advantages of the nanoparticle coating with polysaccharides are their protection by steric forces. These forces protect nanoparticles against nonspecific interactions with proteins and against organ and tissue targeting in a specific manner due to their recognition and mucoadhesive properties (Ma et al. 2008). Polysaccharide coatings can then be considered as a promising alternative to PEG coatings, since they can be modified by specific targeting molecules (Lemarchand et al. 2004), thus improving the active targeting ability of nanoparticles. This is in contrast to pegylated nanoparticles, which require specific modification on their surface to enable molecular recognition and attain active targeting (Ma et al. 2008). Modification of such ligands to their surface is difficult due to lack of available groups. It is therefore more advantageous to use polysaccharide coatings, as they present many specific receptors in certain cells or tissues (Lemarchand et al. 2004). In addition, the polysaccharide-coated delivery system retains encapsulated drugs at the target site (Lemarchand et al. 2004). It is well known that polysaccharide-coated nanoparticles fabricate by dissolving in the aqueous phase in which they are prepared and/or by nanoprecipitation or solvent-emulsion evaporation (Lemarchand et al. 2004). It is worth noting that chitosan and its derivative, a main polysaccharide representative, are widely used by numerous scientific groups as coating agents for nanoparticles because of their high affinity for cell membranes and their positive charge, allowing strong adsorption into the nanoparticles (Janes et al. 2001a).

Dextran and heparin have been used to coat the surface of several nanoparticles for biomedical applications, such as cancer treatment, (Angelopoulou et al. 2012) having been proven to induce a decrease in protein adsorption and promote enhancement of nanocarrier blood-residence time.

In addition to the increasing new methods for cancer diagnosis and treatment, known as theranostics, advanced technologies for tumor imaging and early detection are also being explored by different groups (Metaxa et al. 2014).

11.1.1 Modification with Conjugated Drugs

Polysaccharides are extremely amenable to modification. There are many examples of modified polysaccharides used in drug-delivery systems, indicating that polysaccharides are easily modified. Among them, glucose-based polysaccharides, such as amylose, amylopectin, glycogen, and cellulose, offer an abundance of free reactive hydroxyl groups. Other polysaccharides possess both hydroxyl and carboxylic acid moieties that can be readily modified. A review recently published focused on the derivatization of alginate, a polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid with 1,4 linkages. Modification of alginate can be used to induce a variety of different physiological behaviors. For instance, oxidation of hydroxylic groups enhances their biodegradability, while sulfonation generates a heparin-like polysaccharide with increased blood flow.

Modification of chitosan has also been extensively reviewed. Specifically, modification of the primary amino segment with various alkyl groups can be used to improve solubility and bioactivity (Zhang et al. 2013). Many types of modified drug

polymers are synthesized by insoluble drugs and water-soluble polymers that are chemically conjugated to the drug via a biodegradable spacer. These spacers are selected to be stable in the bloodstream but cleaved at the target site by hydrolysis or enzymatic degradation, thus improving local drug release and avoiding serious side effects. Such drug conjugates can be selectively accumulated at the tumor site by the enhanced permeability and retention (EPR) effects, followed by the above-described mechanism. Based on this concept, several polymer–drug conjugates have recently entered into phase I/II clinical trials. The representative example is *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-based drug conjugates, such as HPMA copolymer–doxorubicin conjugate (PK1) and HPMA copolymer–doxorubicin conjugate containing galactosamine as a targeting moiety (PK2) developed for treating primary or secondary liver cancer. Based on this data, many types of chitosan-conjugated drugs have been investigated. A characteristic example, doxorubicin (Dox) conjugated with specific modified glycol chitosan through carbodiimide chemistry, was reported by Son (Son 2003; Table 1).

11.1.2 Mechanism of Action

Active Targeting Agents

The major problem with traditional cancer chemotherapeutics is the high toxicity due to indiscriminate distribution of drugs to disease and healthy cells alike following systemic administration. Anticancer drugs are poorly solubility in water, as described previously, and thus require organic solvents or detergents for clinical application, resulting in undesirable side effects such as venous irritation and respiratory distress (Zhang et al. 2013). Therefore, designing a distinct carrier system that encapsulates a large quantity of drugs and specifically targets tumor cells is indispensable for successful cancer therapy. Micelles of appropriate size, charge, and shape will facilitate improved delivery. However, this may not be enough due to defenses within the human body. To enhance target-site accumulation and uptake, additional targeting agents are often incorporated into micelle systems. Active targeting agents used in polysaccharide-based micelles can be divided into three categories: peptides, small molecules, and polysaccharides. Peptides include octreotide targeting for somatostatin receptors on tumor cells, the A54 hepatocarcinoma-binding peptide (Yuan et al. 2011), and arginylglycylaspartic acid [Arg-Gly-Asp (RGD)] containing peptide for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Zhang et al. 2013; Du et al. 2011, 2012). Small molecules include glycyrrhetinic acid, a liver targeting ligand; vitamin E succinate-specific toxicity to tumor cells (Wydra and Rudolph 1992); and folic acid with a high affinity to the folate receptor, which is overexpressed on tumor cells (Metaxa et al. 2012).

Through peptide and small-molecule conjugation, nanostructuring systems have shown higher tumor accumulation and cellular uptake than blank nanostructures. It is worth mentioning that polysaccharides themselves can act as active targeting agents due to their bioactivity. As described in the literature, hyaluronan (HA) has a high affinity to the HA receptor on liver sinusoidal endothelial cells and the CD44 receptor that is overexpressed on tumor cells and inflamed synovial fibroblasts.

Table 1 Polysaccharides as pharmaceutical components (Maier et al. 2013)

Polysaccharide	Main chains	Side chains	Source	Comments
Amylose	α-1,4 D-glucose	–	Storage polysaccharide in plants	Unbranched; one of the constituents of starch; used in traditional pharmacy as tablet excipients, degraded by *Bacteroids*, *Bifidobacterium*
Arabinogalactan	β-1,4 and β-1,3 D-galactose	β-1, s and β-1,3 L-arabinose and D-galactose	Plant cell walls	Neutral pectin; hemicellulose; used as thickening agent in food industry, degraded by *Bifidobacterium*
Carrageenan	Galactose residues which are sulfated and alternatively linked in α-1,3 and β-1,4 linkage	The 4 linked residues can be 3,6 anhydro-D-galactose and 3-linked residues are at least partly 4 sulfated	Sea weed extract	–
Chitosan	Deacetylated β-1,4 *N*-acetyl-D-glucosamine	–	Shell of marine invertebrates	Deacetylated chitin; used in medicine as tablet component and absorption enhancing agent, degraded by *Bacteroids*
Chondroitin sulfate	β-1,3 D-glucuronic acid and *N*-acetyl- D-glucosamine	–	Epithelial cells	Mucopolysaccharide, connected to a protein core; contains various amounts of sulfate ester groups at the 4- or 6-position, degraded by *Bacteroids*
Dextran	α-1,6 D-glucose	α-1,3 D-glucose	Bacterial (*Leuconostoc* and *Streptococcus*)	Used in medicine as plasma expander, degraded by *Bacteroids*
Furcelleran	Composed of D-galactose and 3,6-anhydro-D-galactose, with sulfate ester groups on both sugar components	–	Sea weed extract (*Furcelleria fastigiata*)	–

Therefore, HA has been extensively investigated as an active targeting agent to tumor tissue and liver in many micelle systems (Crini 2005). In addition, heparin-based nanostructures have demonstrated enhanced inhibition of tumor growth and angiogenesis (Huang et al. 2012), while cellulose (Hsieh et al. 2008), chitosan (Ilium 1998), and pullulan-based systems promoted drug absorption across the small intestine due to enhanced mucoadhesion (Zhang et al. 2013).

Passive Targeting

The EPR concept dates back to the late 1970s, when Maeda et al. (see [60]) investigated the selective accumulation of macromolecular drugs in tumor tissues. The specific passive accumulation of macromolecules was attributed to defective tumor vasculature with disorganized endothelium at the tumor site and a poor lymphatic drainage system. Since then, researchers have capitalized on this concept for the delivery of various drugs by conjugating them with polymers or encapsulating them within nanoparticles. Nowadays, it is evident that long-circulating macromolecules (polymer–drug conjugates) and nano-sized particulates (such as micelles, nanospheres, and liposomes) accumulate passively in tumors due to the EPR effect (Son 2003). Hydrophilic drug-delivery systems are less readily absorbed by normal tissues. However, they can accumulate in cancerous tissues through the EPR effect. Cancerous tissues exhibit defective, leaky hypervasculature and deficient lymphatic drainage (Son 2003), which results in passive accumulation of nanometer-sized entities in these tissues. This EPR effect serves to localize drugs to cancerous tissues and decrease their toxicity on normal tissues.

Polysaccharides are promising alternatives to PEG because they contain a variety of functional groups (hydroxyl, amino, and carboxylic acid) that can be used for drug conjugation, nanostructure coating, and self-assembly. The advantage of polysaccharide use is the resultant surface charge in carriers that can be used to engineer biointeractions, such as cellular uptake or glomerular filtration. There are two types of charged polysaccharides, cationic and anionic, due to the fact that polysaccharides are polyelectrolytes (Luo and Wang 2014). Cationic polysaccharides promote endocytic uptake by cells, whereas anionic polysaccharides could increase bioavailability by reducing excretion through the glomerular capillary wall. Negatively charged species are less filterable because of the negative charge of the glomerular membrane. Some charge-reversible and pH-responsive polysaccharides (Jeanes et al. 1961) discussed in this chapter can be used to achieve both these functionalities (Fig. 5).

Toxicity of Modified Polysaccharides Used in Drug Delivery Systems

It is well known that many natural polysaccharides are considered as being nontoxic, biologically compatible, polymers (Park et al. 2003; Janes et al. 2001b; Thanou et al. 2001), which can be used broadly as drug-delivery systems. Among them are many types that are approved for dietary applications in Japan, Italy, and Finland, and it was approved by the US Food and Drug Administration (FDA) for use in wound dressings. However, certain modifications could make them more or less toxic, and any residual reactants should be removed carefully. The use of

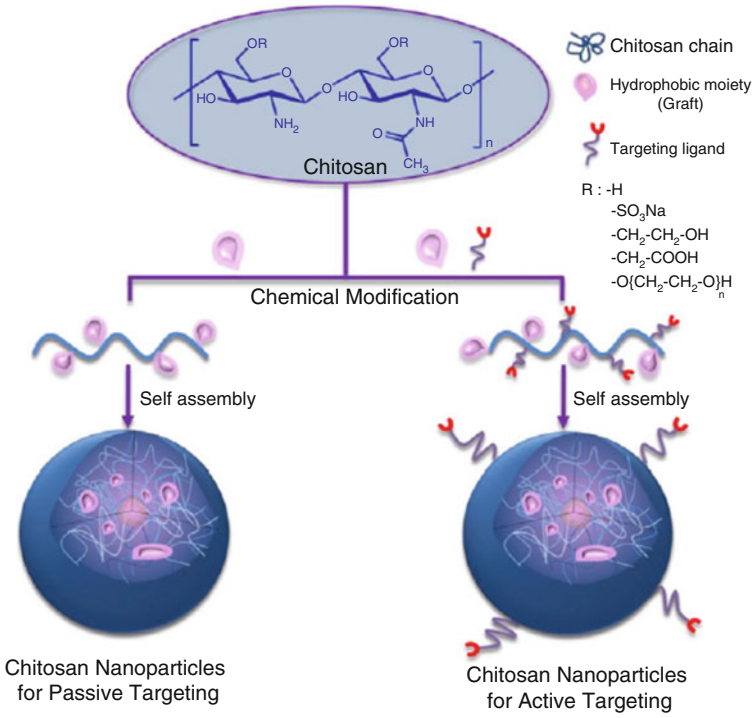

Fig. 5 Self-assembled chitosan nanoparticles for passive or active cancer targeting (Patel 2010)

hydrophilic polymers as drug carriers also helps lower drug toxicity. Zhang et al. prepared pH-responsive chitosan-based microgels (<200 nm diameter) by ionically cross-linking *N*-[(2-hydroxy-3-trimethyl ammonium)propyl chitosan chloride in the presence of tripolyphosphate (Zhang et al. 2006). These microgels were loaded with methotrexate and conjugated to apo-transferrin. The authors demonstrated that the conjugated microgels exhibited a significant increase in HeLa cell mortality compared with nonconjugated microgels. This was ascribed not only to receptor-mediated endocytosis of conjugated microgels, but also to pH-mediated release of methotrexate from microgels due to their swelling at the intracellular level. Chitosan-based micelles demonstrated to be a relatively safe carrier for oral formulation (Sonaje et al. 2011). Chitosan-based micelle systems have also been investigated for applications in antivirus (Huang et al. 2011), antithrombogenicity (Lin et al. 2011), and antiplatelet aggregation (Jiang et al. 2012; Fig. 6).

Aiming at increasing local drug release through polysaccharides, another type of modification was performed by Pankhurst et al. (2003) and Salata (2004). Magnetic targeting, an attractive physical technique, presents substantial attention for drug-delivery applications. Here, therapeutic agents to be delivered are either immobilized on the surface or encapsulated into the magnetic micro- or nanoparticulate

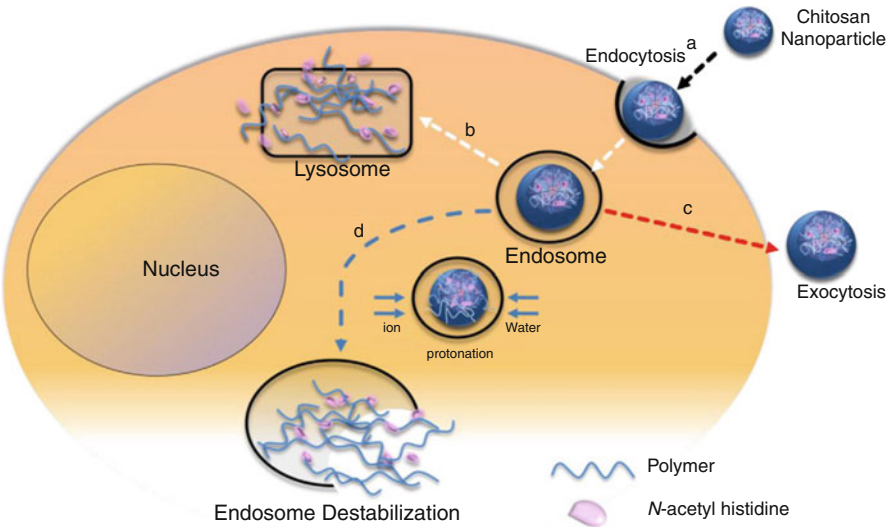

Fig. 6 A proposed model for cellular internalization and drug release of polysaccharide nanoparticles (Patel 2010)

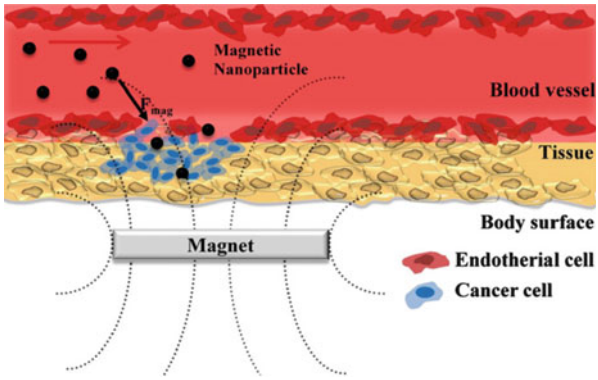

Fig. 7 Magnetic micro- or nanoparticulate polysaccharide carriers (Patel 2010)

polysaccharide carriers. These magnetic carriers, upon intravenous administration, concentrate at the specific site of interest (tumor site) using an external high-gradient magnetic field (Fig. 7). After accumulation of the magnetic carrier at the target tumor site in vivo, drugs are released from the magnetic carrier and effectively taken up by the tumor cells. A smart magnetic nanoparticle based on chitosan modification has been performed aiming at applying this type of targeting, thus increasing in vitro drug release.

In vitro Toxicity

Many groups investigated the toxicity of polysaccharides and their derivatives used in drug-delivery systems. Factors being considered are polymer molecular weight,

hydrophilicity, biocompatibility, and the conjugate system. Schipper et al. observed the effects of chitosan samples characterized by different molecular weights and drug delivery on CaCo-2 cells, HT29-H, and in situ rat jejunum. Toxicity was dependent on drug delivery and molecular weight. At high drug delivery, toxicity was related to molecular weight and concentration; at lower drug delivery, toxicity was less pronounced and less related to molecular weight. Nevertheless, most chitosans tested did not significantly increase dehydrogenase activity in the concentration range tested (1–500 g/ml) on CaCo-2 cells. The in situ rat jejunum study showed no increase in lactate dehydrogenase (LDH) activity with any chitosan sample tested (50 g/ml) (Zhang et al. 2007).

Red blood cell hemolysis assay is a study that reveals material safety. No hemolysis was observed (<10 %) over 1 h and 5 h with chitosans of <5 kDa, 5–10 kDa, or >10 kDa at concentrations up to 5 mg/ml. Furthermore, no red blood cell lysis was observed with paclitaxel–chitosan micelles at 0.025 mg/ml (Qu et al. 2009). In order to assess the intrinsic cytotoxic behavior of ulvan extracts, different standard cytotoxic strategies were followed, including evaluation of cellular viability by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (MTS) test and number, through double-stranded DNA (dsDNA), and total protein quantification. Furthermore, biological performance of ulvan was evaluated and compared to HA, a nontoxic, well-established polysaccharide, as control material due to its application in the biomedical field (Alves et al. 2013).

Polysaccharide-anchored liposomes are well documented and mostly studied as a model to for cell–cell adhesion; recently, however, they are being studied also for varied therapeutic potentials. Considering the potential of natural or hydrophobized polysaccharides, methods have been developed to link polysaccharides to liposome surfaces (Sihorkar and Vyas 2001).

Polysaccharide-anchored liposomes have been studied as stable and targetable drug carriers adaptable in effective chemotherapy, particularly for introducing chemotherapeutics into target cells or tumor cell lines. These systems possess a unique targeting ability to specific tissues, such as alveolar macrophages and other macrophages of the RES (Moreira et al. 1996). Polysaccharide-anchored liposomes could be employed as carrier constructs onto which a site-specific sensing molecule(s) like monoclonal antibodies (MAb) against tumor surface antigens could be physically or chemically attached (Sato and Sunamoto 1992). As a characteristic example, chitosan presents biological characteristics, such as low or no toxicity, biocompatibility, biodegradability, low immunogenicity, and antimicrobial properties. It can be hydrolyzed by lysozyme, and the degraded products of chitosan (amino sugars) are also nontoxic, nonimmunogenic, and noncarcinogenic, being completely absorbed by the human body (Wilson et al. 2010). Its rare positive charge converts chitosan into a special polysaccharide, as it provides strong electrostatic interaction with negatively charged mucosal surfaces and macromolecules such as DNA and RNA (Felt et al. 1998; Morille et al. 2008), which is an attractive feature for treating solid tumors (Li et al. 2009).

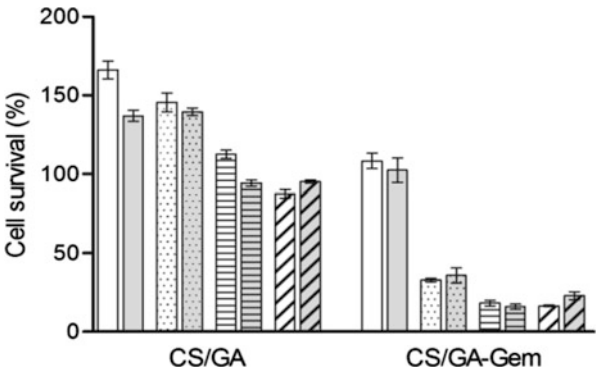

Fig. 8 Percentage of cell survival by treatment with free and chitosan-conjugated gemcitabine

The in vitro cytotoxic effects on a pancreatic-cancer cell line upon treatment with gemcitabine entrapped in prepared chitosan/glucoamylase (CS/GA) and polysorbate-80-coated CS/GA nanoparticles were assessed, comparatively to the free drug, in terms of cell survival and growth. Bare CS/GA (+p80 0.20) nanoformulations showed no deleterious effect on cell viability in this human pancreatic cell line. Moreover, paclitaxel (PTX)-loaded *N*-octyl-*N*-phthalyl-3,6-*O*-(2-hydroxypropyl) chitosan (OPHPC) micelles (PTX-OPHPC) with well-defined spherical shape and homogeneous distribution exhibited drug-loading rates ranging from 33.6 % to 45.3 % and entrapment efficiency from 50.5 % to 82.8 %. In cellular-uptake studies, PTX-OPHPC brought about a significantly higher amount of PTX accumulated in human breast adenocarcinoma cell line [Michigan Cancer Foundation-7 (MCF-7) cells] compared with Taxol®. Moreover, cellular uptake of PTX in PTX-loaded FA-OPHPC micelles (PTX-FA-OPHPC) was 3.2-fold improved in comparison with that of PTX-OPHPC. Results revealed that OPHPC micelles might be promising drug carriers for promoting PTX cellular uptake and that FA-OPHPC micelles could be used as potential tumor-targeted drug vectors (Qu et al. 2013).

The attained results (Fig. 8) showed that free gemcitabine presents the lowest IC50 and GI50 values. Nonetheless, CS/GA and p80-coated CS/GA nanoparticles loaded with gemcitabine were also effective at decreasing cell survival and inhibiting cell growth.

Microbial Toxicity

Interestingly, chitosan and its derivatives seem to be toxic to several bacteria (Miyazaki et al. 1992), fungi (Miyazaki et al. 1992), and parasites. This pathogen-related toxicity is an effect that could be beneficial in the control of infectious diseases. Bacterial inhibition took place in acidic solutions pH 5–5.3, and a 87.92 % kDa drug-distribution chitosan was more effective than a 532 kDa 73 % drug-distribution chitosan against both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Antimycotic effects against *Candida albicans* and *Aspergillus niger* was observed in a lipid emulsion of the same chitosans (Patel et al. 2010).

However, none of these studies proposed a mechanism of action for the observed inhibitory effect (Chourasia and Jain 2004).

Among the different approaches to achieve targeted drug release to the colon, the use of polymers especially biodegradable by colonic bacteria holds great promise. Polysaccharidases are bacterial enzymes that are available in sufficient quantity to be exploited in colon-targeting drugs. Based on this approach, various polysaccharides have been investigated for colon-specific drug release. These polysaccharides include pectin, guar gum, amylose, inulin, dextran, chitosan, and chondroitin sulphate. This family of natural polymers has an appeal to drug delivery, as it comprises polymers with a large number of derivatizable groups, a wide range of molecular weights, varying chemical compositions, and – for the most part – low toxicity and biodegradability and yet have high stability. The most favorable property of these materials is their approval as pharmaceutical excipients (Chourasia and Jain 2004).

11.1.3 Cancer Cell Toxicity

In vivo Toxicity

Living organisms are rich in carbohydrates. The wide chemical and functional diversity of these molecules is related to a rather large source base, which ranges from microbial to animal origin. Carbohydrates that form the structural basis of algae represent a unique group of gel-forming and viscous polymers, which are found neither in higher plants nor are digestible by nonruminant animals (Aiping et al. 2006) Industrial and technological applications of these marine carbohydrates include, among others, the food industry and aesthetics or biomedical devices. In fact, the use of natural-origin polymers on the development of novel biomaterials has already proven their feasibility and applicability, particularly in medical-related areas, including tissue-engineering applications (Dash et al. 2011).

Additionally, there are many references related to liposomes coated with various oligosaccharides and yeast-derived mannan, showing they drastically enhance induction of ovalbumin (OVA)-specific delayed-type footpad swelling response in Balb/c mice, with a peak at 24–48 host challenge. The mannan-coated liposome was included in the study as a reference to compare the effects of various neoglycolipids for their augmentation of a delayed response type. It is possible that the receptor–mannose interaction of oligomannose- or mannan-anchored liposomes might have augmented the processing of OVA reconstituted in these liposomes. In addition, mannose residues may possess some other activity, such as stimulation of IL-12 release, culminating in the activation of T lymphocytes. Only those neoglycolipids (oligosaccharides) with mannose residues at nonreducing termini were effective. However, these authors suggested that instead of using mannan, which can elicit antibody and B-cell mitosis (immunogenic) and toxic effects on i.v. administration, safer neoglycolipids – such as oligomannose – that are ubiquitously found in the body should be used as an adjuvant for the induction of cell-mediated immunity.

Recently, Venketesan and Vyas (Venketesan 2000) rationalized the role of polysaccharide-anchored liposomes in enhancing the immunogenicity of the

model antigen after oral administration. Results were compared in terms of serum immunoglobulin (Ig)G and IgA titers against bovine serum albumin (BSA) administered as control, in both plain and anchored formulations. Coating liposomes with these hydrophobized polysaccharides can be performed by incubation of aqueous solutions of polysaccharide derivatives with preformed liposomal dispersion. In some cases, cholesterol-substituted polysaccharide was used to conjugate sensory devices like sialic acid derivative or an IgM fragment. The sialic-acid-conjugated cholesterol-substituted polysaccharide or immunopolysaccharide derivatives were subsequently anchored over liposomes with the dispersion–incubation technique under optimized standard conditions. The earliest biologically active substance of marine origin was a toxin named holothurin, which was extracted by Nigrelli et al. from a marine organism, the *Actinopyga agassizi* (Nigrelli et al. 1967).

Holothurin showed some antitumor activities in mice. Since then, the search for drugs and natural products of interest from marine organisms has continued. The field of natural polysaccharides of marine origin is already large and expanding. Seaweeds are the most abundant source of polysaccharides, as are alginates, agar, agarose, and carrageenans. However, the literature lacks in vivo data in terms of drug delivery from these dosage forms, which makes it difficult to be conclusive in terms of their effectiveness as drug carriers at this stage. Since some work has been done on in vitro–in vivo correlations with chemically cross-linked chitosan hydrogels with successful and sustained drug delivery in animals, it is anticipated that optimized chitosan-based polyelectrolyte complexes may also perform up to expectation for in vivo drug delivery (Ladaviere et al. 2007; Du et al. 2011).

Bioavailability and Metabolism

In contrast to many synthetic polymers, polysaccharides have very low (if any) toxicity levels. For example, among the resorbable polymers, dextran is a natural polysaccharide that has gained interest in biomedical applications owing to its cell biocompatibility and nontoxic and antibacterial properties (Angelopoulou et al. 2012). The degradation products of polysaccharides are metabolized by human enzymes, mainly lysozyme (Angelopoulou et al. 2012). Dextrans are biopolymers composed of glucose with α-1,6 linkages, with possible branching from α-1,2, α-1,3, and α-1,4 linkages, which exhibit low toxicity and high biocompatibility. Consequently, dextrans have formed the basis of biocompatible hydrogels for controlled prolonged therapeutic release (Podder et al. 1988). Likewise, dextran has exhibited biocompatibility when formulated into microspheres, as suggested by a lack of inflammatory response following subcutaneous injection into rats (Sato and Sunamoto 1992; Saito et al. 2003; Jones 1994). Also, owing to their native presence within the body, most polysaccharides are subject to enzymatic degradation. Through enzyme catalysis, polysaccharides can be broken down to their monomer or oligomer building blocks and recycled for use as storage, structural support, or even cell-signaling applications (Nurkeeva et al. 2003). For example, glycosidases are common, constituting 1–3 % of the human genome (d'Ayala et al. 2008), and can readily catalyze the hydrolysis of many different glycosidic linkages (Nurkeeva et al. 2003). In contrast to

glycosidase, other enzymes are more polysaccharide specific. Hyaluronidase, for instance, specifically degrades the polysaccharide HA by cleaving β-1,4 linkages between D-glucuronic acid and D-*N*-acetylglucosamine, particularly in regions of high HA concentration (Necas et al. 2008). Of note, some polysaccharides are particularly susceptible to degradation by lysosomal enzymes following endocytosis, including glycosidases, esterases, and proteases (Stern 2003). For example, lysozyme, *N*-acetyl-β-D-glucosaminidase, and a range of proteases play a role in the degradation of chitosan (PÉrez and Samain 2010a; Williamson et al. 2002) Thus, enzymatic degradation provides a mechanism of release for therapeutics associated with polysaccharide-based carrier systems. Recently reported is the synthesis of a D-glucose-sensitive dextran hydrogel containing concanavalin A demonstrating that diffusion of proteins (insulin, lysozyme, and BSA) through this gel varied with D-glucose concentration (Zhang et al. 2005). Dextran-based hydrogel incorporating carboxyl groups was prepared using carbodiimide chemistry and cast into membranes. Zhang et al. presented a novel pH-responsive dextran hydrogel produced by the intermolecular cross-linking of CM dextran using carbodiimide chemistry and *N*-hydroxysuccinimide (NHS) as a biocompatible dextran drug-delivery system (Angelopoulou et al. 2012). The diffusion rate of proteins through the membranes is influenced by pH, increasing as pH increases, due to loosening of the gel structure resulting from mutual repulsion of ionized carboxyl groups. Protein diffusion rates through hydrogel membranes are also sensitive to changes in ionic strength. This response appears as a bell-shaped curve, increasing at low ionic strengths, and then decreasing as ionic effects swamp the charge-repulsion effects. The hydrogel is formed by esteric bonds between hydroxyl and carboxyl in CM-dextran in the presence of *N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and NHS. The formed ester bonds can be frocked, and they release the desired entity (Angelopoulou et al. 2012).

Other Biological Activities

According to the literature (Zhang et al. 2013), many scientific approaches aim at achieving high therapeutic efficacy through new drug-delivery systems, which increase circulation time in the body, ensuring an effective concentration at the target site. However, results are not convincing regarding immune-system toxicity, and studies are ongoing regarding free drugs and various carrier systems for interaction with plasma proteins causing rapidly clearance. To minimize clearance and prolong drug circulation, PEG has been conjugated or coated on many drug-delivery systems. For example, studies show that PEG protected octyl-succinyl-chitosan from plasma protein (Qu et al. 2009) and that PEG modification could prolong HA-ceramide circulation (Cho et al. 2012). Moreover, PEG could inhibit liver uptake of HA-conjugated micelles, thereby increasing systemic circulation (Le Droumaguet et al. 2011). Another example of the PEG-conjugated polysaccharide system is PEGylated chitosan nanoparticles, which have been investigated as carriers for diverse, small-molecular drugs, such as paclitaxel, camptothecin, methotrexate, and all-trans retinoic acid (ATRA) (Sun et al. 2012). The effect of PEG conjugation

on PTX-loaded *N*-octyl-sulfate chitosan nanoparticles was investigated by Qu et al. (2009). Despite demonstrable improvements relative to unmodified systems, there are potential drawbacks of PEG usage, such as the nonbiodegradable PEG backbone, continuous accumulation in the body, and possible induction of an immune response In addition, the PEG coating may interfere with cellular uptake of drugs because PEG has been reported to reduce drug–cell interaction and hinder drug release from carrier systems (Luo and Wang 2014). Other hydrophilic, biodegradable, nontoxic, and nonimmunogenic molecules are being sought as an alternative. For example, polysialic acid (PSA) meets all of the latter criteria, and – of equal importance – PSA has no known receptors in the human body, suggesting the possibility for further improvement in circulatory stability. A series of PSA-protein conjugates has been investigated, which shows prolonged circulation of insulin (Zhang et al. 2013), asparaginase (Jain 2007), and catalase (Cho et al. 2012). Recently, Zhang et al. synthesized PSA-based micelle systems for future applications in drug delivery (Zhang et al. 2013).

Clinical Trials

Early results from clinical trials suggest that micelles should be designed and developed with careful attention toward material selection. Ideally, micelles developed for drug delivery should be biodegradable and have high stability, high biocompatibility, and low immunogenicity. Natural polysaccharides meet the latter requirements and can be used to develop micelles in lieu of synthetic polymers. In addition, polysaccharides can be readily modified and exist in positive-, negative-, or neutral-charge states (Zhang et al. 2013). Finally, some polysaccharides are bioactive and can be used to augment the therapeutic efficacy of an associated drug or can enhance the targeting ability of a carrier system (Joke Vandorpe et al. 1997; Gref et al. 1994). Despite these advantages, polysaccharide-based micelle systems are still under development, and outcomes have not met clinical requirements.

12 Conclusions

Nowadays, polysaccharides are the focus of attention in many areas of research. Polysaccharides of marine origin are playing a central role, as the marine environment represents an open source of attractive molecules with potential applicability in many fields of interest. Examples of these are chitin and its derivative chitosan, alginate, carrageenan, agar, and collagen, among others. Since Ehrlich suggested the concept of a “magic bullet” (Torchilin 2010; Park et al. 2010), many research scientists have attempted to develop drugs that selectively destroy disease cells but are not harmful to healthy cells. However, prior to any application development, every polysaccharide should undergo cytotoxicity screening. For the development of such targeted delivery systems, chitosan and its derivatives possess various advantages, such as biocompatibility, biodegradability, mucoadhesivity, and other

unique biological properties. Over the last decade, increasing attention has been paid to the development of systems to deliver drugs for long periods at controlled rates. Some of these systems can deliver drugs continuously for >1 year. However, little effort has been given to developing systems for the controlled release of nucleic acids. Recently, a novel gene-transfer method that allows prolonged release and expression of plasmid DNA in vivo in normal adult animals was established. In this system, a biocompatible natural polymer such as collagen or its derivatives acts as the carrier for the delivery of DNA vectors. The biomaterial carrying the plasmid DNA was administered to animals, and once introduced, gradually released plasmid DNA in vivo. A single injection of plasmid DNA biomaterial produced physiologically significant levels of gene-encoding proteins in the local and systemic circulation of animals and resulted in prolonged biological effects.

Chitosan has been the subject of interest for its use as a polymeric drug carrier material in dosage-form design due to its appealing properties, such as biocompatibility, biodegradability, low toxicity, and relatively low production cost from abundant natural sources. However, one drawback of using these natural polysaccharides in modified-release dosage forms for oral administration is its fast dissolution rate in the stomach. Since chitosan is positively charged at low pH values (below its pKa value), it spontaneously associates with negatively charged polyatomic ions in solution to form polyelectrolyte complexes.

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Chitosan-Based Polysaccharide Biomaterials

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Jayachandran Venkatesan, Baboucarr Lowe, Ramjee Pallela, and Se-Kwon Kim

Contents

Abstract

In recent years, significant developments have been achieved in the field of tissue engineering. Biopolymers and bioceramics are known to play major role in the construction of artificial organs. Chitosan is one of the biopolymers extensively studied for tissue engineering, drug delivery, and wound healing applications. Chitosan is biocompatible and biodegradable. Its pore-forming ability also enhances efficient cell growth and differentiation. In the present chapter, we have discussed about chitosan and its usage toward bone and skin tissue engineering, drug delivery, and wound healing applications.

Keywords

Biomaterials • Chitosan • Hydroxyapatite • Drug delivery • Tissue engineering

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1 Introduction

Chitosan is a partially deacetylated derivative of chitin, commonly found in crustaceans. It is a linear polysaccharide consisting of β-(1–4)-linked D-glucosamine residues with variable number of randomly located *N*-acetylglucosamine groups. Chitosan's molecular weight may range from 50 to 1,000 kDa depending on the source and preparation method employed (Francis Suh and Matthew 2000). Chitosan is normally insoluble in aqueous solutions above pH 7. But, it can be soluble below pH 5. Molecular structure of chitosan is shown in Fig. 1.

Artificial skin, surgical sutures, artificial blood vessels, controlled drug release, contact lens, eye humor fluid, bandages, burn dressing, blood cholesterol control, anti-inflammation, tumor inhibition, bone healing treatment, wound healing accelerator, hemostatic agent, antibacterial, antifungal, and weight loss are important biomedical applications of chitosan (Pighinelli and Kucharska 2013; Şenel and McClure 2004). Chitosan is biologically renewable, biodegradable, biocompatible, nonantigenic, nontoxic, and biofunctional material. It has a hydrophilic surface promoting cell adhesion, proliferation, and differentiation and evokes minimal foreign body reaction on implantation (Li et al. 2005).

2 Tissue Engineering

Tissue engineering is one of the interdisciplinary research fields to construct the artificial organs. Combining knowledge of living cells as well as their genetic composition to aid in the development of biologically active substitutes for the use as biomaterial implants to foster tissue growth and/or repair the damaged sites, which ensure their active biological functions in a host system (Shalumon et al. 2009). Chitosan polysaccharide is extensively used in skin, bone, cartilage, liver, nerve, and blood tissue engineering to make artificial organs. Porous chitosan scaffolds are commonly prepared by freezing and lyophilization methods. Madihally and Matthew (1999) prepared porous chitosan scaffold for bone tissue engineering (Di Martino et al. 2005; Kim et al. 2008; Madihally and Matthew 1999). Chitosan-based biocomposites with collagen were examined ultrastructurally and characterized for their ability to regulate cellular activity. Scanning electron microscopy indicated that the addition of chitosan greatly influences ultrastructure and changes the orientation of the cross-linking of collagen fibers,

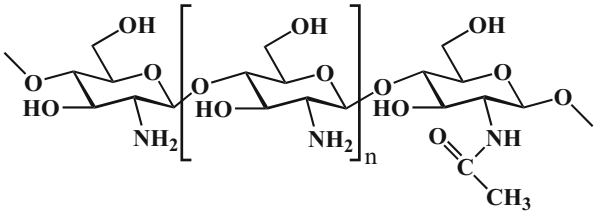

Fig. 1 Molecular structure of chitosan

thereby reinforcing the overall structure to increase pore size (Tan et al. 2001). Huang et al. (2005) prepared the chitosan-gelatin-based scaffold, which has gained much attention in tissue engineering. Degradation results from these studies showed that gelatin-containing chitosan scaffolds represent faster degradation rate and significant loss of material than chitosan. Three-dimensional chitosan and chitosan-gelatin scaffolds supported fibroblast viability equally (Huang et al. 2005).

Homogeneous blends of 25 %, 50 %, and 75 % PCL (polycaprolactone) composites were formed. Increased viability and redistribution of actin fibers was observed on blends formed with 50 % PCL and 75 % PCL relative to individual polymers. 50:50 blends when processed at 55 °C in an oven showed significant improvement in mechanical properties as well as support for cellular activity relative to chitosan (Sarasam and Madihally 2005). Chitosan-gelatin 2D as well as 3D scaffolds have exhibited greater degradation rate with minimal immunological response in the presence of lysozyme, which is an important dimension for their successful use in tissue engineering applications (Huang et al. 2005). The antimicrobial resistance of chitosan provides a stable microclimate for wound healing and repair of damaged tissue (Jayakumar et al. 2010). Chitosan has extensively studied materials for biomedical applications by Asian researchers (Khor and Lim 2003).

2.1 Skin Tissue Engineering

The skin is an important organ with wide range of cellular, molecular, physiological, as well as metabolic functions. Hence, it poses as a complex biological interface to repair and stabilize its roles (barrier formation, pigmentary defense against UV irradiation, thermoregulation, mechanical, and aesthetic functions), when it got damaged. Skin tissue engineering seeks to resolve this efficiently, by developing and functionalizing biomaterial constructs to ensure complete regeneration (Haipeng et al. 2000; Kim et al. 2008; Lee et al. 2003). Ma et al. (2003) developed a scaffold with chitosan and collagen for skin tissue engineering. In vivo animal tests revealed that the scaffold could sufficiently support and accelerate the fibroblast infiltration from the surrounding tissue. Immunohistochemistry analysis of the scaffold embedded for 28 days indicated that the biodegradation of the 0.25 % glutaraldehyde-treated scaffold is a long-term process (Ma et al. 2003). Adekogbe and Ghanem (2005) fabricated and characterized DTBP (dimethyl 3–3, dithiobis' propionimidate) cross-linked chitosan scaffolds for use in skin tissue engineering (Adekogbe and Ghanem 2005). Chitosan sugar residues have been reportedly modified to induce specific cell recognition for use in tissue engineering, such as the synthesis of sugar-bound chitosan, and present unique possibility of extracellular matrix attachment in hepatocyte (Li et al. 2000; Madihally and Matthew 1999). It is also reported elsewhere that an unwoven cloth of chitin fiber and a film of chitosan or N-carboxybutyl chitosan promote the healing of skin wound when applied in wound dressing (Ma et al. 2001). Chitosan oligosaccharide stimulatory effects on macrophages function as chemoattractants for neutrophils both in vitro and in vivo early events essential for wound healing (Shi et al. 2006; Yoshimoto et al. 2003).

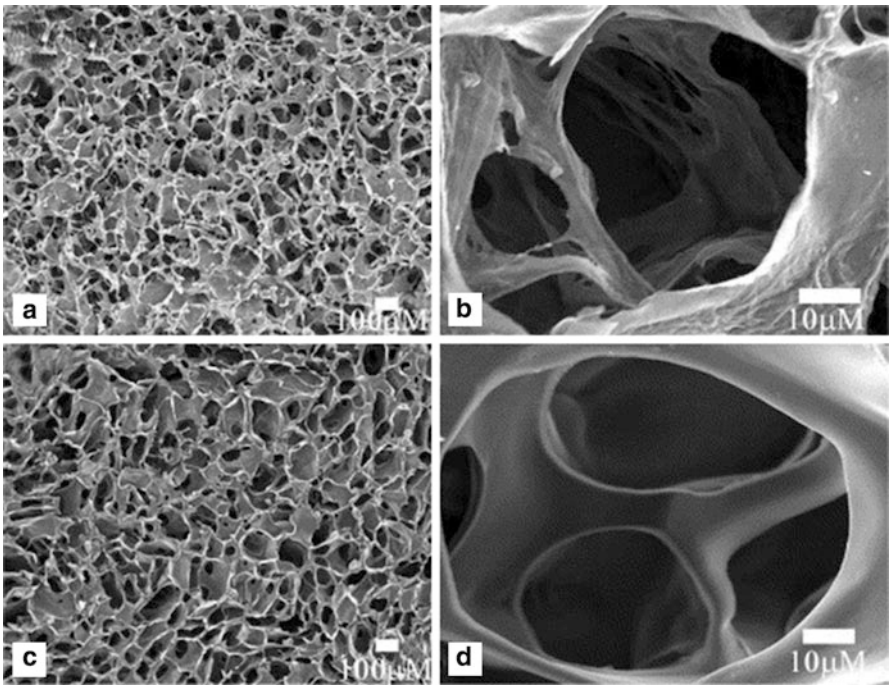

Fig. 2 Scanning electron micrographs (**a** and **b**) chitosan-alginate scaffold and (**c** and **d**) pure chitosan scaffold (Reproduced the figure from Li et al. (2005))

2.2 Bone Tissue Engineering

Scaffolds, cells, and growth factors are playing major role in the construction of bone tissue. Varieties of synthetic and natural polymers have been extensively used for the scaffold fabrication. The typical synthetic polymers are poly(glycolic acid), poly(L-lactic acid), and poly(DL-lactic-*co*-glycolic acid) and the natural polymers are chitin, chitosan, and alginate.

Chitosan is one of the extensively used natural polymers in bone tissue scaffold fabrication. Seol et al. (2004) reported that chitosan sponges can be used as tissue-engineering scaffold with a 100–200 μm pore diameter, which allowed significant cell proliferation (Seol et al. 2004). Cell density, alkaline phosphatase activity, and calcium deposition were monitored for up to 56 d culture. Histological results corroborated that bone formation within the sponges had occurred (Seol et al. 2004). Li et al. (2005) represented the biodegradable porous scaffold of chitosan and alginate polymers with significantly improved mechanical and biological properties. The porosities of pure chitosan and chitosan-alginate scaffolds in this study were determined to be 84.86 ± 2.18 % and 91.94 ± 0.90 %, respectively (Fig. 2). The compressive yield strength was determined to be 0.1257 ± 0.015 MPa and 2.567 ± 0.41 MPa, respectively, for pure chitosan and chitosan-alginate scaffolds.

Similarly, the Young's modulus is 0.467 ± 0.022 MPa and 8.167 ± 1.57 MPa for chitosan and chitosan-alginate scaffolds, respectively. Cell attachment on chitosan and chitosan-alginate scaffolds is increased with time (1, 3, and 7 days). The in vivo study showed that the hybrid scaffold had a high degree of tissue compatibility. Calcium deposition occurred as early as the fourth week after implantation (Li et al. 2005).

However, the mechanical strength of polymer-based scaffolds is often not suitable for mimicking the natural bone. Thus, a combined form of bioactive polymer, carbon materials, and bioceramic-based scaffolds is often used for bone tissue engineering (Lee et al. 2014; Sowmya et al. 2013; Venkatesan and Kim 2010, 2012, 2014; Venkatesan et al. 2012a, b, 2014a, b, c). Zhang and Zhang (2001) prepared chitosan-calcium phosphate composite scaffold for bone tissue engineering (Zhang and Zhang 2001).

Zhang et al. (2008) reported chitosan-hydroxyapatite nanocomposite, which was prepared by in situ coprecipitation with electrospinning process. Poly(ethylene oxide) (PEO) was used as a fiber-forming additive to develop a chitosan-hydroxyapatite nanofiber with a diameter 214 ± 25 nm (Fig. 3). Significant bone formation was observed on chitosan-hydroxyapatite then compared to electrospun chitosan scaffold with human fetal osteoblast (hFOB) (Zhang et al. 2008).

Thein-Han and Misra (2009) reported chitosan-nanohydroxyapatite (chitosan-nHA) in the form of porous scaffold which was prepared by freeze-dry method with pore size ~50–120 μm. The compression modulus of hydrated chitosan scaffolds was increased on the addition of 1 wt.% nanohydroxyapatite from 6.0 to 9.2 kPa in high-molecular weight scaffold. Higher cell proliferation (1.5 times) was observed in the chitosan-nanohydroxyapatite composite scaffold than compared to chitosan scaffold alone (Fig. 4) (Thein-Han and Misra 2009).

Chitosan has been combined with various polymers (Jiang et al. 2006, 2010; Kim et al. 2008; Liuyun et al. 2009; Rezwan et al. 2006; Tan et al. 2001; Wang and Stegemann 2010) and ceramics (Cai et al. 2009; Katti et al. 2008; Kong et al. 2006; Lee et al. 2000; Muzzarelli 2009; Oliveira et al. 2006; Peter et al. 2010; Swetha et al. 2010; Venkatesan et al. 2011; Yin et al. 2003; Zhang et al. 2003, 2008; Zhang and Zhang 2001) and used for bone tissue construction. γ-PGA/chitosan (gamma-poly(glutamic acid)/chitosan) composites showed enhanced serum adsorption, cell attachment, and proliferation in matrix surfaces becoming an important milestone in tissue engineering (Hsieh et al. 2005). Chitosan materials are found to evoke a minimal foreign body reaction as well as fibrous encapsulation with accelerated angiogenesis effectively contributing to the healing process. Chitosan and chitosan fragments on stimulatory immune response are reported to induce local cell proliferation and integration of the material implant (Drury and Mooney 2003; Francis Suh and Matthew 2000). Lysozyme is responsible for the primary degradation of chitosan in vivo and targets acetylated residues (Hirano et al. 1989). Chitosan with hydroxyapatite is one of the better materials to mimic the natural function of bone. Phosphorylated chitosan to calcium-phosphated cement through a creeping substitution process is reportedly used to fill bony defects of tibia and radius in vivo, showing a higher modulus, osteoconductivity,

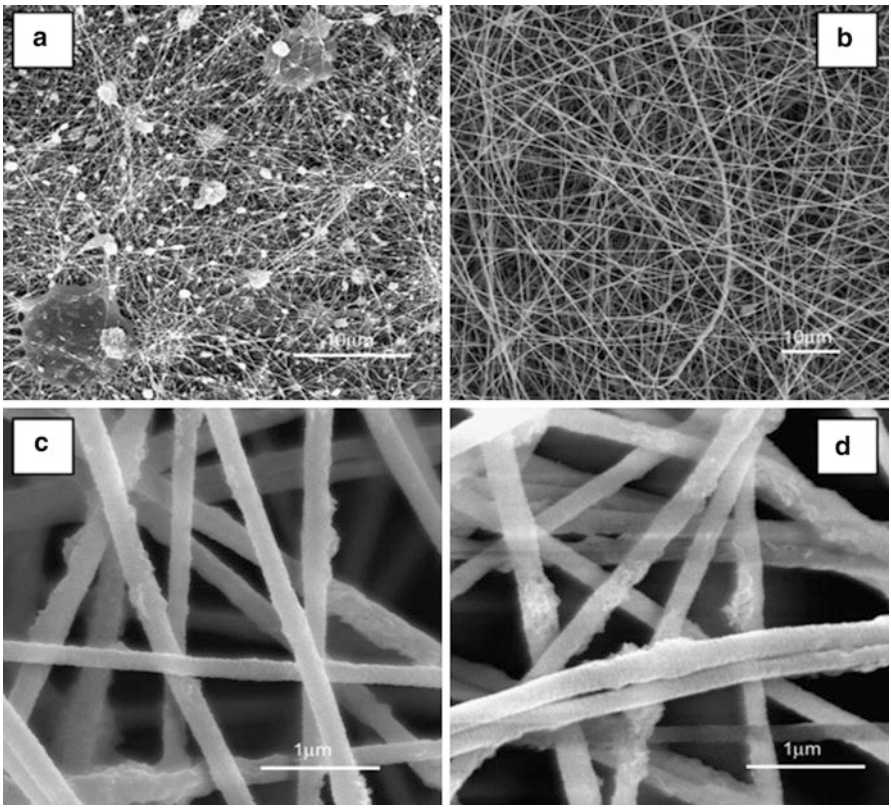

Fig. 3 Field emission scanning electron microscopy images of electrospun chitosan-hydroxyapatite nanocomposite nanofibers: doped with 20 wt% conventional PEO with a moderate molecular weight (**a**), doped with 10 wt% ultrahigh-molecular weight poly(ethylene oxide) at low magnification (**b**), at higher magnification (**c**), and after alkali treatment (**d**) (Reproduced the figure from Zhang et al. (2008))

bioresorbability, and biocompatibility (Di Martino et al. 2005; Rezwan et al. 2006). However, much research has is needed to further develop and functionalize chitosan for clinical trials.

3 Drug Delivery

Significant amount of research has been conducted on chitosan biomaterial for drug delivery. Chitosan can be used to deliver drugs into various administration routes such as oral, buccal, nasal, transdermal, parenteral, vaginal, cervical, intrauterine, and rectal (Felt et al. 1998). Chitosan can be modified into nanoparticles, microspheres, sponges, membranes, and rod shapes (Bhattarai et al. 2010). Ko et al. (2002) reported the preparation of chitosan microparticles using tripolyphosphate (TPP) by an ion cross-linking method with particle range from 500 to 710 μm. Felodipine drug was

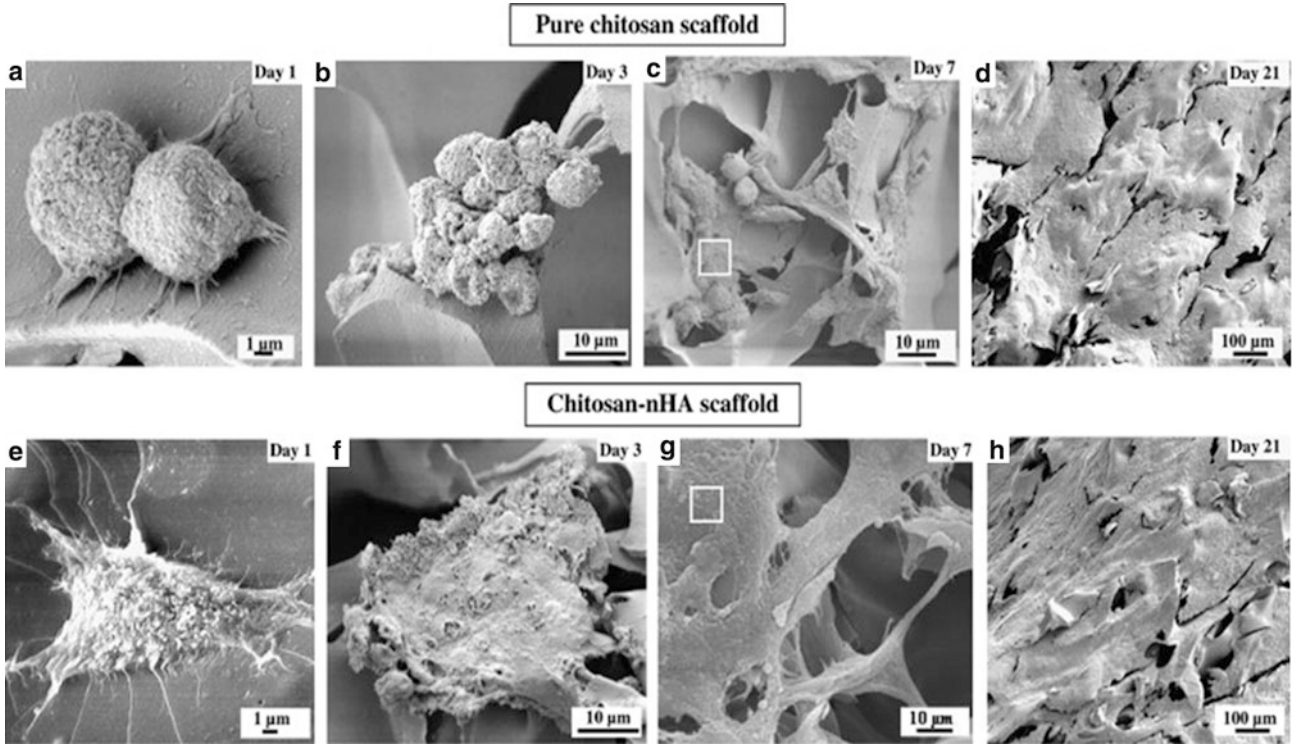

Fig. 4 Scanning electron microscopy images of pre-osteoblasts seeded on high-molecular weight chitosan and chitosan-nHA scaffolds. Pre-osteoblasts on chitosan surface after (**a**) day 1, (**b**) day 3, (**c**) day 7, and (**d**) day 21 of cell culture and on chitosan-nHA surface after (**e**) day 1, (**f**) day 3, (**g**) day 7, and (**h**) day 21 of cell culture (Reproduced the figure from Thein-Han and Misra (2009))

used as model in this study, and chitosan prepared with lower pH resulted in slower felodipine release from chitosan microparticles. Comparatively, with decreasing molecular weight and concentration of chitosan solution, release behavior was increased (Ko et al. 2002). Jameela and Jayakrishnan (1995) prepared chitosan microparticles by glutaraldehyde cross-linkage. Mitoxantrone was incorporated into the chitosan microspheres, where 25 % of the incorporated mitoxantrone drug was released over 36 days from the microspheres (Jameela and Jayakrishnan 1995).

Different kinds of methods have been extensively studied to develop micro- and nanoparticles of chitosan for drug delivery purposes including emulsion cross-linking, spray drying, emulsion-droplet coalescence method, ionic gelation, reverse micellar method, as well as sieving methods. Furthermore, colon-targeted, mucosal, cancer, gene, topical, and ocular deliveries have been extensively studied with chitosan (Agnihotri et al. 2004).

Lorenzo et al. (1998) reported the system consists of chitosan microcores entrapped with acrylic microsphere. Sodium diclofenac drugs were incorporated with chitosan microcores. The modification of chitosan microcores became a novel approach representing great potential in colonic drug delivery (Lorenzo-Lamosa et al. 1998). Chemical modification of chitosan is a possible alternative to associate drugs and used them as controlled release vehicles (Prabaharan and Mano 2004). Another author, Chen et al. (2004) developed a novel pH-sensitive hydrogel with water-soluble chitosan derivative (*N*, *O*-carboxymethyl chitosan) and alginate blended with genipin for controlling protein drug delivery (Chen et al. 2004). Nifedipine was embedded in a chitosan matrix to develop a prolonged release form. The release rate of nifedipine from chitosan matrix was slower for beads than granules (Chandy and Sharma 1992).

Transdermal drug delivery system could also be prepared using chitosan polymer gel (Thacharodi and Rao 1995). Meanwhile, extensive reviews have been written on chitosan for ocular drug delivery (Alonso and Sánchez 2003). Senel et al. (2000) reported chitosan gel for peptide drug delivery, where the hydrogel was prepared with 2 % chitosan in dilute lactic acid together with the transforming growth factor (Şenel et al. 2000). A new thermosensitive hydrogel was designed and prepared by simply mixing *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride and poly(ethylene glycol) with a small amount of α-β-glycerophosphate and used for nasal drug delivery system (Wu et al. 2007). Ionic interactions between positively charged amino groups in chitosan and the negatively charged mucus gel layer make it mucoadhesive. The favorable properties like biocompatibility, biodegradability, pH sensitiveness, mucoadhesiveness, etc. have enabled these polymers to become the choice of pharmacologists for use as oral delivery matrices for proteins (George and Abraham 2006).

4 Wound Dressing

Chitosan and chitins are the most promising biomaterials in wound dressing application. The adhesive nature of chitin and chitosan, together with their antifungal and bactericidal potentials as well as their permeability to oxygen, is a very

important property associated with the treatment of wounds and burns. Different derivatives of chitin and chitosan have been prepared for this purpose in the form of hydrogels, fibers, membranes, scaffolds, and sponges (Jayakumar et al. 2011). Mi et al. (2001) reported the asymmetric chitosan membrane prepared by immersion-precipitation phase conversion method. The asymmetric chitosan membrane showed controlled evaporative water loss and excellent oxygen permeability and promoted fluid drainage considerably. Wound covered with the asymmetric chitosan membrane is hemostatic and healed quickly. Histological examination confirmed that epithelialization rate was increased and the deposition of collagen in the dermis was well organized by covering the wound with this asymmetric chitosan membrane (Mi et al. 2001). Mi et al. (2002) developed bilayer chitosan for wound dressing, consisting of a dense upper layer (skin layer) and a sponge-like lower layer (sub-layer), which is very suitable for use as a topical delivery vehicle of silver sulfadiazine (AgSD) in controlling wound infections. Physical characterization of the bilayer wound dressing showed that it has excellent oxygen permeability, controls water vapor transmission rate, and promotes water uptake capability. AgSD dissolved from bilayer chitosan dressings to release silver and sulfadiazine. The release of sulfadiazine from the bilayer chitosan dressing displayed a burst release on the first day and then tapered off to a much slower release (Mi et al. 2002). A novel wound dressing composed of nano-silver and chitosan was fabricated using a nanometer and self-assembly technology. Sterility and pyrogen testing assessed biosafety, and efficacy was evaluated using Sprague-Dawley rats with deep partial-thickness wounds (Lu et al. 2008). Novel wound dressings composed of chitosan film and minocycline hydrochloride (MH) were prepared using commercial polyurethane film (Tegaderm) as a backing material (Aoyagi et al. 2007). Chemical modification of chitosan into *N*-carboxybutyl chitosan was used for wound management (Biagini et al. 1991).

Chitosan enhances the functions of inflammatory cells such as polymorphonuclear leukocytes (PMN) (phagocytosis, production of osteopontin, and leukotriene B4), macrophages (phagocytosis, production of interleukin (IL)-1, transforming growth factor β1, and platelet-derived growth factor), and fibroblasts (production of IL-8). In spite of application of chitosan to various species, this finding is observed only in dogs. Secondly, intra-tumor injection of chitosan on tumor-bearing mice increases the rate of metastasis and tumor growth. Therefore, it is important to consider these effects of chitosan, prior to drug delivery (Ueno et al. 2001). Chitosan membrane, prepared with a 75 % degree of deacetylation and a thickness of 10 μm, was used in non-mesh or mesh form. After 10 days, the chitosan-dressed area had been healed more promptly compared with the Bactigras dressed area. The data confirm that chitosan mesh membrane is a potential substitute for human wound dressing (Azad et al. 2004).

Chitosan fibers with different degrees of acetylation were prepared by controlling the ratio between the amount of acetic anhydride and the weight of the fibers during the acetylation process. Results showed that the partially acetylated chitosan wound dressing had a much higher absorption capacity than the original untreated chitosan samples (Qin 2008).

The chitosan hydrogel could completely stop bleeding from a cut mouse tail within 30 s of UV irradiation and could firmly adhere two pieces of sliced skins of

mouse to each other. Full-thickness skin incisions were made on the back of the mice, and subsequently, an Az-CH-LA aqueous solution was added into the wound and irradiated with UV light for 90 s. The application of the chitosan hydrogel significantly induced wound contraction and accelerated wound closure and healing (Ishihara et al. 2002), in both db/db and db/+mice. However, the addition of FGF-2 in the chitosan hydrogel further accelerated wound closure in db/db mice, although not in db/+mice (Obara et al. 2003).

Ueno et al. (1999) developed cotton fiber-type chitosan for wound healing and was evaluated histologically and immunohistochemically. Immunohistochemical typing of collagen I, III, and IV showed increased production of type III collagen in the chitosan group (Ueno et al. 1999).

The biochemical significance of the chitin/chitosan products was found to accompany their commercial viability in terms of time taken to heal and clinical labor costs (Muzzarelli et al. 2007). To measure this efficiently, a study was conducted to ascertain the mechanical and bioadhesive strength and biological evaluations of chitosan films for wound dressing (Khan et al. 2000).

Ong et al. (2008) developed chitosan containing different amounts and types of polyphosphate polymers and their hemostatic efficacies evaluated in vitro. The optimal chitosan-polyphosphate formulation (ChiPP) accelerated blood clotting, increased platelet adhesion, generated thrombin faster, and absorbed more blood than chitosan. Silver-loaded ChiPP exhibited significantly greater bactericidal activity than ChiPP in vitro, achieving a complete kill of *Pseudomonas aeruginosa* and a >99.99 % kill of *Staphylococcus aureus* consistently. The silver dressing also significantly reduced mortality from 90 % to 14.3 % in a *P. aeruginosa* wound infection model in mice (Ong et al. 2008). Analysis on the accelerating effects of open wound healing by chitin and chitosan was carried out in dogs (Okamoto et al. 1995).

5 Conclusions

Chitosan is one of the promising biomaterial for use in tissue engineering, drug delivery, and wound healing applications due to its biocompatible, biodegradable, and nontoxic nature. Chitosan can be modified into any form such as microsphere, hydrogel, porous scaffold, and nanofibers, thus expanding its biomedical applications into another area: biosensors construction.

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Structure and Bioactivities of Fungal Polysaccharides

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Contents

Abstract

Edible fungi have been consumed as medicines and food in Asian countries since time immemorial. Health benefits of edible fungi are mainly associated to polysaccharides, which are major bioactive components. Contemporary phytochemical and pharmacological studies have proved that polysaccharides are one of the major active ingredients in different types of edible and medicinal fungi. It has been demonstrated that polysaccharides exhibit significant biological activities, including immunomodulation, antitumor, antioxidant, anticancer, and antiviral activities. The purpose of the present review was to summarize previous and current references regarding structural characterization and biological activities of different fungal polysaccharides.

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1 Polysaccharides in Fungi

Polysaccharides are complex organic molecules, which are essential for pathogenic mechanisms as well as immune response during fungal infections (Roeder et al. 2004).

1.1 Structure of Polysaccharides

Unlike mammalian cells, fungi have a cell wall and a complex compartment, which are mainly composed of polysaccharides (Nimrichter et al. 2005). Glucans, chitin, and mannans (polymers consisting of repeating units of glucose, *N*-acetyl glucosamine, and mannose, respectively) are particularly abundant in the fungal cell wall. Polymeric structures of polysaccharides are composed of at least ten monosaccharides, which are sequentially connected by glycosidic bonds (Bertozzi and Rabuka 2009; Mulloy et al. 2009; Stanley and Cummings 2009). The characteristic branched or linear structures can be categorized when a monosaccharide constituent of a polysaccharide is involved in more than two glycosidic bonds. The polysaccharides consist of homopolymers, "polymer composed of identical monosaccharide," or heteropolymers, "polymer composed of two or more types of monosaccharides." Classic approaches for the determination of polysaccharide structure are chromatographic methods in association with spectrometric and spectroscopic techniques. These approaches allow determination of sequence and composition, anomeric configuration, type of glycosidic linkage, and presence of substituent (Mulloy et al. 2009).

The cellular structural components of polysaccharides are found in three domains of life. Several studies have shown that in microorganisms, these polysaccharides play crucial roles in the development of the cell envelope (Roberts 1996; Nimrichter et al. 2005). Both in prokaryotic and eukaryotic microbial pathogens, polysaccharides are important cell wall components (Roberts 1996; Nimrichter et al. 2005). This surface distribution is in agreement with the fact that host pathogenic interactions are influenced by polysaccharide molecules directly (Moxon and Kroll 1990; Smith 1990; Levitz 2004; Zaragoza et al. 2009).

1.2 Types of Polysaccharides

Polysaccharides are built up from monosaccharides which are ubiquitous biopolymers. They belong to carbohydrates (sugars), 99 % of which are found in plants. Sometimes, polysaccharides are not pure, but they are associated with other

polysaccharides, polyphenolics, or proteins either by covalent or by non-covalent bonds. Normally, there are two types of polysaccharide products in the markets: water extract and diluted alkali extract, out of which, the former is more prevalent. Despite their origin, the antitumor effect of polysaccharides is generally attributed to their 1,3-β-glucan structures (Yoshioka et al. 1985) or β-glucan protein complex (Kawagishi et al. 1990).

1.2.1 Cellulose

Cellulose is the most abundant carbohydrate in nature. The structural components of plants are formed primarily from cellulose, such as wood is largely composed of cellulose and lignin, while paper and cotton are nearly of pure cellulose. Cellulose is a polymer made with repeated glucose units bonded together by beta-linkages. In human and some animals, cellulose remains undigested due to the absence of cellulase enzyme required to break the β-linkages. However, certain animals such as termites can digest cellulose, because the bacteria possessing the enzyme are present in their gut. Cellulose is insoluble in water and does not change color when mixed with iodine. However, on hydrolysis, it yields glucose. About ca. 40 % of carbon in plants (10.5×10^{10} t) is actually present as cellulose. The annual regeneration of cellulose by biosynthesis (photosynthesis) is ca. 1.3×10^{9} t. Approximately, one tree generates ca. 14 g of cellulose/day.

In plants, cellulose functions as a fiber component of highly efficient biological compound materials, e.g., wood (Whistler and BeMiller 1993). Primary cell wall contains 8 % cellulose, while the remaining 92 % is hemicellulose and pectin, while the secondary cell wall contains 95 % of cellulose. Naturally, cellulose fibers are impure. Cotton fiber consists of 94 % cellulose, and its annual production is 20×10^{6} t (nearly equal to synthetic textile fibers). Annual production of cellulose for paper and cardboard manufacture is greater than 100×10^{6} t. Some bacteria produce highly pure cellulose (Lehmann 1996). In the case of animals, cellulose occurs only in some tunicates.

1.2.2 Pectin

Pectin is present in primary cell walls and in the nonwoody parts of terrestrial plants. It is a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues.

1.2.3 Glycogen

Glycogen is the analogue of starch, a glucose polymer in plants, and is sometimes referred to as an animal starch, having a similar structure to amylopectin but more extensively branched and compact than starch. Glycogen serves as the secondary long-term energy storage in animal and fungal cells with the primary energy stores being held in adipose tissue. Glycogen is produced primarily in the cells of the liver and the muscles and produced in the process of glycogenesis within the brain and stomach as well. The glycogen polymer consists of α-(1-4) glycosidic bonds linked with α-(1-6)-linked branches. Many cell types have glycogen which is found in the form of granule in the costal cytoplasm and plays an important role in the glucose cycle.

Glycogen forms an energy reserve that can be quickly mobilized to meet a sudden need for glucose, but that is less compact and more immediately available as an energy reserve than triglycerides (lipids).

1.2.4 Starches

Starches which are the most important storage saccharide in plant cells occurred as large aggregates or granula (energy storage). Extreme hydration of starch molecules is due to external hydroxyl groups which undergo hydrogen bonding with water. Its worldwide huge production was 8×10^6 t which was recorded in 1976. By using acids or enzymes (glucosidases), degradation to glucose finally changed into sugar.

1.2.5 Chitin

Chitin is an important naturally occurring polymer, which forms structural component of many animals such as exoskeletons. Chitin is a primary constituent of crustacean shells, insect cuticles, and fungal cell walls (Ruiz-Herrera 1992). Chitosan, on the other hand, although not a component in animal species, is a major structural biopolymer in the cell walls of fungi, such as *Mucor*, *Absidia*, and *Rhizopus* genera (Ruiz-Herrera 1992; Khor 2001). Chemically, chitin is closely related to chitosan (a more water-soluble derivative of chitin) and cellulose in that it is a long unbranched chain of glucose derivatives. Both materials contribute structure and strength, which provides protection to the organism.

Chitin is biodegradable in nature, and its decomposition may be catalyzed by chitinase enzymes, secreted by microorganisms (bacteria and fungi) and some plants. Microorganisms get simple sugars during decomposition of chitin by releasing enzymes, which break down the glycosidic bonds in order to convert it to simple sugars and ammonia. Chitin and its deacetylated product, chitosan, have received much interest for potential application in agriculture, biomedicine, biotechnology, and food industry due to their biocompatibility, biodegradability, and bioactivity (Kurita 1998; Felse and Panda 1999; Kumar 2000; Tharanathan and Kittur 2003; Synowiecki and Ali-Khateeb 2003). The production of chitin and chitosan from fungal sources has gained increased attention in recent years due to their potential advantages over the current source. For example, crustacean waste supplies are limited by seasons and fishing industry locations, whereas fungal mycelium can be obtained by simple fermentation regardless of geographical location or season (White et al. 1979).

Furthermore, fungal mycelia have lower levels of inorganic materials compared to crustacean shells, and no demineralization treatment is required during processing (Teng et al. 2001). Considering the significant amounts of fungal-based waste materials accumulated in the mushroom production and fermentation industries and the expenses involved in managing the waste, production of highly functional value-added products may provide a profitable solution to the industry. *Agaricus bisporus* is one of the most popular mushrooms worldwide and especially in the United States. Depending on the size of the mushroom farm, the amount of waste, which mainly consists of stalks and mushrooms with irregular dimensions

and shapes, ranges between 5 % and 20 % of production volume (personal communication). In the United States alone, this results in 50,000 metric ton of waste material per year with no suitable commercial use. On the other hand, citric acid is the most widely used organic acid in the food, beverage, and pharmaceutical industries as an acidifying or flavor-enhancing agent and is commercially produced by submerged fermentation with *Aspergillus niger*. The annual world requirements for citric acid are estimated at 4×10^5 t (Kristiansen et al. 1999), which results in 8×10^4 t of *A. niger* mycelium waste per year (Ali et al. 2002).

2 Bioactive Fungal Polysaccharides

One of the most common and well-studied medicinal fungal polysaccharides is lentinan, a glucan elaborated by the edible mushroom *Lentinus* (or *Lentinula*) *edodes*, also known as the shiitake mushroom. It is composed of a main chain of β-(1,3)-D-glucose residues to which β-(1,6)-D-glucose side groups are attached (one branch to every third main chain unit) (Giavasis 2013; Wasser 2002; Mizuno and Nishitani 2013; Thakur and Singh 2013; Ganeshpurkar et al. 2010). A similar polysaccharide, schizophyllan (also called sizofiran), is produced by the edible mushroom *Schizophyllum commune*. These are probably two of the most well-studied immunomodulating microbial β-(1,3)-D-glucans, and both have been commercialized as novel therapeutics in cancer treatment, while *Lentinus edodes* is the most common edible mushroom in Japan (Giavasis 2013; Wasser 2002; Mizuno and Nishitani 2013; Thakur and Singh 2013; Ganeshpurkar et al. 2010). *Ganoderma lucidum* is another well-studied medicinal mushroom of the class *Basidiomycetes* which has been used in traditional East Asian medicine as a dry powder or as a hot water extract (a type of bitter mushroom tea).

Another edible and medicinal mushroom, *Agaricus blazei*, originating from Brazil, is the source of several antitumor polysaccharides contained in its fruit body (Giavasis 2013; Wasser 2002; Mizuno and Nishitani 2013). These include α-β-(1,6),β-(1,3) glucan, acidic β-(1,6),α-(1,3) glucan, and β-(1,6),α-(1,4) glucan. By contrast to most mushroom glucans, the above glucans have a main chain of β-(1,6) glucopyranose, instead of the more common β-(1,3)-linked main chain (Giavasis 2013; Wasser 2002; Mizuno and Nishitani 2013). The fruit body also contains an antitumor water-soluble proteoglycan α-(1,4) glucan main chain and β-(1,6) glucopyranoside branches at a ratio of 4:1 (Mizuno and Nishitani 2013; Fujimiya et al. 1998), as well as two immunostimulating hetero-glucans containing glucose, galactose, and mannose, one consisting of glucose and ribose and a xyloglucan (Giavasis 2013; Mizuno and Nishitani 2013).

Other immunostimulating biopolymers from *Basidiomycetes* include grifolan, a gel-forming β-(1,3)-D-glucan with β-(1,6) glucosidic bonds at every third glucopyranosyl residue, found in the edible fungus *Grifola frondosa* and Krestin, a proteoglycan with a β-(1,3)-D-glucan chain produced by the edible mushroom *Coriolus versicolor* (also known as *Trametes versicolor*), which has been commercialized in Asia, as an effective immunostimulative drug (Laroche and

Michuand 2007; Hobbs 2004). Another type of edible medicinal mushroom is the *Tremella* group of mushrooms (*T. mesenterica*, *T. fuciformis*, *T. aurantia*, *T. cinnabarina*) which are jelly mushrooms with an unusually high polysaccharide content (60–70 % of the fruiting body, as opposed to 10–30 % in other mushrooms). *Tremella* acidic polysaccharide is a glucuronoxylomannan composed of a linear backbone of α-1,3-linked D-rhamnose, to which xylose and glucuronic acid side chains are attached (Lu et al. 2006).

Scleroglucan is an extracellular glucan excreted by mycelia of *Sclerotium glucanicum* or *S. rolfsii* (Survase et al. 2007; Bluhm et al. 1982). It has a β-(1,3)-linked backbone, to which single D-glucosyl side group is linked via β-(1,6) linkage to every third or fourth unit of glucosyl backbone. Although scleroglucan is known and used mostly as a viscosifier for enhanced oil recovery, it also possesses antitumor and antiviral activity (Giavasis et al. 2002).

3 Health Benefits of Fungal-Based Polysaccharides

3.1 Immuno-functions

Depending on their structural particularities, cell wall polysaccharides function as regulators of virulence or activators of innate immunity (Roeder et al. 2004; Zaragoza et al. 2009). It has been reported that well-studied immune working polysaccharides produced by fungus have α- and β-glucans (Brown et al. 2003; Hohl et al. 2005; Bittencourt et al. 2006; Rappleye et al. 2007; Wheeler et al. 2008; van de Veerdonk et al. 2009; Chai et al. 2011) and complex mannans (Leitao et al. 2003; Cambi et al. 2008; van deVeerdonk et al. 2009). *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *C. gattii* are some of the fungal pathogens in which the immune functions of polysaccharides are available (Monari et al. 2006; Rappleye et al. 2007; Cambi et al. 2008; Chai et al. 2011).

Capsular polysaccharides are among the earliest microbial virulence determinants, which were reviewed in the classic Griffith's experiment (Smith 1990). This study established a direct relationship between the presence of protection against host defense and polysaccharide capsules in bacterial pathogens. Afterwards, over several decades, the linkage between capsular polysaccharides and microbial virulence has been joined together (Moxon and Kroll 1990; Monari et al. 2006; Vecchiarelli 2007; Zaragoza et al. 2009). Although it is clear that in some cases, these structures work in favor of the host (Mazmanian and Kasper 2006; Pletz et al. 2008; Kumar et al. 2009), consequently, microbial polysaccharides may work in favor of the pathogen or induce immune responses that promote infection control, depending on their chemistry and/or structural aspects.

Candida albicans, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *C. gattii* are examples of fungal pathogens in which the immune functions of polysaccharides are known in great detail (Monari et al. 2006; Rappleye et al. 2007; Cambi et al. 2008; Chai et al. 2011). *Cryptococcus*

neoformans and *C. gattii* are the etiologic agents of cryptococcosis, a disease that presumably begins in the lung. In immunocompromised individuals, *C. neoformans* can disseminate to the central nervous system and other organs (Bicanic and Harrison 2004). *C. gattii*, on the other hand, can cause disseminated disease in immunocompetent individuals (Byrnes et al. 2011). In Brazil, cryptococcosis is the fungal disease with the highest mortality rates among HIV-positive individuals. *C. neoformans* and *C. gattii* have polysaccharide capsules surrounding the cell body. Bacterial capsules are common in bacterial pathogens but relatively rare and still poorly defined among eukaryotic organisms. For construction of the capsule, yeast cells secrete polysaccharides into the extracellular environment by mechanisms that involve the release of vesicles for subsequent polysaccharide incorporation into the cell surface, for distal capsular enlargement (Zaragoza et al. 2006; Rodrigues et al. 2007).

Additionally, both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system and therefore might be used to treat a variety of diseases (Wasser and Weis 1999). Mushrooms which appear to enhance or potentiate resistance to disease states are being sought for the treatment of cancer, immunodeficiency diseases (including AIDS), or generalized immunosuppression after drug treatment (Pujol et al. 1990; Clericuzio et al. 2004). The mycelia of *Irpex lacteus* contain many nutrition compounds, such as protein, polysaccharides, adenosine, glycoprotein, and cordycepic acid (Yang et al. 2005).

3.2 Anticancer/Antitumor Potential

Cancer is one of the major causes of human death worldwide. There are many anticancer therapies available, including chemotherapy and anticancer drugs. The great majorities of them are known to be cytotoxic to cancer cells but are also toxic to normal cells and harmful to the immune system (Yang et al. 2007). Thus, the discovery of a new safe compound, capable of potentiating immune function, has become an important goal of research in the biomedical sciences. Recently, mushrooms have attracted much attention in the biochemical and medical fields due to their useful therapeutic effects (Balkwill 2009). It is well documented that various mushroom-derived polysaccharides are used as remedies and prevention agents for cancer and immune disorders and so on (Goodridge et al. 2009; Kim et al. 2004). For instance, several polysaccharides isolated from *Lentinus edodes*, *Schizophyllum commune*, *Coriolus versicolor*, *Phellinus linteus*, etc., have been shown to possess immunostimulatory activities (Kim et al. 1996; Ooi and Liu 2000; Zheng et al. 2005).

Carcinogenesis is a process which normally takes several years during which progressive genetic changes occur, leading to malignant transformation. Cancer prevention is the best intervention in this process before an invasive disease develops. Over the last half century, our understanding of carcinogenesis has grown enormously, owing largely to recent technology, allowing exploration of

molecular pathways, cancer-associated genes, and tissue architecture and the use of drugs, biological compounds, and nutrients to prevent the development of cancer, i.e., to inhibit, delay, or reverse carcinogenesis (Kellof and Sigman 2000). Mushroom polysaccharides were likely to affect primarily the promotion and progression stages, according to the referred model of carcinogenesis. There are advantages of using mushrooms as sources of bioactive compounds, rather than plants. For example, the fruiting body can be produced in much less time, and the mycelium may also be rapidly produced (in a liquid culture that can be manipulated to produce optimal quantities of active products or from mycelial biomass and supernatant of submerged cultures using bioreactors).

Edible mushrooms have been consumed by the oriental world for a long history for their flavor, nutrition, and biological functions to satisfy the mouth feel and human body immune systems. *Agaricus blazei* Murill has been considered antitumor and anticarcinogenic, and extensive research has been carried out to explain its polysaccharide structures and their relationship with antitumor activity (Kobayashi et al. 2005; Mizuno et al. 1990).

It has been reported that polysaccharide glucan protein complex, presenting α- and β-glycosidic linkage with β-glucans being predominant over α-glucans, antitumor activity (Gonzaga et al. 2005). A lot of mushrooms have been studied for their antitumor activities (Yoshioka et al. 1985), and their extractions are used clinically as a drug for therapy (Liu et al. 1996; Ohno et al. 1986). *Ganoderma* is a therapeutic fungal bio-factory (Russell and Paterson 2006), which can inhibit tumor cell proliferation and induce their death (Xie et al. 2006). The methanolic extract of *Ganoderma lucidum* exhibited antimutagenic activity and hepatic damage effect, which is caused by benzopyrene (Lakshmi et al. 2006).

Majority of the *Basidiomycetes* mushrooms contain biological active protein-bounded polysaccharides or polysaccharides in fruit bodies, cultured mycelium, or culture stock, which exert antitumor and immunomodulating activity (Wasser 2002). A fungal polysaccharide "Huaier" obtained from *Trametes robiniophila* Murr, a sandy beige (light-grayish-brown) mushroom grown on the trunks of trees such as *Sophora japonica*, belonging to *Hymenomycetes-Basidiomycota* (Anithworth et al. 1973), has been applied in traditional Chinese medicine (TCM) for approximately 1,600 years (Li et al. 2006), first recorded by Shi-Zhen Li, who was a famous Chinese scientist in Ming dynasty. In recent decades, Huaier has been found and used as a complementary agent for cancer therapy. The water extract from Huaier is mainly a polysaccharide protein which has been proved to be the main active ingredient in respect of anticancer effects and immunity-enhancing actions (Cui et al. 2007). As an anticarcinogenic medicine, *T. robiniophila* has been used for the treatment of liver cancer with satisfactory results. Accumulated evidences suggest that the antitumor mechanism of Huaier may be associated with the inhibition of the growing of endothelial cells, interference with tumor angiogenesis (Xu et al. 2003), system immune activation (Chen et al. 2004; Jia et al. 2009), induction of tumor cell apoptosis, and suppression of tumor cell proliferation (Zhu et al. 2008). Irrespective of its efficient treatment for liver cancer, so far, there is no information available about the anticancer effects of Huaier on

cholangiocarcinoma, and its underlying mechanism of action is still largely unknown. As we know, cholangiocarcinoma is a malignant tumor arising from the bile duct epithelial cells or cholangiocytes of the intrahepatic and extrahepatic biliary system (de Groen et al. 1999; Shaib and El-Serag 2004), characterized by a poor prognosis, high recurrence rate, and resistance to radiotherapy or chemotherapy due to its special biological characteristics. More recently, some species of edible higher *Basidiomycetes* have been found to markedly inhibit the growth of different tumor cell lines. There are approximately 200 species of higher *Basidiomycetes* that were found to have this activity (Poucheret et al. 2006).

The cholangiocarcinoma is considered to be a multidrug and radioresistant tumor and has a high mortality ratio (Sirica 2005). The increasing global incidence of this tumor requires the urgent need for novel and effective therapeutic agents. Recently, much attention has been focused on medicinal plants to discover novel anticancer agents that lack the toxic effects associated with current therapeutic agents (Schwartsmann et al. 2002). However, since the last century, the Second Affiliated Hospital of Harbin Medical University had succeeded in administering Huaier extract to patients who suffered from cholangiocarcinoma to prolong their survival or reduce the recurrence rate. For the development of hospital pharmaceutical preparation to be an open access drug, it is intended to make a series of experiment-based research for identifying its efficiency on cholangiocarcinoma. From that observed study, they extracted a water-soluble polysaccharide from that fungus and preliminarily test its immunomodulatory and tumor-inhibitory activities in vitro.

3.3 Biological Activities

Polysaccharides and their conjugates, which are used in food industry and medicine for a long time, have attracted much attention in recent years because of various biological activities like antitumor and immunomodulating (Shao et al. 2004), antioxidant (Xu et al. 2009), anticoagulant, and antiviral activities (Pushpamali et al. 2008; Athukorala et al. 2006). A lot of mushrooms have been studied for their biological activities (Yoshioka et al. 1985). Extractions of these mushrooms have been used clinically as therapeutic drugs (Liu et al. 1996; Ohno et al. 1986). Modern scientific studies on the above called "medicinal mushrooms" have expanded exponentially during the last two decades, and scientific explanations to show how compounds derived from mushrooms function in humans are increasingly being established (Zaidman et al. 2005). Fungal fruiting bodies, fungal mycelium, and the culture fluid in which the mycelium has been cultivated may all be explored for biological activity. Polysaccharides are the most potent macro-fungi-derived substances from lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*) (Chihara et al. 1970), and yiner (*Tremella fuciformis*). However, the polysaccharides from *Irpex lacteus* have been seldom reported.

Many active compounds have been identified in *Polygonatum odoratum* such as mucous polysaccharides and homoisoflavanones (Tomshich et al. 1997),

azetidine-2-carboxylic acid (Baek et al. 2012; Kim et al. 2006), saponin (Deng et al. 2012), and steroidal compounds (Sugiyama et al. 1984). Among them, polysaccharides are abundant in *Polygonatum odoratum* and considered as one of the most important bioactive components with antitumor, antidiabetic, and antioxidant functions. Extraction of polysaccharides from *Polygonatum odoratum* is an important approach to further research or scale application. Tomshich et al. (1997) reported extraction and purification of polysaccharides from ten medicinal plants including *Polygonatum odoratum*, and several reports were documented on extraction and bioactivities of polysaccharides from this species in recent years (Baek et al. 2012).

3.4 Antimicrobial Effects

Several fungal biopolymers are known to be active against bacterial and viral infections in vitro or in vivo, due to the stimulation of phagocytosis of microbes by neutrophils and macrophages. For instance, lentinan is active against tuberculosis and *Listeria monocytogenes* infection, as well as *Salmonella enteritidis* and *Staphylococcus aureus* infection (Sasidhara and Thirunalasundari 2012; Mattila et al. 2000). Interestingly, lentinan and an acidic proteoglycan from *G. lucidum*, as well as glucans from *G. frondosa* and *T. versicolor*, have been used as anti-HIV drugs, provoking an increased host resistance to HIV virus and limiting the toxicity of conventional anti-HIV drugs, a common drug used against AIDS virus (Markova et al. 2002; Lindequist et al. 2005). Different studies suggest that the antioxidant and anticoagulant abilities of polysaccharides are strongly dependent on the degree of sulfation, molecular weight, position of sulfate groups on the sugar backbone, sugar composition, and glycosidic branching (Rioux et al. 2007; Wang et al. 2010; Jiao et al. 2011; Jimenez-Escrig et al. 2012).

Research on antimicrobial activity of polysaccharides may contribute to valuable new information for future antibiotic development. Many authors have previously investigated the antimicrobial activity of polysaccharides from a wide range of organisms such as seaweeds, mushroom, and durian fruit (Lipipun et al. 2002; Zhu et al. 2012).

The results discussed above imply new concepts about the structure and function of fungal polysaccharides. These studies raised an important question: Do polysaccharide samples of identical composition but variable size and degree of polymerization present distinct biological functions? The findings discussed above imply new concepts about the structure and function of fungal polysaccharides. Besides structural aspects traditionally studied, such as sequence analysis, compositional determination, anomeric configuration, type of glycosidic linkage type, and presence of substituent, it becomes clear that other structural parameters, including molecular diameter and degree of polymerization, must be considered for functional studies. In fact, the conclusion that polysaccharide functions are influenced by these additional parameters was further supported by Cordero et al. (2011).

Using static and dynamic light scattering, viscosity analysis, and high-resolution microscopy of *C. neoformans* polysaccharides, this study demonstrated that spatial conformation (branching) influences phagocytosis, nitric oxide production by macrophage-like cells, and susceptibility to reactive oxygen species, serology, and clearance during infection. These previously unexplored parameters can generate new insights, for example, on immunogenicity assays of polysaccharides, as well as their use in therapy or prevention of diseases. Studies in this area are still embryonic, and unclearly, much remains to be discovered. For conceptual validation, future studies must include evaluation of the relationship between immune activity and molecular dimensions of polysaccharides synthesized by other organisms, as well as of fungus.

4 Conclusion

This review paper explores the functional properties of different fungi, as well as outlines the potential uses as active ingredients in medicinal purposes. In addition, here we discussed the bioactivities and biological activities and properties of fungal polysaccharides. The potent biological activities of different fungal polysaccharides may represent an interesting advance in the search for functional applications in the relevant medicinal uses including cure of different diseases such as cancer, HIV, and AIDS. Edible mushrooms have been consumed by the oriental world for a long history for their flavor, nutrition, and biological functions to satisfy the human body defense systems. There are different types of fungal polysaccharides which are very beneficial for our health in the form of oral uses such as mushrooms and also in the form of medicinal uses which is described in the chapter. Several studies have shown that in microorganisms, those polysaccharides play crucial roles in the development of the cell envelope (Roberts 1996; Nimrichter et al. 2005). It has been reported that many fungi used for medicinal purposes which has been discussed in the above chapter should need more analysis for further research.

For example, *Agaricus blazei* Murill has been considered as antitumor and anticarcinogenic, and extensive research has been carried out to elucidate their polysaccharide structure and its relationship to the antitumor activity (Kobayashi et al. 2005; Mizuno et al. 1990). Capsular polysaccharides were among the earliest microbial virulence determinants, which are reviewed in the classic Griffith's experiment (Smith 1990). As an anticarcinogen medicine, Huaier has been used for the treatment of liver cancer with satisfactory results. In recent decades, Huaier has been found and used as a complementary agent for cancer therapy. Another example is *Ganoderma* which is a therapeutic fungal bio-factory (Russell and Paterson 2006). *Agaricus blazei* Murill has been considered to have antitumor and anticarcinogenic activities. Interestingly, lentinan and an acidic proteoglycan from *G. lucidum*, as well as glucans from *G. frondosa* and *T. versicolor*, have been used as anti-HIV drugs, provoking an increased host resistance to HIV virus and limiting the toxicity of conventional anti-HIV drugs, a common drug used against

AIDS virus (Markova et al. 2002; Lindequist et al. 2005). Further research on the structural bioactivities is suggested to be needed.

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Biopolysaccharides for Skin Hydrating Cosmetics

60

Mayuree Kanlayavattanakul and Nattaya Lourith

Contents

Abstract

Skin hydrating polysaccharides are derived from several natural sources. This biopolymer is constructed with simple sugar building blocks that are easily hydrated in an aqueous environment, thereby creating the gel structure called hydrogel or hydrocolloid. Polysaccharide that is a biodegradable polymer is excellent in compatibility with the biological tissues and largely meeting the consumers' preferences toward natural products. In this chapter, applications of biopolysaccharides in cosmetics are subjected to be summarized especially skin hydrating biopolysaccharide that is incorporated as the active ingredient in cosmetic product. Mechanisms of skin hydrating effect and *in vivo* instrumental evaluation are addressed. Commercial biopolysaccharides are summarized for further reference in addition to some potential candidates, particularly botanical polysaccharides. The potential biopolysaccharides, with *in vitro* activities, are additionally included. Health benefits of this biopolymer in sufficiently suppressing dryness of the skin and potentially protecting from and/or treating wrinkles of the skin acting as antiaging ingredients are highlighted.

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1 Introduction

Polysaccharides are composed of multiple saccharides forming a large branched or unbranched chain. They are simply classified as homopolysaccharide (same monosaccharide unit) and heteropolysaccharide (different monomeric units). Thus, they are accounted as polymers. This naturally derived polymers constructed with simple sugar building blocks are hydrated in an aqueous environment, thereby creating a gel structure called hydrogel or hydrocolloid. This system, in which water is immobilized by insoluble polymers, is imparting moisturizing effect consequently. The moist gels are highly compatible with the biological tissues and are biodegradable according to their natural occurrence (Brode 1991), classifying them as biopolymer. These are inexpensive and vastly available from natural sources which enlighten their importance in health benefit applications, including cosmetics. These strengthen the consumers' preferences toward the safety and efficacy of biopolysaccharides. In this chapter, application of polysaccharides in cosmetics will be classified into functional and active polysaccharides. Functional polysaccharides in cosmetics are claimed on the basis of their functionalities in the formulation technology such as film former, gelling agent, thickener, suspending agent, conditioner, and emulsifier, of which it mainly relies on the physicochemical properties of the biopolymer. On the other hand, cosmetic active polysaccharides are role by the ability of hydrogel or hydrocolloid mobilizing water to the contacted skin (Goddard and Gruber 1999). The skin hydrating benefit is the main object to be exclusively discussed herein. Mechanisms of this skin hydrating agent are included with *in vivo* efficacy evaluation. Furthermore, biopolysaccharides with skin hydrating effect, including those of potential ones with in vitro activities, are also included, of which botanical polysaccharides are mainly the focus in this chapter. However, preparation, characterization and physicochemical analysis of polysaccharide, and modification will be out of the scope of this chapter. Antioxidant and antibacterial activities of biopolysaccharides will also not be included, as well as their application as delivery systems.

2 Classification of Polysaccharide in Cosmetics

Polysaccharides applied in cosmetics are classified on the basis of their actions in the products that are functional and active polysaccharides.

2.1 Functional Polysaccharide

Polysaccharides are incorporated into cosmetic product to function as gelling agent, viscosity adjuster, thickener, and emulsifier as well according to its polymerized network holding water by means of its swelling capability. Therefore, functional polysaccharide is traditionally classified on the basis of its electrochemical charges in the structure as follows.

2.1.1 Anionic Polysaccharide

Cosmetically interesting anionic polysaccharide is predominantly comprised of a group of naturally occurring materials. A majority of this group is xanthan gum with a β-(1–4)-D-glucopyranose glucan skeleton. It is naturally synthesized and composed in the cell wall of bacteria and is isolated by bacterial fermentation. In water, xanthan polymerizes forming viscous liquids which crystallized giving the unique ability to emulsify the suspensions. This pseudoplastic fluid, with a shear-thinning property, is commonly used in cosmetic preparation to enhance the stability against freeze-thaw challenge.

2.1.2 Cationic Polysaccharide

This cosmetic important group of polysaccharide mainly relied on synthetically altered polyglycans. They have unique advantage to bind tightly with protein (negative charge) of the human skin and hair. Cationic polysaccharide therefore has been found to be very useful as film-forming agent popularly applied for damage controls in hair and skin-conditioning products in addition to the vast application in hair fixative preparations. Among natural-derived cationic polysaccharides, chitosan is the main commercialized polyglycan with a cationic nature. This seminatural polysaccharide is partially deacetylated (>50 %) of naturally occurring chitin to improve solubility.

2.1.3 Nonionic Polysaccharide

This polysaccharide is not charged and thus less affected by negatively or positively charged compounds as surfactants. Starch as one of the most used and least expensive natural nonionic polysaccharides is mainly used as thickener. Guar gum is another nonionic polysaccharide which has found broader appeal as a natural thickener isolated from seed of plants in the Leguminosae family. Guar gum consists of two different sugars, which are mannose and galactose. Its chelating effect with metal ions enables gel thickening in alkaline media. Seminatural nonionic polysaccharides are mainly ethers of cellulose- or guar-based materials, for example, hydroxycellulose, methylcellulose, etc., that are widely used as thickeners and film formers in nail products.

2.1.4 Amphoteric Polysaccharide

Positive and negative charges are present in the same molecule naming the group of this biopolymer to amphoteric polysaccharides. There are few naturally

occurring amphoteric polysaccharides used in cosmetics. The most frequently applied are seminatural derivates which is carboxymethyl chitosan.

2.2 Active Polysaccharide

In addition to the above functioning polysaccharides, application of these biopolymers acting as active ingredient is widely adopted in cosmetics. Polysaccharides are pharmaceutically and cosmetically classified on the basis of skin hydrating effect as follows.

2.2.1 Cellulose

Cellulose is the most abundant renewable polymer composed of repeating units of monosaccharide that is known as cellobiose. This anionic water-insoluble polymer is extracted from natural source, mainly plants with hemicelluloses, lignin, and other extractives. It is a hygroscopic material that is implied in skin-conditioning products particularly skin hydrating cosmetics.

Although plant is the major producer of cellulose, microorganisms such as algae, bacteria, and fungi are of economically important sources as manipulation of the cellulose structure with different properties and functions can be controlled by means of the biosynthetic modification especially in skin tissue-repairing products (Fu et al. 2013). Cellulose ethers are the modified cellulose and enlarge cellulosic application in cosmetics with optimal physicochemical properties available for a wider choice of dosage forms particularly those of methyl and ethyl cellulose derivatives (Chen et al. 2012; Vandamme et al. 2002).

2.2.2 Starch

Starch is a glucose polymer biosynthesized in plants for energy preserving aspect. It is the modified flour with a primary constituent of carbohydrates that differentiated by the proportion of amylose and amylopectin varied by source in similar to size, structure, and pharmaceutical property. Commercial highly pure starch is mainly isolated from cereal (wheat, rice, corn, oat, sorghum, and barley), legume (lentil, bean, and pea), and tuber of potato and tapioca. Similar to cellulose, modified starches are widely used in cosmetics, for example, hydrolyzed corn starch, hydrolyzed wheat starch, hydroxypropyl corn starch, sodium carboxymethyl starch, etc. In addition, those of novel application forms of starch in microcapsules, nanoparticles, and composites are recently emerging in the cosmetic industry (Rodrigues and Emeje 2012).

2.2.3 Pectin

Pectin is a non-starch water-soluble linear polysaccharide found in higher plants, particularly in fruits and vegetables. Commercialized pectin mostly relied on food industry by-products, e.g., apple pulp and citrus peel. Its structure governs property with the appointed various applications depending on its source.

Table 1 Gum in skin nourishing cosmetics

Gum	CAS number	Mannose-galactose
Guar hydroxypropyltrimonium chloride	65497-29-2	2:1
C18-22 hydroxyalkyl hydroxypropyl guar		
Locust bean hydroxypropyltrimonium chloride		4:1
C. spinosa gum	39300-88-4	3:1
C. spinosa hydroxypropyltrimonium chloride	742071-24-5	
T. foenum-graecum hydrolyzed trimonium chloride	742071-24-6	1:1
Cassia gum		5:1

2.2.4 Gum

This plant hydrocolloid can be either anionic or nonionic polysaccharides including salt polysaccharide. It is biosynthesized as plant protector, following an injury or attack. This class of polysaccharide has long been served in pharmaceutical applications which are gum arabic or acacia gum from *Acacia* sp., gum tragacanth from *Astragalus gummifer*, gum karaya from *Sterculia urens*, and gum ghatti from *Anogeissus latifolia*, of which gum acacia and tragacanth were reported for their emollient property (Raymond et al. 2003) contributing to their hydrating effect.

In addition to the above gum, those of legume polysaccharides which are *Cyamopsis tetragonoloba* or guar gum, hydroxypropyl guar, C18–22 hydroxyalkyl hydroxypropyl guar, guar hydroxypropyltrimonium chloride, hydroxypropyl guar hydroxypropyltrimonium chloride, carboxymethyl hydroxypropyl guar, hydrolyzed guar, *Ceratonia siliqua* gum, locust bean hydroxypropyltrimonium chloride, hydrolyzed *C. siliqua* gum extract, *Caesalpinia spinosa* gum, *C. spinosa* hydroxypropyltrimonium chloride, hydrolyzed *C. spinosa* gum, *Trigonella foenum-graecum*-hydrolyzed trimonium chloride, cassia gum, and cassia hydroxypropyl trimonium chloride are used and commonly called galactomannans. Those of guar, carob, tara, fenugreek, and cassia gums with skin-conditioning effects are summarized in Table 1.

2.2.5 Mucilage

This viscous polysaccharide is extractable from Leguminosae plant such as okra, psyllium, and flax. Mucilage is traditionally applied in topical products as functional ingredient. This water-soluble polysaccharide, similar to gum, is widely used in cosmetics due to its high water absorption capacity.

2.2.6 Seaweed Polysaccharide

Alginates are one of the most well-known marine polysaccharides prepared from brown seaweeds in the Phaeophyceae family, out of which *Macrocystis pyrifera* is a major commercialized seaweed for alginate production. In addition, *Laminaria hyperborea*, *L. digitata*, and *L. japonica* are becoming important sources of good quality alginates.

Marine red algae (Rhodophyceae family) largely produces carrageenans. Carrageenan is a hydrocolloid that can be classified into κ-, ι-, and λ-carrageenans.

Red-purple algae of the Rhodophyceae additionally synthesizes agar of which *Gracilaria* and *Gelidium* spp. are the major grown genera for agar production.

2.2.7 Microbial Polysaccharide

This class is interchangeably known as exopolysaccharide. Xanthan gum produced by *Xanthomonas campestris* is highlighted as the main significant bacterial exopolysaccharide accounted for more than 6 % of the total market value of these categorized polysaccharides (Imeson 1997). Pullulan, a water-soluble extracellular polysaccharide, from *Aureobasidium* especially *A. pullulans* and gellan from *Auromonas elodea* are additional microbial polysaccharides that are also widely used in cosmetics. The principal commercialized pullulan is produced by Hayashibara, the Japanese company, that deals in this polysaccharide preparation since 1976. In addition, dextran, glucan, and fructan are microbial producible and largely used in cosmetics. Those of skin hydrating microbial polysaccharides that act as humectants used in cosmetics are included in Table 2 with the incorporated content as exemplified in Table 3.

Table 2 Microbial polysaccharides in moisturizing cosmetics

Polysaccharide	Strain	CAS number
Xanthan gum	*Xanthomonas campestris*	11138-66-2
Xanthan gum cross polymer		
Xanthan gum hydroxypropyl trimonium chloride		
Sclerotium gum	*Sclerotium rolfsii*, *S. glucanicum*	39464-87-4
Biosaccharide gum-1		223266-93-1
Biosaccharide gum-2		758716-52-8
Biosaccharide gum-3		896736-76-8
Biosaccharide gum-4		283602-75-5
Biosaccharide gum-5		
Pseudoalteromonas exopolysaccharides	*Pseudoalteromonas*	
Dextran sulfate	*Leuconostoc mesenteroides*	9042-14-2
Beta-glucan	*Aureobasidium pullulans*, *Agrobacterium biobar*, *A. radiobacter*	55965-23-6, 53238-80-5
Beta-glucan hydroxypropyltrimonium chloride		
Beta-glucan palmitate		
Hydrolyzed beta-glucan		
Oxidized beta-glucan		
Alcaligenes polysaccharides	*Alcaligenes latus*	188846-47-1

Table 3 Microbial polysaccharide content in cosmetics

Microbial polysaccharides	Concentration (%) in cosmetics		
	Leave-on	Dermal contact	Baby products
Xanthan gum	0.001–6	0.001–6	0.2–0.6
Xanthan gum cross polymer	0.03–5	NR	
Biosaccharide gum-1	0.002–6	0.002–6	NR
Biosaccharide gum-2	1	1	
Biosaccharide gum-4	0.004–5	0.00001–5	
Dextran sulfate	0.01–0.1	0.01–0.1	
Sclerotium gum	0.003–2	0.003–2	
Hydrolyzed sclerotium gum	1	1	
Beta-glucan	0.0002–0.1	0.0002–0.1	
Alcaligenes polysaccharides	0.3	0.005–0.3	

NR no reported uses

2.2.8 Animal Polysaccharide

Chitin is the second most abundant biopolymer after cellulose with the similar structure formed in the exoskeleton of arthropods or in the cell walls of fungi and yeast including other lower plants and animals for reinforce and strength proposes. Chitosan is a chitin derivative which is vastly applied in pharmaceutics and cosmetics. Partial deacetylation of chitin in basidic condition or by enzymatic hydrolysis conducted by a chitin deacetylase yielded the modified compound chitosan. Chitin is not only used for chitosan production but also a raw material for glucosamine and oligosaccharide manufacturing (Sandford 2003). Significant water evaporation reduction of the skin treated with high molecular weight chitosan (10^4 to 10^6 Da) was reported (Dee et al. 2007). This skin hydrating effect is regulated by occlusion of chitosan on skin layer. Furthermore, chitin is claimed to reduce a risk of scar formation (McCarthy 1996) with an ability to enhance synthesis of skin matrix acting as the mucopolysaccharide templates as examined in mouse (Bakkers et al. 1997; Varki 1996).

In addition to chitin and chitosan, mucopolysaccharide is produced from the vertebrate particularly in mammalian. Mucopolysaccharides are the term designated to hexosamine-containing polysaccharides present in animal tissue in free sugar polymeric form or that bind with proteins (Meyer 1938). These heteropolysaccharides are different from cellulose, starch, and chitin that are based on a single monosaccharide by their structural repeating units of disaccharide. Hyaluronic acid or hyaluronan (HA) is a natural polysaccharide found in the intercellular matrix of most vertebrate connective tissues including the skin. Together with other glycosaminoglycans (GAGs) that are dermatan sulfate, chondroitin sulfate, and keratin sulfate are represented as the prominent fluid in the skin responsible for skin elasticity. HA is found in the dermis at a greater amount than the epidermis (≈0.5 and 0.1 mg/g wet tissue) (Koshiishi et al. 1999). This polysaccharide therefore mainly regulates elasticity of the skin. Commercialized HA isolated from the

synovial fluid, umbilical cord, skin, and rooster comb of animal source is available with various molecular weights, the highest being 5,000 kDa (Milas et al. 2001). This biodegradable viscoelastic polymer has several health benefits according to its physiological roles leading to versatile applications including cosmetics (Manuskiatti and Maibach 1996).

However, recent advance in biotechnology enables production of specific polysaccharides from the certain strain of microorganism that is more economically feasible with a shorter time of production of these biopolymers and within a reduced space. In addition, nanotechnology is highly emerging in cosmetics recently including skin hydrating products that nanovesicles and cubosomes showed superior efficacy over the casual cosmetic formulation (Esposito et al. 2007) including the liquid crystal moisturizer (Tsai et al. 2010). In addition, penetration enhancement would be greater than topical application of the classical product with massage that was misunderstood to improve the efficacy of the skin hydrating formulation (Hamed et al. 2012).

3 Skin Hydrating or Moisturizing Effect

Dryness of the skin aesthetically draws back the individual's confidence with adverse experiences as follows:

1. Sensory characteristics: feels dry, uncomfortable, painful, and itchy, stings, and tingles
2. Visible characteristics: redness, a lackluster surface, dry white patches, flaky appearance, cracks, and even fissures
3. Tactile characteristics: rough and uneven

Dryness of the skin further accumulates into a reduction of elasticity and wrinkles of the skin consequently as evidenced by biomechanical properties of the skin (Choi et al. 2013a). Therefore, application of skin hydrating cosmetics is not only to hydrate the skin, but their pleiotropic skin benefits ultimately enhance aesthetic preference of the skin (Rawlings et al. 2012). That is recently known as corneotherapy (Klingman 2011).

Moisturizers can immediately prevent excessive water loss from the skin, principally via their occlusive elements. The stratum corneum's barrier function prevents entry of foreign substances and the loss of internal substances, including water. The water content of the stratum corneum (SC) should be greater than 10 % in the skin having a normal appearance and not feeling rough, scaly, or dry. Ideally, SC should have 20–35 % water content. Moisturizers serve to return water content to the skin with the humectants and occlusive ingredients preventing transepidermal water loss (TEWL) (Flynn et al. 2001). Skin hydration is regulated by the SC components in particular corneocytes and natural moisturizing factor (NMF) and intercellular lipid bilayer matrix. NMF is a combination of several compounds created in the skin and comprising approximately 20–25 % of the keratinous layer

in which it retained the water content (Shai et al. 2009). A defective skin barrier function causes inflammation of the skin in addition to impairment of filaggrin initiating atopic dermatitis resulting in skin dryness. Filaggrin forms NMF in SC and is essential during formation of the cornified enveloped corneocytes (Rawlings and Harding 2004) that links between the adjacent corneocytes by corneodesmosomes. Filaggrin deficiency, therefore, abnormalizes the skin barrier in turn and severely enhances skin dryness causing atopic dermatitis accordingly (Wolf and Wolf 2012). In addition to protease degrading filaggrin, corneodesmosome degradation by proteolytic enzymes also enhances skin dryness causing desquamation that is visually noticed as skin flakes (Harding et al. 2000). Protection from excessive water and electrolyte loss from the skin is regulated by SC intercellular lipid. The occlusive oily layer therefore effectively suppresses TEWL by hydrogen bonding with water at the polar head lipids that are in orthorhombic (solid) and hexagonal (gel) packing.

Moisturizers can be considered safe in comparison with traditional drug used by dermatologists. However, inconvenient skin reactions from topical preparation may be encountered. Therefore, moisturizers that are usually free from irritating substances are highly in demand (Lodén 2004) particularly natural moisturizer. Thus, this chapter is exclusively devoted for skin hydrating biopolysaccharides.

3.1 Mechanism of Moisturizer

Moisturizing product is formulated and expected to suppress TEWL and retain or increase the content of water in SC in order to maintain the skin barrier. Although function mechanism of this active cosmetic category is complicated, simple classification on the basis of the actions would be by:

1. Occlusive effect: TEWL in SC which physically blocks thereafter the percolation of water from the inner viable epidermis is protected. In addition, emollience or lubricance that smoothens the skin by filling up the spaces between skin flakes is enhanced to retain the skin hydration. Occlusive ingredients generally used in personal care products are petrolatum, mineral oil, paraffin, and squalene including vegetable and animal fats.
2. Humectant effect: skin hydration in SC is improved by an attraction of water from viable skin tissues remoisturizing the skin from the inside out. Commonly used humectants in cosmetics are glycerin, propylene glycol, urea, sodium lactate, sorbitol, and pyrrolidone carboxylic acid (PCA).

Skin hydrating cosmetics usually combine the moisturizing effects from occlusants and humectants. Synergistic effects by water retaining in the skin and the prevention of TEWL compulsively sustain the skin barrier, therefore largely contributing to hydrating efficacy. Although skin moisturizing efficacy could be monitored by sensory evaluation, instrumental analysis would accurately reflect the efficacy with the practical validation between different studies.

3.2 Evaluation of Skin Hydrating Efficacy *In Vivo*

Skin barrier is a physical property referring to water content in the skin that can be assessed by several techniques. This chapter will be devoted to those of noninvasive methods that are the agreed standardized methods used in clinical evaluation of skin hydrating or moisturizing products which are TEWL, skin hydration, and skin image. The devices based on spectroscopic and microwave principles are not included.

3.2.1 TEWL

Transepidermal water loss is the outward permeation of condensed water through SC by diffusion, of which perspiration including other forms of water loss is excluded from TEWL. This transcutaneous water loss is generally measured by open and closed chamber methods that are commercially available. The open chamber type relies on Fick's diffusion law indicating the quantity being transported per specific area and time. TEWL is thereafter processed and shown in $g/m^2/h$. Although this method allows continuous measuring of TEWL, opening of the probe to the surrounding atmosphere turbulences the result. The closed chamber devices determining skin water flux density are therefore developed to limit the atmosphere influence, which are the condenser chamber and unventilated chamber. The unventilated closed chamber can occlude the skin and might be improper for continuous measurement as the normal evaporation of the skin is blocked. Therefore, the ventilated ones are implied by using dry or moistened carrier gas for continuous determination of TEWL.

TEWL conferring skin barrier function is routinely tracked by Tewameter® (Courage + Khazaka, Germany), the open chamber type, and unventilated closed chamber type, that is, VapoMeter® (Delfin, Finland) including the condenser closed chamber, AquaFlux® (Biox, UK). In addition, DermaLab® (Cortex, Denmark) and Evaporimeter® (ServoMed, Sweden) sharing the same principle with Tewameter® are also used. In the meantime AS-CT1® (Asahi, Japan) that is an unventilated closed chamber type is additionally used besides VapoMeter®.

In addition to direct measurement of skin water loss rate, SC water content or skin hydration can be determined indirectly by means of electrical properties of the skin.

3.2.2 Skin Hydration

The skin water content in terms of SC hydration reflecting skin barrier is measured on the basis of electrical capacitance, conductance, or impedance. These fundamentals rely on the dielectric medium nature of SC. The instrument is therefore developed on the basis of this electrical concept of the skin. Measurement of skin permeability to alternating electric current (impedance) reflects electromagnetic interaction with skin dipoles and electrolytes (Kajs and Gartstein 1991). A low resistance but high impedance correlates with a greater skin hydration (water content). Skicon® (IBS, Japan) operates at 3.5 MHz with the result expressed in microsiemens (μs) ranging from 0 to 1,999 μs. This conductance instrument consists of the concentric interdigital electrodes liberating a direct galvanic contact between the probe and skin surface. The noninvasive capacitance-based devices are based on the difference

of the dielectric constant of water and other substances brought in the electrical measurement field using low operating frequency (up to 1 MHz) (Darlenski et al. 2009). Examples of the capacitance principal are Corneometer® (Courage + Khazaka) and Nova® dermal phase meter or DPM (Nova, USA). The measurements are shown in arbitrary unit (AU). Corneometer® contains an interdigital grid of gold electrodes covered with a low dielectric vitrified material. The frequency shift of the oscillating system is detected referring to skin capacitance that is in contact with the probe ranging from 0 to 120 AU. Nova® DPM has two concentric brass ring electrodes separated by an isolator with a distance between the inner and outer probes of 1 mm and readout measurement ranging from 90 to 999 AU. In addition, Moist Sense® (Moritex, Japan) is also used to assess the relative skin moisture value with 0 to 99 AU. Furthermore, other devices measuring skin conductance, ASA-M2® (Asahi) in addition to Skicon®, are also used. In the meantime, Moisture Meter SC® (Delfin, Finland) exterminating SC hydration by means of skin capacitance and DermaLab Moisture Unit® (Cortex) sharing the same principle with Nova® DPM are also used to verify skin hydrating efficacy of topical products.

Skin hydration measurement is therefore assessable by capacitance- and conductance-based device regarding to their correlation. In addition, there are validations of different instruments that are developed by different manufacturers. For instance, Corneometer® and Skicon® are strongly correlated as examined *in vivo* ($r = 0.97$). However, conductance method is influenced by electrolytes with a lack of sensitivity at low hydration. In contrary, the sensitivity of the capacitance instrument is limited at high hydration values (Clarys et al. 2012).

Capacitance imaging (CI) of the skin surface is developed on the basis of silicon image sensor (SIS) technology that is previously developed for security reasons by fingerprint recoding. The resulting capacitance map is interpreted in terms of skin hydration/dryness. This skin recoding called SkinChip® (L' Oréal, France) composed of 92,160 microcapacitors on a 1.8×1.28 cm plate plugged directly to the USB port is recently proposed to determine skin hydration *in vivo* (Lévêque et al. 2006).

3.2.3 Skin Image

In addition to the above noninvasive techniques that determine skin condition relevant to cutaneous moisture, skin flakes resulting from desquamation are collected and analyzed by D-Squame® (CuDerm, USA). It is the test kit that validated with the above instruments' applicability for clinical efficacy evaluation of skin moisturizing products. Skin dryness resulting from desquamation is visually scaled following the application of the D-Squame® tape onto the skin with a moderate pressure using the thumb or fingertips. Scaliness of the skin is divided into a 1–5 score referring normal to very dry skin. The desquamated corneocytes collected by the stripping tape can be additionally analyzed using an image analyzer (Black et al. 2006; Gasser et al. 2004). Furthermore, the skin image that is directly taken from the volunteer's skin by Visioscan® (Courage + Khazaka) on SESc recording parameter alternatively used scaliness to assess skin hydrating efficacy of the cosmetic product. In addition, skin replica is also analyzed upon scaliness of the skin by Visiometer® (Courage + Khazaka).

3.2.4 *In Vivo* Assessment of TEWL, Skin Hydration, and Skin Image

Although there might be some differences resulting from the different instruments used, standard guidelines by EEMCO (European Group for Efficacy Measurements on Cosmetics and Other Topical Products) recommendation are adopted worldwide that make the results able to be validated in different analytical conditions. In addition, the measuring instruments should be calibrated routinely on the basis of the manufacturer guideline. Furthermore, TEWL is related with SC hydration. These determination concepts are therefore used interchangeably. However, clinical evaluation strictly relies on the same practice.

Prior to starting the measurement, the volunteer or the participant must be acclimatized in the temperature- and humidity-controlled environment to suppress turbulent effect from sweating that alters water evaporation rate of the skin. This volunteer preparation will take 15–30 min at 20–22 °C and relative humidity of 40–60 %. The measurement will be taken in the same climatized room without external air convection turbulence (du Plessis et al. 2013).

Clinical evaluation of skin hydrating efficacy is equivalently measured on the face or volar forearm. Therefore, the volar forearm is generally chosen in skin biomechanical property assessment with a minimal discomfort to the volunteer (Bazin and Fanchon 2006).

3.3 *In Vitro* Hydration Property

Hydration property of polysaccharide is determined by means of water absorption, water-holding capacity, and swelling properties. These parameters confer skin hydrating potential of the polysaccharides that should be conducted prior to clinical evaluation *in vivo* in terms of TEWL and/or SC water content.

Absorption of water refers to the structural network of polymer specifically the volume. Baumann apparatus is generally used to measure water absorption, that is, the kinetics of water uptake. However, measurement of water absorption capacity is routinely calculated as the percentage of weight increases of the sample dried over P_2O_5 *in vacuo* for 24 h by

$$\text{Water absorption capacity } (\%) = 100 \times \frac{(Wn - Wo)}{Wo}$$

where Wn and Wo are the weights of the sample before and after putting the saturated $(NH_4)_2SO_4$ desiccator (81 % RH) and the saturated K_2CO_3 desiccator (43 % RH) at 20 °C after 48 h of the test.

Water-holding capacity is the amount of water that is retained by the polymer at the specific weight commonly 1 g and determined under a specific temperature, humidity, time, and speed of centrifugation. This parameter sometimes is known as water-retaining capacity. However, centrifugation measurement is generally higher than the determination by Baumann apparatus. The standardized protocol determination is studied by hydrating a known polysaccharide weight in a centrifuge tube for 18 h

before pelleting the insoluble residue by centrifugation (3,000 g; 20 min). Following removal of the excess supernatant and/or soluble material, the hydrated polysaccharide residue is weighted prior to repeating the residue weight in the dry form. Consequently, the water retention capacity is calculated using the following equation:

$$\text{Water retention capacity}(g/g) = \frac{\text{Residue fresh weight} - \text{Residue dry weight}}{\text{Residue dry weight}}$$

In addition, the moisture-retention ability of polysaccharide can be calculated as the percentage of residual water of wet sample prepared by adding 10 % water to the sample dried over P_2O_5 *in vacuo* for 24 h by using the equation

$$\text{Water retention capacity}(\%) = 100 \times \frac{Wn}{Wo}$$

where Wn and Wo are the weights of the sample before and after putting the saturated K_2CO_3 desiccator (43 % RH) and the silica gel at 20 °C after 48 h of the test.

Swelling property is assessed by the bed volume technique by swelling the polysaccharides in water overnight using a volumetric cylinder. Generally a known weight of polymer (100–200 mg) hydrated with water in a graduated cylinder (10 ml) is dispersed with gentle stirring, covered, and left for saturation for 18 h at room temperature. Thereafter, the settled volume occupied by polysaccharide is recorded and swelling is calculated by the equation as shown below:

$$\text{Swelling} = \frac{\text{Volume occupied}}{\text{Polysaccharide dry weight}}$$

In addition to the above physicochemical properties that indirectly confer skin hydration potential of the polysaccharides, total polysaccharide content, the sugar constituents, molecular weight, porosity, and particle size also contribute to the moisturizing property.

4 Skin Hydrating Effect of Polysaccharides

Regarding the above classification of moisturizing polysaccharides, those of commercial available skin moisturizing polysaccharides are summarized as shown in Table 4 with the recommended concentrations and useable preparation as available. CAS (Chemical Abstracts Service) number and INCI (International Nomenclature of Cosmetic Ingredients) name are included for further reference.

In addition to the currently used commercialized moisturizing polysaccharides, candidate biopolysaccharide from different sources potential for skin hydrating cosmetics is also included. Those with *in vitro* (water-retaining and water absorption capacities and total polysaccharide content) and *in vivo* (human volunteers or animal model) results from accredited journal and patents are exclusively summarized and alphabetically listed. However, those of mushroom-derived polysaccharides are excluded from this article.

Table 4 Commercialized moisturizing polysaccharides in cosmetics

Name[a]	Supplier	CAS number	INCI name/ composition	Dose (%)
ABS aloe beta-glucan	Active Concepts		*Aloe barbadensis* Yeast β-glucan	1–5
Actiglow® C	Active Organics		Hydrolyzed GAGs	1–35
Actimoist® Bio		7732-18-5, 9067-332-7	Sodium HA	1–10
Actiphyte® Acacia			Acacia gum	5–10
Actiphyte® Algae		92128-82-0	Algae	
Actiphyte® Aloe vera 10-fold		85507-69-3	*Aloe barbadensis*	
Actiphyte® *Spirulina*			*Spirulina maxima*	
Actisea® 100			Algae	1–10
ROVI Sodium Hyaluronate	Air Products		Sodium HA	
Akomarine® Active Complex	Akott		Algae Carrageenan Algin	
Akomarine® Chlorella			*Chlorella vulgaris*	
Akomarine® Gum Complex			Algae	
Akomarine® Kelp			*Laminaria digitata*	
Akomarine® Sea Lettuce			*Ulva lactuca*	
Carboxymethyl yeast beta-glucan (CMG) C90	Angel Yeast Co., Ltd.	9050-93-5	Sodium carboxymethyl β-glucan	5–10
Yeast polysaccharides M60		68876-77-7	Yeast polysaccharides	0.5- 3
Yeast mannoprotein M60				0.05-0.5
PatchH_2O™ A00297	BASF		Algin Sodium HA Pullulan	1–3
Bio-Beta-Glucan	Bioland	160872-27-5	β-Glucan	
Marin moist		92128-82-0	*Laminaria japonica*	
Fructan™		9013-95-0	Fructan	
Nanomoist				
SC-Glucan		5965-23-6, 160872-27-5	β-Glucan	
Ulmus			*Ulmus davidiana*	
Dermatein® Hyaluronic Acid	BioOrganic Concepts	9067-32-7	Sodium HA	
Dermatein® Power		9050-36-6, 9067-32-7	Sodium HA β-Glucan *Aloe barbadensis*/ Guar	0.1–2/1–5

(*continued*)

Table 4 (continued)

Name[a]	Supplier	CAS number	INCI name/ composition	Dose (%)
Echinacea extract	Carruba	90028-20-9	*Echinacea purpurea*	
Wakame extract			*Undaria pinnatifida*	
Hygroplex™ HHG	Chemisches Laboratorium Dr. Kurt Richter GmbH			2–5
Tinocare® GL[b,c]	Ciba	39464-87-4	Sclerotium gum	
Zenzivo™	Clariant		Chitosan	
Pheohydrane®[c]	Codif		Hydrolyzed algin *Chlorella vulgaris*	0.5–1
Pheofiltra® Undaria HG			*Undaria pinnatifida*	2–5
SMW Hyaluronic acid	Contipro Biotech s.r.o.			0.005–0.06
Cromoist HYA	Croda		Hydrolyzed collagen Hyaluronic acid	
Cromoist HWYA		70084-87-6, 9004-61-9	Hydrolyzed wheat protein HA	
Cromoist CS			Hydrolyzed collagen Sodium chondroitin sulfate	
Phytessence Wakame		56-81-5, 7732-18-5, 223751-81-3	*Undaria pinnatifida*	1–7
Phytaluronate®J	DSM	9000-40-2	*Ceratonia siliqua* gum	1–5
Hyasol BT			Sodium HA	3–5
HA-Sol™	Engelhard-BASF		Sodium HA	
ABIL® Filler CL[b,c]	Evonik Goldschmidt	7732-18-5, 22047-49-0, 105524-32-1, 849230-52-0, 64248-79-9, 64-17-5	Sodium HA cross polymer	0.5–5
HyaCare®			Hydrolyzed HA	0.01–0.2
TEGO® Smooth			Hydrolyzed sclerotium gum	1–10
MG-60	Hayashibara International			
Unicerin C-30	Induchem		Cellulose Hydroxypropyl methylcellulose	
Chitosan	Jeen International Corporation	9012-76-4		

(*continued*)

Table 4 (continued)

Name[a]	Supplier	CAS number	INCI name/ composition	Dose (%)
Glucan β	Kaden Biochemicals GmbH	-		-
Collagen-Hyal	Kelisema		Soluble collagen Sodium HA	
Indinyl® CA LS 8998	Laboratories Serobiologiques		*Cassia angustifolia* polysaccharide	2.5–10
PA Reviviscence® LS 9562			*Tamarindus indica* polysaccharide	3–5
Active Powder® Moist LS 9696			*Cassia angustifolia* polysaccharide Gellan gum	3–7
Active Powder® Volu Lips LS 9773			*Pisum sativum* Algae *Cassia angustifolia* polysaccharide Gellan gum Xanthan gum	4–7
Lipo pearl™	Lipo Technologies	61789-91-1, 8042-47-5, 9000-70-8, 12001-26-2, 13463-67-7	Gelatin Cellulose gum	
Phytohyaluronate	Lonza			1–10
Trimoist	Mibelle AG Biochemistry		Sodium carboxymethyl β-glucan	
Chitosan MM 222	Michel Mercier		Chitosan	0.5–1
Chitanide™ 222	M.M.P.		Chitosan succinamide	10
CD-58	Onlystar Biotechnology	1398-61-4	*N*-succinyl chitosan	
NMF-26		9012-76-4	Algae	
Astaxanthin	Oryza Oil & Fat Chemical Co., Ltd.		*Haematococcus pluvialis* Astaxanthin	
Seamollient®	Philip Rockley	7732-18-5, 68917-51-2, 92128-52-0	Algae	10–50
Fructan	Presperse Incorporated	9013-95-0	Fructan	3
Ulmus Extract			*Ulmus davidiana*	
Phycol® FV		57-55-6, 7732-18-5, 84696-13-9	*Fucus vesiculosus*	
Sea Silk		7732-18-5, 92128-82-0	*Ulva compressa Himanthalia elongata*	3–5
Pheofiltrat® Undaria HG			*Undaria pinnatifida*	2–5
Hydrane® BG			*Gigartina stellata*	3–5
Hydractin®	Rahn		*Carica papaya* Algin	2–8

(*continued*)

Table 4 (continued)

Name[a]	Supplier	CAS number	INCI name/ composition	Dose (%)
Ritachitosan LV	RITA Corporation	9012-76-4	Chitosan	
Hydroil	Shanghai Leasun Chemical		HA	3–6
Sodium hyaluronate	Shandong Freda Biochem Co., Ltd.		HA	0.05-0.5
Chitoglycan	Sinerga	83512-85-0	Carboxymethyl chitosan	
Red Alga Gel		68917-51-1, 9000-07-1	Algae	
Fucogel 1000®	Solabia	–	Biosaccharidegum-1	5
Fucogel®				1–20
Hyaluronic acid BT		–	Sodium HA	–
Bashyal	Soliance		Sodium HA	–
Cristalhyal				–
Soligel		–	Rhizobian gum	

[a]All of the listed polysaccharides are used for baby care, cleansing, body care, facial care
[b]Additionally used in makeup cosmetics
[c]Additionally used in sun care

4.1 Animal Polysaccharides

Hyaluronic acid (HA) is the predominant mucopolysaccharide of the skin acting as the key molecule that regulates skin hydration. It is therefore popularly formulated in moisturizing products at a concentration varying from 0.025 to 0.050 %, serving as skin hydrating agent (Stern and Maibach 2008). In addition to the common application of HA in the forms of dermal fillers (e.g., Hylaform®, Restylane®, and Dermalive®), it is topically applied to maintain SC function restoring skin barrier.

Comparative clinical evaluation of skin hydrating effect of HA (anionic polysaccharide) and ceramide in foam and emulsion cream was conducted by a randomized double-blind split body study in 20 female volunteers. The appearances relevant to skin dryness including moisturizing effect were significantly improved ($p < 0.05$) following 2 weeks as rated by the investigator. The improvement was enhanced at the end of the evaluation for 4 weeks ($p < 0.001$), although that of HA foam was more superior over the ceramide cream (1.5-fold at 2 weeks and 1.3-fold at 4 weeks) (Draelos 2011). However, concentrations of moisturizing agents were not addressed. In addition, formulation using the same dosage form including instrumental evaluation would additionally support the outcome of these moisturizers.

Moisturizers containing ceramide are included. Impruv®, Cetaphil RestoraDerm™, CeraVe™, Triceram®, EpiCeram®, Atopiclair®, and MimyX®

are commonly commercialized over-the-counter (OTC) products. GAGs with the molecular weight of 10^3–10^7 Da at the concentration of 0.05–5 % were patented into skin hydrating cosmetics (Bosco et al. 2013).

In addition to the above animal-derived skin hydrating polysaccharide, chitosan modified from shrimp (*Solemocera prominentitis*) chitin with the degree of substitution of 83.2 % and molecular weight of 2,420 KDa was formulated in the moisturizing mask. Skin water-holding capacity of the mask was examined in seven volunteers in a comparison with the control mask containing methylcellulose at the same concentration (2 %). Skin capacitance (Corneometer®) of the shrimp chitosan was better than that of methylcellulose (Chen and Hen 2000). Those of commercialized glycosaminoglycans (GAGs) including HA for skin hydration are included in Table 4.

4.2 Botanical Polysaccharides

4.2.1 *Abelmoschus esculentus*

Okra is regarded as the important source of polysaccharide according to its high content of the fruit mucilage. Its polysaccharides with physicochemical properties found more applications in cosmetics (Camciuc et al. 1998; Sengkhamparn et al. 2010). Moisturizing alcohol-based hand rub containing okra polysaccharide was formulated. This moisturizing product with 0.105 % polysaccharide maintained skin hydration significantly better than the placebo as instrumentally monitored (Moist Sense®) in 20 volunteers who are health-care workers in the hospital. Skin hydration was retained for 210 min of application. Therefore, it was recommended for application encouraging hand hygiene with antiseptic properties (Kanlayavattanakul et al. 2012).

4.2.2 *Aesculus hippocastanum*

Horse chestnut that is adopted in traditional German medicine is one of the important sources of polysaccharides including oligosaccharides that composed more than 50 % in the herb (Thornfeldt 2005). Thus, it is the candidate botanical source of skin hydrating polymers applicable for cosmetics. However, none of horse chestnut polysaccharide moisturizer is presented yet, including the skin hydrating efficacy. Thus, this botanical polysaccharide is worthy to be formulated and challenged for clinical evaluation.

4.2.3 *Aloe vera*

The leaf enriched with polysaccharides is largely implied in skin preparation and popularly sold over the counter (OTC) for skin nourishing effect. Aloe polysaccharide in topical product (0.1, 0.25, and 0.5 %) significantly increased skin capacitance (Corneometer®), although TEWL (Tewameter®) was insignificantly suppressed as examined in 20 volunteers monitored after 1 and 2 weeks of application (Dal'Belo et al. 2006). Aloe polysaccharide is therefore widely commercialized in several trade names as shown in Table 4.

4.2.4 *Aztec chia*

Chia native to Mexico was a polysaccharide gel isolated from the seed. Low molecular weight chia polysaccharide (150–250 KDa) exhibited moisturizing effect. It was therefore formulated with glucan (700–1,300 KDa) and HA (800–1,500 KDa) claiming as skin hydrating cosmetics at 0.5–3 % (Choi et al. 2010).

4.2.5 *Camellia sinensis*

Tea polysaccharide containing 64.27 % of neutral sugar and 27.95 % of uronic acid was shown to be a good moisturizer as it is able to retain water (*in vitro* test) comparable to glycerol at the same tested concentration. It is therefore highlighted as another important bioactives derived from tea in addition to phenolics that are widely formulated in aesthetic products (Wei et al. 2009). Tea polysaccharide with molecular weight range between 100 and 300 KDa was prepared and formulated (0.1–20 %) into moisturizing cosmetics as patented by the Korean cosmetic firm (Kwon et al. 2010).

4.2.6 *Carica papaya*

Papaya fruit is one of the important skin hydrating polysaccharides. Papaya polysaccharide with a molecular weight of 2,540 KDa having 76.9 % of total sugar and 10.92 % of uronic acid was characterized. Galactose (52 mol ratio) and arabinose (21.6 mol ratio) were found as the main monosaccharides followed by glucose, rhamnose, and xylose (11.0, 10.2, and <0.1 mol ratio). This antioxidant polysaccharide absorbed and retained moisture content (in vitro) comparable to HA and glycerol at the same tested concentration (100 mg) (Zhang et al. 2012). Papaya polysaccharide is commercialized by Rahn in Hydractin® (Table 4).

4.2.7 *Durio zibethinus*

Durian is one of the important tropical fruits that is enriched with polysaccharides in the hull. Durian polysaccharide consists of polygalacturonan branched with galactose, glucose, rhamnose, fructose, and arabinose with molecular weight approximately 500–1,400 Da (Hokputsa et al. 2004). The polysaccharide was therefore formulated into gel (10 %) and assessed on its skin hydrating activity in 18 volunteers for 8 weeks. A randomized single-blind split-face placebo-controlled study was directed to apply 0.139 % polysaccharide gel formulation or 0.3 g of product twice daily (morning and evening). Skin water content (Corneometer®) was significantly increased at the first examination following treatment of 4 weeks ($p = 0.024$) and largely achieved ($p = 0.003$) at the end of the study. The results additionally revealed that the efficacy in female volunteers was more superior over male. In addition, the product imparted excellent enhancement of skin hydration in the subjects who are younger than 30 years old than the older (Futrakul et al. 2010).

4.2.8 *Echinacea purpurea*

The root of the herb has polysaccharides which are isolated and characterized. Polysaccharides (xylan and galactan) with molecular weight approximately 35 and

450 KDa were obtained. This polysaccharide with biological activity was suggested to be incorporated in health benefit preparations (Dalby-Brown et al. 2005). The polysaccharide extract was further commercialized and claimed as skin moisturizer as shown in Table 4 by Carruba.

4.2.9 *Malva sylvestris*

Malva nut is widely applied in dermatological use due to its therapeutic effects relevant to the skin (Pieroni et al. 2004). The herb's potential in skin hydration is governed by its abundant mucilage. Polysaccharides in this herb consisted of glucuronic acid, galacturonic acid, rhamnose, galactose, fructose, glucose, sucrose, and trehalose with the minor constituents of uronic acid, arabinose, mannose, xylose, fucose, raffinose, and xylotriose (Barros et al. 2010; Classen and Blaschek 1998). Therefore, Malva mucilage was formulated into skin hydration products (Cauchard et al. 2010; Choi et al. 2005).

4.2.10 *Myrosma cannifolia*

Guapo, native tuber plant of Venezuela, is examined for its potential starch of cosmetic application. Its amylase and amylopectin ratio is corn resembling with optimal physicochemical properties for cosmetics in addition to safety and compatibility in cosmetic base. Its water absorption capacity is adequate and challenge to be verified on its skin hydrating efficacy (Rincón et al. 2005).

4.2.11 *Orchidaceae* sp.

Orchid, a plant of the genus *Odontoglossum* of the family *Orchidaceae* and/or a plant derived from a hybrid plant of the genus *Odontoglossum* and the genus *Cochlioda*, constituted 60–70 % polysaccharide in which mannose is the main neutral sugar (90 %) prepared. Cosmetics with moisturizing efficacy were formulated and claimed at the extract concentration of 0.1–15 % (Sasaki et al. 2013).

4.2.12 *Piptadenia colubrina*

A native leguminous tree of South American rain forest has isolated skin beneficial polysaccharide with the extractive yield of 0.05–0.25 %. The extracted polysaccharide was further formulated into a gel-cream product (5 %). Clinical evaluation on the basis of skin capacitance was examined by randomized single-blind placebo-controlled study in 15 volunteers for 14 days and tracked by Corneometer®. Skin capacitance was significantly achieved. Cellular skin hydrating effect was evidenced by expression of filaggrin enhanced by the polysaccharide in human skin explants (Pereda et al. 2010).

4.2.13 *Tamarindus indica*

Polysaccharide tamarind seed (65–73 % of the kernel) with the molecular weight of 600–750 KDa was formulated into cosmetics (0.1–1 %) with skin hydration activity claimed (Cocchi and Sanso 2010). Tamarind polysaccharide claiming skin moisturizing activity is commercialized with the trade name of PA Reviviscence® LS 9562 by Laboratoires Serobiologiques (Table 4).

4.2.14 *Ulmus davidiana*

The root of *U. davidiana* var. *japonica* that has been used traditionally in Oriental medicine was examined on its skin benefits. The polysaccharide derived from the plant root with molecular weight of 20 KDa consisting mainly of rhamnose (57.37 %) was preliminary water retaining *in vitro* including its safety and activity in human skin fibroblast. The noncytotoxic polysaccharide with water-holding capacity additionally suppressed inflammatory mediators as evidenced by the reduction of PEG2, IL-6, and IL-8 in the cultured cells. Moisturizing activity of the polysaccharide was further assessed in 10 female volunteers. Skin hydrating effect monitored by Corneometer® and VapoMeter® was almost the same as HA that was used as the positive control (Eom et al. 2006). This skin hydrating polysaccharide is further commercialized by Bioland and Presperse Incorporated as shown in Table 4.

4.3 Microbial Polysaccharides

4.3.1 *Aspergillus niger*

The mycelium was biotechnologically controlled to produce chitin-glucan with the ratio of these two polysaccharides between 30:70 and 50:50. The obtaining composite of biopolymer was formulated into oil in water emulsion at 0.5–2.0 % and clinically evaluated in 13 female volunteers for 6 weeks. That of 1.5 % polysaccharide significantly improved skin hydration by the suppression of TEWL (Tewameter®) with no erythema. Confirmatory skin moisturizing effect of the 1.5 % chitin-glucan formulation was further conducted in 20 male volunteers for 16 weeks. This natural polysaccharide significantly improved skin barrier by water-holding capacity in SC with additional effect on skin rejuvenation as skin roughness was decreased (Gautier et al. 2008).

4.3.2 *Aureobasidium pullulans*

β-Glucan from *A. pullulans* was prepared and further incorporated into skin moisturizing cosmetics at the amount of 5–20 % that enhanced skin hydration evidenced in 5 female volunteers (Moriya et al. 2011).

4.3.3 *Gluconacetobacter sacchari*

Bacterial cellulose produced from *Gluconacetobacter sacchari* composited with glycerin (1 %) showed a significant skin moisturizing effect as examined in 15 volunteers who contacted with the product for 24 h. TEWL was suppressed, whereas skin capacitance was increased as shown by Tewameter® and Corneometer® (Almeida et al. 2013).

4.3.4 *Klebsiella pneumoniae*

Polysaccharide from a nonpathogenic strain was formulated (10 %, 40 kDa) and assessed on its skin hydrating efficacy in 40 female volunteers. The formulation

significantly enhanced skin water content following 15 and 30 days of study as evidenced by Corneometer® with an ability to suppress TEWL as shown by Tewameter® (de Cargo and Gaspar 2012).

4.3.5 *Nostoc commune*

Cyanobacterium in *Nostoc* genus particularly *N. commune* largely synthesized the mucilaginous matrix. The polysaccharide plays an important role in the bacterium's defense mechanism, of which *Nostoc* polysaccharide is regarded as the biomedical potential natural product with a wide variety of therapeutic effects applied in traditional medicine of several countries. Furthermore, *Nostoc* polysaccharide showed a comparable in vitro moisture absorption and retention capacities to chitosan and urea studied at the same concentration. Consequently, its skin hydrating efficacy was confirmed in an animal model as the water content in mouse SC was improved (Li et al. 2011).

4.3.6 *Saccharomyces cerevisiae*

Baker's yeast is one of the important sources of glucan, moisturizing polysaccharides in cosmetics (Kanlayavattanakul and Lourith 2008). Glucan derivatives are widely used in topical preparations particularly carboxymethyl glucan. It improved skin barrier function as evidenced in enhancement of skin hydration (Corneometer®) in 5 volunteers whom were directed to apply the product containing carboxymethyl glucan (0.04–0.4 %) twice daily for 2 weeks (Zülli et al. 1998). Modifiers of β-glucan enlarge applications suitable for various preparations including sodium carboxymethyl β-glucan. Nano-cosmetics for eye hydration were formulated in a form of liposome containing 0.1–5 % of the active polysaccharides (Mercuri 2008).

4.3.7 *Zymomonas mobilis*

Levan, fructan polysaccharide, was prepared using *Z. mobilis*. The prepared polysaccharide with a molecular weight of 2,250 KDa was noncytotoxic in human skin cell cultures with the anti-inflammatory effects against IL-1α. Skin moisturizing effect was further evaluated in a comparison with hyaluronan at the same concentration by means of TEWL using VapoMeter® and skin capacitance using Corneometer® in 10 female volunteers. Skin hydrating efficacy of *Z. mobilis* polysaccharide was comparative to that of HA (Kim et al. 2005). This microbial polysaccharide is commercialized by BASF and Bioland (Table 4).

4.4 Seaweed Polysaccharide

In addition to the above skin hydrating polysaccharides from microbial source, the seaweed Rhodophyta (red algae) is an important source of sulfated galactans including carrageenans, whereas the brown seaweed (Phaeophyta) largely produces alginates. Carrageenan is exhibited as the excellent active polysaccharide. κ-Carrageenan was formulated (0.5–10 %) with starch (0.5–20 %) in skin mattifying cosmetics with

the moisturizing feeling (Cassin 2013). Furthermore, anionic polysaccharide with the average size of less than 500 KDa was prepared from cell culture of microalgae of the genus *Parachlorella* or *Chlorella* and commercialized by Codif (Table 4). The polysaccharide is composed of rhamnose, xylose, mannose, glucose, arabinose, and glucuronic acid at 15–55, 3–30, 1–25, 1–45, 0.5–10, 22, and 0.1–15 mol%. The polysaccharide (0.1 %) was formulated into a topical application product claiming safety and efficiency (Coragliotti et al. 2010).

4.4.1 *Gigartina stellata*

This red seaweed with the synonym of *Mastocarpus stellatus* enriched with polysaccharide mainly composed of galactose was revealed as the potential source for skin hydrating product according to the in vitro water absorption with the swelling capacity of 7.20 ± 0.42 ml/g and water retention capacity of 5.42 ± 0.06 g/g (Gómez-Ordóñez et al. 2010). Presperse Incorporated is the company that produces red seaweed polysaccharide as shown in Table 4.

4.4.2 *Himanthalia elongata*

Comparative *in vitro* water retention evaluation of edible seaweed of the Spanish coast was conducted. *H. elongata* was noted as the potential polysaccharide for skin moisturizing effect due to its water retention capacity of 7.26 ± 0.13 g/g and swelling ability of 10.97 ± 0.62 ml/g (Gómez-Ordóñez et al. 2010). Similar to *G. stellata* polysaccharide commercialization, *H. elongata* is commercialized by Presperse Incorporated (Table 4).

4.4.3 *Laminaria* sp.

Hydrocolloids extracted from 12 edible seaweeds including *L. japonica* or kombu were comparatively evaluated on skin moisturizing activity. Skin capacitance (Corneometer®) in 10 female volunteers was superiorly enhanced by *L. japonica* over the others at the same tested concentration (5 %). This edible seaweed extract was further formulated into cosmetic cream at various concentrations (1–15 %). Skin moisturizing effect was found significant and superior with the 10 % hydrocolloid algae as it prolonged the suppression of TEWL (Tewameter®) for 8 h (Choi et al. 2013b). This commercialized polysaccharide (Akott) is included in Table 4. In addition, sweet kombu or *L. saccharina* is additionally highlighted as the suitable moisturizing polysaccharide due to its high water retention and swelling capacities (8.93 ± 0.52 g/g and 10.20 ± 0.37 ml/g) (Gómez-Ordóñez et al. 2010).

4.4.4 *Monostroma nitidum*

These edible green macroalgae called aonori in Japanese were extracted to give humectant mucilage. The mucilage was formulated into cosmetic product in the form of a mask in combination with hydroxyethylcellulose (HEC). The equivocal amount of the algal mucilage and HEC (total 2 %) was found superior over that containing 2 % methylcellulose in terms of moisturizing effect as assessed in 7 volunteers monitored by Corneometer®. In addition, the mucilage functioned as thickening agent (Chen and Chen 2003). It is therefore accountable as multifunctional ingredient.

In addition to the above edible seaweed, *Ulva* sp. producing water-soluble sulfated polysaccharides with (ulvan) 8–29 % of the algal dry weight consisted mainly of rhamnose and is also one of the important sources of moisturizing polysaccharide. *U. lactuca* polysaccharide with variety of health benefits is also commercialized as skin moisturizing agent, Akomarine® Sea Lettuce (Table 4). In addition to *U. lactuca*, *U. compressa* is also claimed as a skin moisturizing polysaccharide (Carruba, Codif, and Presperse Incorporated). Furthermore, *Undaria pinnatifida* or wakame is one of the edible seaweeds producing polysaccharides with SC benefits.

5 Conclusion

Moisturizing or hydrating polysaccharides are derived from several natural sources of which botanical and edible hydrocolloids particularly botanical polysaccharides are largely meeting the consumers' preferences toward natural cosmetic products. In addition, advanced biotechnology preparation of skin hydrating polysaccharides affords those of economically feasible choices with animal and microbial polysaccharides. Furthermore, delivery system employing nanotechnology would enhance the skin hydrating efficacy. Those with *in vitro* water-retaining activity are encouraged for further clinical evaluation including those of candidate herbs or botanical sources enriched with polysaccharide content. Health benefits of this biopolymer in sufficiently suppressing dryness of the skin and potentially protects and/or treats wrinkles of skin acting as antiaging ingredients accordingly.

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Polysaccharides from Medicinal Mushrooms and Their Antitumor Activities

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Haixia Chen and Shuqin Li

Contents

Abstract

Cancer is a main burden of disease worldwide since the past decades. About 11 million cancer cases are added every year. Cancer is also called malignant tumor which developed as abnormal cells in the body. And once the patient is diagnosed with a malignant tumor in the body, the survival ratios are very low. Thus, prevention and curation for tumor have become a significant part in tumor scientific research and disease control all over the world. Surgery, radiotherapy, and chemotherapy are the three dominant methods to treat tumor in recent decades. Among these treatments, chemotherapy is most frequently used. However, as these treatments have serious side effects in tumor carriers, efforts have been made to search other nontoxic biological macromolecules that have antitumor properties. Polysaccharides are one of the most important components in organic compounds and widely involved in many biological phenomena. Mushrooms have been valued as edible and medical provision for mankind for thousands years. Due to its diversity and diverse distributions, its medicinal

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values are utilized by folks, especially in China and Japan. In recent years, special attentions are paid to mushroom-derived polysaccharides. Evidences have shown that mushroom polysaccharides have direct and indirect antitumor activities, mostly by way of immunomodulation. This chapter will discuss several aspects of mushroom polysaccharides such as its bioactivities, antitumor mechanisms, toxicity, and also the clinical trials conducted. The main aims of this review are to summarize the available information about mushroom polysaccharides and give a thorough understanding in this area and thus provide references for research and application in antitumor in the future.

Keywords

Cancer • Medicinal mushroom • Polysaccharides • Antitumor • Mechanism • Bioactivities • Toxicity • Clinical trials

Abbreviations

3D	Three-dimensional
5-FU	5-Fluorouracil
AKT	Protein kinase B
C. versicolor	*Coriolus versicolor*
CCl_4	Carbon tetrachloride
G. frondosa	*Grifola frondosa*
G. lucidum	*Ganoderma lucidum*
GLPP	*G. lucidum* polysaccharides peptide
Gl-PS	*Ganoderma lucidum* polysaccharides
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
INF-γ	Interferon-γ
L. edodes	*Lentinus edodes*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK	Natural killer
P. eryngii	*Pleurotus eryngii*
PAC	Cyclophosphamide
PDP	Polysaccharide from *Pholiota dinghuensis Bi*
PEPE	Polysaccharidic extract of *P. eryngii*
PNPS-1	Polysaccharide isolated from *Pholiota nameko*
PSK	Protein-bound polysaccharide Krestin
PSP	Polysaccharide peptide
PSPC	Polysaccharides-protein complex
RFA	Radiofrequency ablation
ROS	Reactive oxygen species

S-GAP-P	Sulfated polysaccharide
SPG	Schizophyllan
STZ	Streptozocin
TACE	Transcatheter arterial chemoembolization
TNF-α	Tumor necrosis factor-alpha

1 Introduction

Cancer is a generic term for a large group of diseases that can be chronic and are responsible for a large number of deaths worldwide. Although there has been considerable progress in modern cancer therapy research, many problems exist in the treatment of cancer. The whole subject area is still problematic, with difficulties in understanding the molecular behavior of various types of cancers and the numerous side effects experienced by patients from treatments (De Silva et al. 2012). Cancer is characterized by uncontrolled proliferation of anaplastic cells which tend to invade the surrounding tissues and metastasize to other tissues and organs (Zong et al. 2012). In recent years, the treatments of cancer mainly include surgery, radiotherapy, and chemotherapy. These treatments usually exhibit serious side effects, killing both malignant cells and normal cells and bringing great pain to patients. Based on this tough situation, utilization of nontoxic drugs for cancer becomes an urgent demand. Mushrooms have been valued as highly tasty and nutritious food and medicine by many societies for thousands of years throughout the world. Traditionally, mushroom (member of the class Basidiomycetes) has been defined as a fleshy, aerial umbrella-shaped, fruiting body of macrofungi. To ancient Romans they were "the food of the Gods," to the early Egyptians they were "a gift from the God Osiris," and the Chinese considered them as "the elixir of life" (Smith et al. 2002). Medicinal mushrooms have an established history of use in traditional ancient therapies (Wasser 2011). In Asia, especially in China and Japan, several mushrooms are adopted due to its medicinal uses, such as *Ganoderma lucidum*, *Lentinus edodes*, and *P. tuber-regium*. In recent decades, researchers have focused more on the extracts of medicinal mushrooms. Among these extracts, polysaccharides are the most potent substance with antitumor efficacy. It is commonly obtained from fruit bodies, cultured mycelia, or culture broth of various mushrooms from the class of Basidiomycetes. These isolated natural products from mushroom include acidic and neutral ones with different types of glycosidic linkages, some of which are bound to protein or peptide residues such as polysaccharide-protein or polysaccharide–peptide complex which showed higher potent antitumor activities. Based on the summarization of Zhang et al. (2007) and the information we summarized, polysaccharides from 29 species of mushroom are listed in Table 1. The related polysaccharides have been extensively studied in the past 30 decades, and the sources of polysaccharides as well as the types also demonstrated. Furthermore, special attention is paid to the derivatives of polysaccharides in the recent studies. Chemical modification is often carried out to improve the antitumor activity of polysaccharides and their clinical qualities

Table 1 Source and type of some macrofungi polysaccharides

Fungi source	Polysaccharides source	Type of polysaccharides	References
Pleurotus tuber-regium	Sclerotium, mycelium	β-D-Glucan	Zhang et al. 2001, 2006, 2003
Ganoderma lucidum	Fruiting body, culture broth	Heteroglycan, mannoglucan, glycopeptide	Miyazaki and Nishijima 1981; Mizuno 1997
Auricularia auricula	Fruiting body	Glucan	Ukai et al. 1982, 1983
Schizophyllum commune	Mycelium	Glucan, schizophyllan	Yamamoto 1981
Hericium erinaceus	Fruiting body, mycelium	Heteroglycan, heteroglycanpeptide	Kawagishi et al. 1990; Mizuno 1992, 1998
Lentinus edodes	Culture broth, fruiting body	Mannoglucan, polysaccharide-protein complex, glucan, lentinan	Chihara 1969; Chihara et al. 1970; Hobbs 2000
Sclerotinia sclerotiorum	Sclerotium	Glucan, scleroglucan	Palleschi et al. 2005
Polystictus versicolor	Fruiting body, culture broth, mycelium	Heteroglycan, glycopeptide, Krestin (PSK)	Cui and Chisti 2003
Grifola frondosa	Fruiting body	Proteoglycan, glucan galactomannan, heteroglycan, grifolan	Cun et al. 1994; Zhuang et al. 1993, 1994
Inonotus obliquus	Fruiting body, mycelium	Glucan	Kim et al. 2005
Agaricus blazei	Fruiting body, mycelium	Glucan, heteroglycan, glucan-protein, glucomannan-protein complex	Mizuno 1992, 1998
Flammulina velutipes	Fruiting body, mycelium	Glucan-protein complex, glycoprotein	Zeng 1990
Ganoderma applanatum	Fruiting body	Glucan	Nakashima et al. 1979
Polyporus umbellatus	Mycelium	Glucan	Yang et al. 2004
Clitopilus caespitosus	Fruiting body	Glucan	Liang et al. 1996
Pleurotus citrinopileatus	Fruiting body	Galactomannan	Wang et al. 2005
Trametes robiniophila	Mycelium	Proteoglycan	Zhang 1995
Tremella fuciformis	Fruiting body, mycelium, culture broth	Heteroglycan	Huang 1982
Pleurotus ostreatus	Fruiting body	Glycoprotein	Solomko 1992

(*continued*)

Table 1 (continued)

Fungi source	Polysaccharides source	Type of polysaccharides	References
Morchella esculenta	Fruiting body	Heteroglycan	Duncan et al. 2002
Phellinus linteus	Fruiting body	Glucan	Kim et al. 2004
Dictyophora indusiata	Fruiting body	Heteroglycan, mannan, glucan	Hara et al. 1991
Peziza vesiculosa	Fruiting body	Proteoglycan, glucan	Mimura et al. 1985
Tricholoma mongolium	Fruiting body	Glucan	Wang et al. 1996
Cordyceps sp.	Fruiting body, mycelium, culture broth	Glucan, heteroglycan	Hsu et al. 2002
Pleurotus eryngii	Fruiting body	Heteropolysaccharide	Yang et al. 2013
Pholiota dinghuensis Bi	Mycelium	Protein-bound polysaccharide	Gan et al. 2012
Pleurotus nebrodensis	Fruiting body	Beta-glucan	Cha et al. 2012
Tricholoma sp.	Mycelium	Polysaccharide-peptide complex	Wang et al. 1995a

(mostly water solubility). Nie et al. prepared chemically sulfated polysaccharide (S-GAP-P), which was derived from water-insoluble polysaccharide of *Grifola frondosa* mycelia, and investigated it in vitro and vivo. The results demonstrated that S-GAP-P had higher antitumor efficacy and enhanced the peritoneal macrophage phagocytosis in S180-bearing mice (Nie et al. 2006).

Mushroom-derived polysaccharides often contain glucose, galactose, mannose, xylose, ribose, arabinose, and glucuronic acid in their α- or β-backbones and branches. Due to their different combinations, the conformation of polysaccharide might differ from each other in many ways. Polysaccharides from various species of Basidiomycetes which consist of β-1,3 glucopyranoside main chain with β-1,6-linked glucose branches possess antitumor activities. And it is also well known that the biological activities of polysaccharides are influenced by their solubility in water, molecular weight, branching rate, triple helical solution conformation, and β-1,6-bonding system in the β-1,3 backbones (Zaidman et al. 2005).

2 Bioactivities

In the 1960s, mushroom polysaccharides were proved to have antitumor activity for the first time (Zhang et al. 2012). Mushroom polysaccharides have shown widely inhibitory effects towards many kinds of tumors including Sarcoma 180 solid

cancers, Ehrlich solid cancer, Sarcoma 37, Yoshida sarcoma, Lewis lung carcinoma, and so on (Zhang et al. 2007). These polysaccharides mainly involve PSK, lentinan, schizophyllan, and polysaccharide peptide (PSP). They all have been proven to inhibit Sarcoma 180 which has been implanted in mice (Wang et al. 1995b). In addition, other polysaccharides derived from mushroom were also studied. In an investigation, the efficacy of *Ganoderma tsugae* which is a famous traditional medicine was proved; the polysaccharide fractions are possessing a significant antitumor activity against solid tumor Sarcoma 180 with inhibition ratio beyond 50 % (Peng et al. 2005). In a study, the antitumor activity of polysaccharides extracted from *Ganoderma lucidum* on the human breast cancer cell in vitro has been evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Artificial derivatives such as sulfated *Pleurotus tuber-regium* polysaccharides exhibited relatively higher in vitro antitumor activity against human hepatic cancer cell line HepG2 than the native polysaccharides (Tao et al. 2006). It was also demonstrated that polysaccharides of rare species named *Antrodia camphorata* could inhibit the proliferation of U937 cells via activation of mononuclear cells (MNCs) (Liu et al. 2004a). All these results indicated the roles which mushroom-derived polysaccharides played in the inhibition effects on the cancer cells (Zhao et al. 2010).

Other than the purified polysaccharides, the polysaccharides-peptides complex is also a direction in the antitumor study. A polysaccharides-protein complex (PSPC) isolated and characterized from the culture filtrates of a mushroom called *Tricholoma* sp. was proved to significantly suppress the growth of Sarcoma 180 solid tumor in vivo, and the inhibitory ratio is 96 %. No deleterious effect is detected during the experiment (Liu et al. 1996). The protein-bound polysaccharides PSK also evidenced to effect on gastric cancer by immunomodulation (Nio et al. 1991).

3 Mechanism of Action

Numerous studies suggested the mechanisms of antitumor activity of polysaccharides from mushrooms, which included immunomodulation in host organism associated with numerous agents, the inhibition of metastasis, and the promotion of apoptosis in cancer cells. All these mechanism are illuminated as follows.

3.1 Immunomodulation

Mushroom-derived polysaccharides are thought to provide antitumor action primarily by activating the immune response of the host organism rather than attacking the tumor cells directly to achieve their effects. It is well known that mushroom polysaccharides work best when it is used in adjuvant chemotherapies which have high side effects and affect the immune system. Unlike traditional antitumor drugs, they cause no harm and place no additional stress to the body,

acting as biological response modifier in the body and helping to upregulate the suppressed immune system. For example, the Maitake MD-fraction extract from fruit body and cultured mycelia of *G. frondosa* can decrease the cisplatin-induced immunosuppression and nephrotoxicity in mice (Masuda et al. 2009). But polysaccharides did not show massive efficacy while administrated under normal condition (Wasser 2011). Innate immune system and adaptive or acquired immune system are the two major functional units in the human body (De Silva et al. 2012); both of them participate in the action of this macromolecule.

The mechanism that polysaccharides activate innate immune system is assumed to conduct through signal pathway via Toll-like receptors. Dentin-1-deficient mice would cause dramatically reduction in cytokine production in bone marrow-derived dendritic cells (Yamanaka et al. 2012). Macrophage is a pivotal factor in the process, while polysaccharides act as nonself substances in the body to activate the receptors in the macrophage surface. Lentinan can enhance the absolute quantity and phagocytosis of macrophage, which lead to the reduction of tumor size and prolong the living life of tumor cell-inoculated mice (Yang 2002). During the process, polysaccharides can also activate the neutrophil and complement system, which are important in innate immunity. The use of mushroom polysaccharide could lead to the production of cytokine produced by monocyte-macrophages (Wang et al. 1997). Lentinan could trigger a cascade of cytokine release, such as tumor necrosis factor-alpha (TNF-α) and various types of interleukins resulting in the maturation, differentiation, and proliferation of immunocompetent cells for host defense mechanisms (De Silva et al. 2012). And a unique polysaccharide component from *Antrodia camphorata* was evidenced to enhance the concentration of Th1-type cytokines (TNF-α, IFN-γ, and IL-12) and Th2-type cytokines (IL-4 and IL-6) (Liu et al. 2004b).

Natural killer (NK) cells are large granular lymphocyte, and they are considered to be part of the innate defense system. They are able to kill certain tumor cell without prior sensitization. The basal activity of NK cells increases dramatically following stimulation with interferons. In addition, NK cells display Fc-receptors for IgG and are important mediators of antibody-dependent cell-mediated cytotoxicity (Lin 2005). The study of *Pleurotus eryngii* showed that polysaccharides were found to significantly enhance the killing activity of NK cells in tumor-bearing mice, suggesting that it could enhance the specific and nonspecific cytolytic activities against autologous tumor cells (Yang et al. 2013).

Adaptive immune system includes humoral- and lymphocyte-mediated immune responses. Mushroom polysaccharides are known to stimulate B-lymphocyte and T-lymphocyte. Report showed that lentinan was able to restore the suppressed activity of helper T cell in tumor-bearing host to their normal state, resulting in complete restoration of humoral immune response (Ooi and Liu 1999). In addition, the study also indicated that lentinan could promote potentiation of response of precursor T cells. Moreover, it had been reported that the delayed-type hypersensitivity response induced at tumor sites by lentinan and the subsequent infiltration of immune effector cells, such as natural NK cells and cytotoxic lymphocytes, are important mechanisms of antitumor action for lentinan (Zhang et al. 2007).

3.2 Inhibiting Metastasis

Most deaths caused by cancer are not due to primary tumor growth but dissemination of tumor cells to secondary sites by a series of events known collectively as metastatic cascade (Jeon et al. 2011). Metastasis is responsible for 90 % of deaths caused by cancer which comprises three independent processes including tumor growth, angiogenesis, and invasion. All three processes may be suppressed by polysaccharides via the inhibition of AKT signaling (Patel and Chen 2012; Dai et al. 2010). Tumor growth and metastasis have been found to be associated with angiogenesis. *G. lucidum* polysaccharide peptide (GLPP) was tested for its anti-angiogenic properties using chick chorioallantoic membrane assay. The result shows that GLPP might be a potent inhibitor of angiogenesis and subsequent metastasis (Weng and Yen 2010).

3.3 Inducing Apoptosis

Aside from preventing metastasis, mushroom polysaccharides also have shown efficacy of inducing apoptosis in tumor cells. By upregulating the immune system as mentioned above, mushroom polysaccharides can inhibit cancer cell proliferation, arrest cell cycle in various checkpoints, and enhance apoptosis. In previous studies, polysaccharide PSK was demonstrated to act as a growth inhibitor for pancreatic cancer cells, known otherwise to be highly resistant to conventional chemotherapies (Rosendahl et al. 2012). By upregulating cell cycle regulatory p21WAF/Cip1 and proapoptotic protein Bax levels, PSK could arrest pancreatic cancer cell cycle and induce apoptosis. Similar study also showed that *Phellinus gilvus* could exhibit antitumor effects by significantly increasing the melanoma apoptosis rate (Bae et al. 2005)

4 Toxicity

One of the benefits of mushroom polysaccharides in antitumor treatment is that this agent exhibits modest side effects. Other methods such as surgery, radiotherapy, or chemotherapies show numerous adverse actions that may affect patients' mood and quality of life and often inevitably cause at least one form of therapy-associated tissue injury or systematic side effect, such as hepatotoxicity (McWhirter et al. 2013), mucositis (Thorpe et al. 2013), late gastrointestinal and urogenital side effects (Schmid et al. 2012), skin reaction, fatigue, pain, etc. (Ragaz et al. 1997). In Tong's study, using a cytotoxicity assay, the two fractions of *Pleurotus ostreatus* polysaccharides had obvious effects on the inhibition of the growth of Hela cells, suggesting they might be a potential candidate for cancer therapy. Meanwhile, the inhibition ratio of human embryo kidney 293 T cell was significantly lower than the control group treated with 5-FU, which killed human normal cell when killing tumor cell. But no antiproliferative

effects of these polysaccharides on human embryo kidney 293 T cells were observed, implying that they had no direct cytotoxicity to noncancerous cells (Tong et al. 2009). In an antiviral clinical trial study, lentinan, which is isolated from *L. edodes*, was reported to have generally good tolerability. During the trial, only a few patient drop out because of side effect or personal preferences. And most side effects resolved promptly after the discontinuation of medication, and all of them were relieved within 24 h (Roupas et al. 2012). In addition, a *Maitake* extract clinical phase I/II trial was deployed in breast cancer patient, and there were only a little number of patients who withdrew prior to the completion of the study due to the side effects, such as mild nausea and joint swelling or grade I allergic reaction (Deng et al. 2009a). These studies proved that mushroom polysaccharide is a promising and safe medicinal agent in medication of antitumor therapy.

5 Other Bioactivities

5.1 Antioxidant Activities

Antioxidation is another complimentary characteristic that mushroom polysaccharide possesses. As we have learned, oxidation is essential to many organisms for the production of energy to fuel biological processes. However, reactive oxygen species (ROS) are often overproduced under pathological conditions (Guo et al. 2010), thus leading to increased risk of chronic disease. Antioxidants have been shown to inactivate ROS and thus protect from oxidative damage. Among various natural substances, polysaccharide extracts from mushrooms may prove to be one of the useful candidates in search for effective, nontoxic substances with free radical scavenging activity. And polysaccharide extracts from *Jisongrong* mushrooms were reported to have scavenging effects on superoxide and hydroxyl radicals (Zhou and Chen 2011). In a report which studied four widely used mushrooms, the radical scavenging ability of semi-purified polysaccharide extract from *G. lucidum* can be up to 94.8 % when the concentration was set at 2.5 mg/mL, which was also the maximum efficacy among the four polysaccharides. The study also reported that the radical scavenging ability of polysaccharides was associated with the concentration of available hydroxyl group, and antioxidant activity was in a strong relationship with the monosaccharide ratios (Kozarski et al. 2012). In our previous studies, polysaccharides from mushroom *Inonotus obliquus* showed good antioxidant activities in the in vitro assays including 1,1′-diphenyl-2-picrylhydrazyl (DPPH) assay, ferric reducing power assay, and lipid peroxidation inhibition assay (Ma et al. 2013). Antioxidant activities after physical modifications and chemical modification have also been tested, such as modification by acid or alkali hydrolysis and thermal and ultrasonic treatment. The result showed better antioxidant effect after these treatments (Ma et al. 2012; Zhang et al. 2013).

5.2 Antiviral Effects

Antiviral efficacy is also one of the notable abilities of mushroom polysaccharide. For example, it is known that HIV is a virus which caused immunodeficiency problem in human and no efficient treatment is available in the world at the moment. A polysaccharide extracted from *Grifola frondosa* was reported to act as adjuvant in HIV infection therapy. In a study, about 85 % of HIV-infected patients exhibit an upregulated sense of well-being associated with a series of symptoms and secondary disease caused by HIV (Gu et al. 2006). This character of mushroom polysaccharides provides a way for future HIV treatment and also finds a new way for antivirus study on the basis of traditional therapies.

5.3 Hypoglycemic Effects

Diabetes mellitus is a serious chronic metabolic disease, which now afflicts 3 % of global population. Diabetes mellitus is a chronic condition characterized by high blood glucose levels that result from insulin resistance in peripheral tissues or impaired insulin synthesis in the pancreas. In addition to impairment in glucose and carbohydrate metabolism, the disease causes abnormalities in the metabolism of lipids and proteins (Liu et al. 2013). Though immunomodulation and antitumor properties of mushroom polysaccharides were firstly studied, more and more attention is paid on their hypoglycemic effects in recent studies. There were already investigations that demonstrated that *Ganoderma lucidum* polysaccharides (Gl-PS) exhibited potential antihyperglycemic effects in rats. And in Zheng et al.'s study, the mechanism of hypoglycemic effect exhibited by Gl-PS was also discussed. The results showed that after 8 weeks' oral administration, plasma concentrations of fasting glucose, triacylglyceride, total cholesterol, and nitric oxide significantly decreased compared with the control. In addition, the Gl-PS had a hypoglycemic effect in STZ-induced diabetic rats through preventing apoptosis of pancreatic β-cells and enhancing β-cells regeneration (Zheng et al. 2012). The mushroom polysaccharides might become potential nontoxic candidates for hyperglycemic treatment.

5.4 Anti-inflammatory Effects

Inflammation is a multiple and complex process enacted by activated immune-associated cells, such as macrophages, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (Wu et al. 2014). *Geastrum saccatum*, a mushroom cultivated in Brazil, was produced under natural conditions in the unexplored reserve of Mata. An extract of *Geastrum saccatum* which contained high amount of glucose and traces of galactose was reported to possess an anti-inflammatory activity. The research studied the inhibition activity of this extract on the ear edema induced by croton oil. The results showed that administration of glucan extract reduced edema (Guerra Dore et al. 2007).

5.5 Hepatoprotective Effects

Liver is an important organ for detoxification. However, liver diseases are becoming a serious health problem worldwide. Most of the hepatotoxins induce tissue injury after having been metabolized to free radicals and cause subsequent cell damage through mechanism of covalent binding and lipid peroxidation (Nitha et al. 2013). Hepatoprotective effect is also one of the characters of mushroom polysaccharides which can be promising hepatoprotective drugs. *Pleurotus eryngii* is an edible mushroom which has biological functions as described in Chinese traditional medicine. Chen et al. investigated hepatoprotective effects of water-soluble polysaccharidic extract from *P. eryngii* (PEPE) by using in vivo mouse models and in vitro biochemistry assays (Chen et al. 2012). The results indicated that PEPE enhanced the antioxidant activity in a liver-injury mouse model and effectively prevented excessive lipid formation in liver tissue. This result suggested that PEPE could be a valuable functional food additive for hepatoprotective treatments. Hepatoprotective effects were also found in mycelial polysaccharide from *Pholiota dinghuensis Bi* (PDP). Biochemical assay and histopathological analysis showed that crude PDP exerted significant hepatoprotective effect in a dose-dependent manner against carbon tetrachloride (CCl_4)-induced acute liver injury (Gan et al. 2012).

5.6 Anti-thrombus Effects

Anti-thrombus effects are rarely reported in mushroom polysaccharides. Only Huang noted its anti-thrombus effects in his studies on a mushroom polysaccharides extracted from *Tremella fuciformis* (Huang 1982). Thus, anti-thrombus effect could be another direction in the mushroom polysaccharide research.

5.7 Hypolipidemic Effects

Hyperlipidemia is a key factor in the development of some serious disorders, such as atherosclerosis, fatty liver disease, and cardio-cerebrovascular disease (Qiu et al. 2013). Lowering of high serum and liver lipid level plays a significant role in prevention, arrest, and even reversion of atherosclerosis (Li et al. 2010). Several mushroom polysaccharides possess hypolipidemic effect as reported. In Chen's research eight mushroom polysaccharides were subjected to macrophage-derived foam cell system to screen for and identify the biologically functional polysaccharides. These eight mushrooms included *Pleurotus eryngii, Lentinus edodes, Pleurotus ostreatus, Pleurotus nebrodensis, Hypsizygus marmoreus, Flammulina velutipes, Ganoderma lucidum,* and *Hericium erinaceus.* According to the results, different types of edible mushrooms had diverse polysaccharide components. Among them in selected edible mushrooms, *P. eryngii* polysaccharide had the strongest ability to inhibit lipid accumulation (Chen et al. 2013). *Pholiota nameko*

is a widely cultivated mushroom in China and Japan. The hypolipidemic effects of polysaccharide isolated from *Pholiota nameko* (PNPS-1) were studied. Hyperlipidemic Wistar rats were treated with PNPS-1 and the results evidently demonstrated that PNPS-1 significantly possessed cholesterol-lowering effects on the serum of hyperlipidemic rats (Li et al. 2010). Similar result can be obtained in other mushroom polysaccharides. All these results indicated that mushroom polysaccharides could be a promising agent for hyperlipidemic therapy.

6 Clinical Trials of Mushroom Polysaccharides

Although there are approximately 1,400 species of mushrooms described and many mushroom polysaccharides have been studied, only a few of them have clinical uses such as lentinan from *L. edodes*, schizophyllan from *S. commune*, polysaccharides from *G. frondosa*, and compounds from *T. versicolor* (protein-bound polysaccharide Krestin/PSK and polysaccharide peptides/PSP) (Chatterjee et al. 2011).

6.1 Lentinan

Polysaccharide works best when used as adjuvant chemotherapies. In this respect, lentinan has been studied best both in animal model and in human clinical practices (Wasser 2011). Yang et al. had evaluated the efficacy of the combination of transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA), and lentinan in treated hepatocellular carcinoma (HCC) patients. The results showed that tumor necrosis was significantly higher in the combination group than those purely treated with TACE or RFA or the combination of TACE and RFA. And the tumor recurrence rate was also lower than other groups. Lentinan was showed to be helpful to the patients (Yang et al. 2008).

6.2 Schizophyllan

Schizophyllan (SPG) is another β-glucan derived from the culture broth of *Schizophyllum commune* fries. Remarkable improvement of schizophyllan in the conjugation with chemotherapies was also reported. In the study of Masaki Inoue and Yoshiaki Tanaka, 68 ovarian cancer patients were randomly assigned in four groups treated with, respectively, cisplatin, adriamycin, and cyclophosphamide (PAC) or a PAC plus SPG. The results revealed the fact that SPG could activate host immunity which had been damaged by chemotherapy and, as a result, could improve the long-term prognosis (Inoue et al. 1993). As to the gastric cancer, SPG seemed to function in the control or elimination of microscopic metastatic foci and/or small-sized macroscopic foci which were present in the stage III patients (Fujimoto et al. 1984).

6.3 Protein-Bound Polysaccharide Krestin (PSK)

PSK is a protein-bound polysaccharides extracted from the mycelia of *Coriolus versicolor* which is well known by its immunity-enhancing properties. Similar to lentinan and schizophyllan mentioned above, PSK was also tested as an adjuvant chemotherapy for cancer patient. For example, in a long-term study in Japan, patients with curatively resected colorectal cancer were orally administrated with mitomycin C intravenously on the day of and the day after surgery, followed by oral 5-fluorouracil (5-FU) administration for over 6 months compared with patients receiving PSK for over 3 years on the basis of control group. The results assured the efficacy of PSK as an adjuvant chemotherapy on curatively resected colorectal cancer (Mitomi et al. 1992).

6.4 Polysaccharides from *Grifola frondosa*

Polysaccharides extracted from *Grifola frondosa* (*Maitake* extract) are also an important agent in cancer therapy. In Gary Deng's study, this agent was subjected to a phase I/II trial in breast cancer treatment. The results showed that at an intermediate dose, oral administration of polysaccharides from *G. frondosa* could upregulate the production of IL-2, IL-10, TNF-α, and IFN-γ by subsets of T cells in patients (Deng et al. 2009b). This indicated its promising immunomodulating role in cancer therapy.

7 Conclusions

Polysaccharide is a macromolecule which is notably well known by its conformational diversity due to its primary structure consisted of different monosaccharides and also due to its special 3D structures. Such variability provides the mushroom polysaccharide with antitumor activities armed with various mechanisms. The antitumor efficacy is well established by many studies and the results confirmed its feasibility in antitumor treatment. Its unique property also compensates the deficiency of chemotherapies, which in a way prolongs the patients' life and alleviates the pain during the curing process. In the past decades, such polysaccharides had provoked tremendous interests in researchers to study its antitumor efficacy, and numerous mushroom polysaccharides or their derivatives were extracted and subjected to studies both in vitro and vivo. Depressively, among this group, an accountable number of polysaccharides were approved for commercial production and have been used on cancer patients, such as lentinan, schizophyllan, PSK, polyporus polysaccharide. As such resource is a promising and new direction in cancer therapies, there is an urgency to fully tap its potential and it requires more advanced investigation, such as the more elucidating antitumor mechanism and the relationship between structure and activities and also the use of high-resolution equipment to define the 3D structure of polysaccharides.

Mushroom has a long-established history both in food and medicinal uses; the recognition of the ability of mushroom-derived polysaccharides is of much significance. Challenges may lie ahead, but surely it will start a new era of medicine.

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Resistant Starch as Functional Ingredient

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Abstract

Resistant starch (RS) is a recently recognized source of fiber and is classified as a fiber component with partial or complete fermentation in the colon, producing various beneficial effects on health. RS also offers an exciting new potential as a food ingredient, mainly as a prebiotic ingredient. RS can be fermented by human gut microbiota and provides the colonic microbiota with a fermentable carbohydrate substrate. As a functional fiber, its fine particles and bland taste make the formulation of a number of food products possible with better consumer acceptability and greater palatability than those made with traditional fibers. Technically, it is possible to increase the RS content in foods by modifying the processing conditions such as pH, heating temperature and time, the number of heating and cooling cycles, freezing, and drying. RS shows improved

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crispness and expansion in certain products, which have better mouthfeel, color, and flavor than products produced with traditional insoluble fibers.

Keywords
Prebiotic properties • Resistant starch • Fermentation properties • Functional ingredient • Polysaccharides

Abbreviations
RS Resistant starch

1 Introduction

Polysaccharides in foods consist of starch and plant cell wall constituents (Perera et al. 2010; Sánchez-Zapata et al. 2009, 2012). Starch is the major source of carbohydrate in the human diet (Ratnayake and Jackson 2008). It occurs in many plant tissues as granules, usually between 1 and 100 μm in diameter, depending upon the plant source. Chemically, starches are polysaccharides composed of α-D-glucopyranosyl units linked together with α-D-(1–4) and/or α-D-(1–6) linkages and are comprised of two molecular types: amylose, the straight chain polyglucan comprised of approximately 1,000, α-D-(1–4) linked glucoses, and amylopectin, the branched glucan, comprised of approximately 4,000 glucose units with branches occurring as α-D-(1–6) linkages (Haralampu 2000; Sharma et al. 2008).

X-ray diffraction differentiates various patterns and structures of starch. Two crystalline structures of starch have been identified (an A and B-type), which contain differing proportions of amylopectin. A-type starches are found in cereals, while B-type and amylose-rich starches are found in tubers. A third type called C-type appears to be a mixture of both A and B forms and is found in legumes (Fuentes-Zaragoza et al. 2010). In general, digestible starches are broken down (hydrolyzed) by the enzymes α-amylases, glucoamylase, and sucrase-iso-maltase in the small intestine to yield free glucose that is then absorbed (Nugent 2005). However, not all starch in the diet is digested and absorbed in the small intestine (Ratnayake and Jackson 2008).

On the basis of its digestibility, starch was classified into three groups, namely, readily digestible starch, partially resistant starch, and resistant starch (Englyst and Cummings 1987). According to this classification, starch that is not accessible to digestive enzymes, such as that protected by hard to digest coatings in whole and coarsely milled grains, starch in raw banana and potato exhibiting B- or C-type X-ray diffraction patterns, and retrograded amylopectin, is considered to be partially resistant starch. Retrograded amylose that can be solubilized in potassium hydroxide and which subsequently becomes susceptible to hydrolysis by amyloglucosidase is referred to as resistant starch (Perera et al. 2010).

Resistant starch (RS) refers to the portion of starch and starch products that resist digestion as they pass through the gastrointestinal tract. RS is an extremely broad and diverse range of materials and a number of different types exist (RS1-4).

At present, these are mostly defined according to physical and chemical characteristics (Nugent 2005).

Resistant starch is the fraction of starch that is not hydrolyzed to D-glucose in the small intestine within 120 min of being consumed, but which is fermented in the colon. Many studies have shown that RS is a linear molecule of α-1,4-D-glucan, essentially derived from the retrograded amylose fraction, and has a relatively low molecular weight (1.2×10^5 Da) (Tharanathan 2002).

Currently, naturally occurring resistant starch (such as found in whole grains, legumes, cooked and chilled pasta, potatoes, rice, and unripe bananas) is considered dietary fiber, while resistant starches added to foods for health benefits are classified as functional fiber under the American Association of Cereal Chemists (AACC) and the National Academy of Sciences (NAS) definition (Sajilata et al. 2006).

The increased awareness of consumers concerning the relationship between food, lifestyle, and health has been one of the reasons for the popularity of food rich in fiber; so resistant starch (RS) has gained importance as a new source of dietary fiber (Fuentes-Zaragoza et al. 2010). The general behavior of RS is physiologically similar to that of soluble, fermentable fiber, like guar gum. The most common results include increased fecal bulk and lower colonic pH (Slavin et al. 2009). Additional observations suggest that resistant starch, such as soluble fiber, has a positive impact on colonic health by increasing the crypt cell production rate or decreasing colonic epithelial atrophy in comparison with non-fiber diets. There are indications that resistant starch, like guar, a soluble fiber, influences tumorigenesis and reduces serum cholesterol and triglycerides. Overall, since resistant starch behaves physiologically as a fiber, it should be retained in the total dietary fiber assay (Haralampu 2000). The recent increased interest in RS is related to its effects in the gastrointestinal tract, which in many ways are similar to these of dietary fiber. Like soluble fiber, RS is a substrate for the colonic microbiota, forming metabolites including short-chain fatty acids (SCFA), i.e., mainly acetic, propionic, and butyric acid. Butyric acid is largely metabolized by the colonocyte and is the most import energy source for the cell (Elmstahl 2002). RS consumption has also been related to reduce postprandial glycemic and insulinemic responses, which may have beneficial implications in the management of diabetes (Tharanathan and Mahadevamma 2003). Therefore, there is wide justification for assuming that RS behaves physiologically like fiber (Sajilata et al. 2006).

The advent of novel and innovative products is a strategic field in the food industry. With an increasing trend toward health and nutrition, consumers are not merely interested in traditional nutritional aspects of the food, but are also concerned with supplementary health merits derived from its regular ingestion. In response, food manufacturers, researchers, and producers have aimed to improve the digestion and health benefits of starch as it is the main carbohydrate in the human diet and serves as an energy source (Viuda-Martos et al. 2010; Sánchez-Zapata et al. 2013). Due to its physiological effects, RS is considered to be a promising and innovative food ingredient, which could be parallel with dietary fibers (Fuentes-Zaragoza et al. 2011; Li et al. 2011).

2 Resistant Starch Classification

RS escapes digestion in the small intestine and passes to the large intestine where it is fermented by bacteria in healthy individuals. Prospective physiological benefits and high quality of the final food products make RS much important to the food formulators and nutritionists. Importance of RS is disease prevention including modulation of glycemic index, diabetes, cholesterol lowering capability, and weight management (Aktar Nasrin and Anal 2014). RS has physicochemical properties, particularly the low water holding capacity, stability in high processing temperatures, bland flavor, and white color, which provide better appearance, texture, and mouthfeel than do conventional fiber sources and improve expansion and the crispness in certain food applications. Native starches are modified with chemical, physical, and enzymatic methods for the formation of RS indigestible residues. RS is the fraction of starch which is not hydrolyzed to D-glucose in the small intestine within 120 min of being consumed, but which is fermented in the colon. Many studies have shown that RS is a linear molecule of a-1,4-D-glucan, essentially derived from the retrograded AM fraction, and has a relatively low MW (1.2×105 Da). RS is an extremely broad and diverse range of materials and a number of different types exist. At present, these are mostly defined according to physical and chemical characteristics (Haralampu 2000; Sharma et al. 2008). Resistant starch is generally categorized in five forms based on the nature of starch and type of sources RS1-RS5 (Fuentes-Zaragoza et al. 2011).

Type 1 (RS1) includes physically inaccessible starch that is locked within cell walls and food matrixes, thus preventing amylolysis. Milling and chewing can make these starches more accessible and less resistant. RS1 is heat stable in most normal cooking operations, which enables its use as an ingredient in a wide variety of conventional foods.

Type 2 (RS2) is composed of native starch granules from certain plants containing uncooked starch or starch that was gelatinized poorly and hydrolyzed slowly by R-amylases (e.g., high-AM cornstarches) (Hernández et al. 2008; Ratnayake and Jackson 2008; Sanz et al. 2009; Yao et al. 2009). RS2 describes native starch granules that are protected from digestion by the conformation or structure of the starch granule. This compact structure limits the accessibility of digestive enzymes (has low bioaccessibility) and various amylases and accounts for the resistant nature of RS2 such as ungelatinized starch. In the diet, raw starch is consumed in foods like banana (Sajilata et al. 2006). A particular type of RS2 is unique as it retains its structure and resistance even during the processing and preparation of many foods; this RS2 is called high-AM maize starch (Wepner et al. 1999).

Type 3 (RS3) refers to retrograded or crystalline non-granular starch formed after cooking, like the starch found in cooked and cooled potatoes, bread crusts, cornflakes, and retrograded high-AM maize starch. RS3 refers to non-granular starch-derived materials that resist digestion (Wepner et al. 1999; Sanz et al. 2009; Yao et al. 2009). RS3 is of particular interest, because of its thermal stability. This allows it to be stable in most normal cooking operations and enables

its use as an ingredient in a wide variety of conventional foods. During food processing, in most cases in which heat and moisture are involved, RS1 and RS2 can be destroyed, but RS3 can be formed (Faraj et al. 2004). Storey et al. (2007) classified a soluble polysaccharide called "retrograded resistant maltodextrins" as type 3 RS. They are derived from starch that is processed to purposefully rearrange or hydrolyze starch molecules, and subsequent retrogradation, to render them soluble and resistant to digestion. This process results in the formation of indigestible crystallites that have a molecular similarity to type 3 RS but with a smaller degree of polymerization as well as a lower MW (Storey et al. 2007), converting a portion of the normal α-1,4-glucose linkages to random 1,2-, 1,3-, and 1,4-α or β linkages (Mermelsteim 2009).

Type 4 (RS4) includes chemically modified or re-polymerized starches (e.g., chain linkage altered dextrins, ethers, or esters) used by food manufacturers to alter the functional characteristics of the starch (Ratnayake and Jackson 2008; Sanz et al. 2009; Yao et al. 2009) and includes starches which have been etherized, esterified, or cross-bonded with chemicals in such a manner as to decrease their digestibility (Nugent 2005). RS4 can be produced by chemical modifications, such as conversion, substitution, or cross-linking, which can prevent its digestion by blocking enzyme access and forming atypical linkages (Sajilata et al. 2006; Kim et al. 2008).

Type 5 (RS5) is an AM-lipid complexed starch (Jiang et al. 2010; Fuentes-Zaragoza et al. 2011), which is formed from high-AM starches that require higher temperatures for gelatinization and are more susceptible to retrograde (Cummings and Stephen 2007). In general, the structure and amount of starch–lipid in foods depend on their botanical sources (Siswoyo and Morita 2003). Also, Frohberg and Quanz (2008) defined as RS5 a polysaccharide that consists of water-insoluble linear poly-α-1,4-glucan that is not susceptible to degradation by alpha-amylases. They also found that the poly-α-1,4-D-glucans promote the formation of short-chain fatty acids (SCFA), particularly butyrate, in the colon and are thus suitable for use as nutritional supplements for the prevention of colorectal diseases.

3 Methods of RS Determination

Determination of RS in food ingredients and processed foods has become vital to the provision of nutritional information to consumers and others (Fuentes-Zaragoza et al. 2010, 2011; Perera et al. 2010). To enable effective use of the research output on RS for food processing and nutritional applications, analytical procedures for the determination of RS need to be compared. At present, significant differences exist among procedures with respect to sample preparation, the enzymes used, and the establishment of experimental conditions that mimic gastrointestinal digestion of starch. Ongoing improvements in analytical methodology are essential, but frequent modifications in protocols reduce the availability of comparable data to assess the efficacy of methods and the nutritional quality of foods. Added to this,

food analyses used in the various procedures have differed in their genetic origin, composition, and processing history.

In vitro methods for the analysis of RS in foods are based conceptually on the gastrointestinal digestion of starch in foods. Enzyme hydrolysis is a common feature of all methods, combined with chemical hydrolysis or gravimetric isolation. Differences in sample preparation are significant and range from mechanical methods (milling, grinding, and homogenization) to mastication.

The type of RS quantified is dependent on the protocol. Most methods are focused on the determination of total RS, but specific methods have been developed to quantify RS1, RS2, RS3, RS4, or RS5. Methods for the determination of RS proposed by Englyst et al. (1982) and Englyst et al. (1992) are the basis for this discussion.

Significant modifications proposed by other authors are compared and contrasted. As proposed by Englyst et al. (1982), RS and other nonstarch polysaccharides are first separated from the enzyme-hydrolyzable starch. Then, RS is solubilized in alkali and thereby separated from the other enzyme-resistant polysaccharides. Even though Englyst et al. (1982) labeled this fraction as RS, the analytical protocol used reveals that it measures RS3 only. Briefly, in this method 100–200 mg of homogenized wet sample or ground dry sample is mixed with sodium acetate buffer at pH 5.4 and heated for 1 h at 100 °C to gelatinize starch. Having included homogenization and boiling steps, this protocol eliminates the contribution of RS1 and RS2 to the final RS value. An enzyme mixture containing α-amylase, pullulanase, and amyloglucosidase is added and hydrolysis is carried out for 16 h at 40 °C. Absolute ethanol is added to terminate enzyme activity and to precipitate nonhydrolyzed starch. The pellet is collected after centrifugation and washed twice with 80 % ethanol. The residue is dried with acetone and then treated with 2 M potassium hydroxide for 30 min at room temperature to solubilize retrograded starch (RS3). An aliquot of alkali digest is mixed with 2 M acetic acid and amyloglucosidase and the contents are incubated at 65 °C for 1 h. After cooling and centrifuging, neutral sugars (glucose, galactose, mannose, xylose, and arabinose) in the supernatant are analyzed by gas–liquid chromatography (GLC). The quantity of glucose detected by GLC represents the amount of RS (RS3) in the sample.

The above procedure was modified later by the authors (Englyst et al. 1992) to measure total glucose, free glucose, total RS, and RS1, RS2, and RS3 as separate fractions. The determination of total RS is carried out in stepwise fashion. Total glucose (TG) and free glucose (FG) contents are determined, in order that total starch (TS) may be calculated as $TS = (TG - FG) \times 0.9$. In this protocol for TS determination, RS1 and RS2 are eliminated by mincing/milling/homogenizing the food sample and subsequently heating the sample at 100 °C for 30 min, respectively. Any retrograded starch that contributes to RS3 is eliminated by treating the sample with 7 M potassium hydroxide at 0 °C. The rapidly digestible starch (RDS) and slowly digestible starch (SDS) contents of the sample are determined separately, and total RS is calculated as $RS = TS - (RDS + SDS)$. In this method, 0.8–4.0 g of sample is minced only to minimize structural changes. The low

viscosity of samples leads to the release of considerably higher amounts of glucose during enzyme hydrolysis, especially in the absence of other polysaccharides, than is the case with the in vivo digestion of starch. Thus, guar gum is added to increase the viscosity. Glass beads are added to samples to ensure proper mixing and to break cell walls during enzyme hydrolysis. For the determination of RDS and SDS, samples are equilibrated with 20 mL of acetate buffer at 37 °C, and then starch is hydrolyzed with an enzyme mix containing pancreatin as a source of α-amylase (30,000 BPU/g), amyloglucosidase (400 AGU/mL), and invertase (3,000 EU/mL). Englyst et al. (1992) proposed that enzyme hydrolysis be carried out at 37 °C instead of at 40 °C, as previously used in the Englyst et al. (1982) protocol, to better mimic human physiological conditions of digestion. A significant reduction in the duration of enzyme digestion is used in the Englyst et al. (1992) protocol. After 16 h of sample digestion with uses sequential removal of aliquots of enzyme digest at 20 min (as the rapid release of sugars ended at 20 min of digestion with pancreatin) and at 120 min from the beginning of the digestion. The rationale to terminate enzyme hydrolysis at 120 min is that the release of glucose reached a plateau at 120 min. The glucose content of the 20 min enzyme digest (G20 fraction) represents RDS and is calculated as RDS = (G20 − FG) × 0.9. The difference in glucose contents between the 120 min and 20 min digests represents SDS and is calculated as SDS = (G120 − G20 − FG) × 0.9. Instead of directly measuring glucose originating from RS as in Englyst et al. (1982), this method indirectly estimates total RS as RS = TS − (RDS + SDS). As 37 °C is the highest temperature at which a sample is treated in this protocol, any RS2 in the food sample would not be hydrolyzed by α-amylase. RS1 also is preserved in the unhydrolyzed fraction as the sample is only minced, and no milling or homogenization is employed. As a result, this indirect measurement of RS includes any RS1, RS2, and RS3 in the sample.

Englyst et al. (1992) devised protocols for determination of RS1, RS2, and RS3. By definition, RS1 is the fraction of starch that is inaccessible to enzymes due to physical impediments, e.g., large-sized particles and cell walls. The analytical procedure for RS1 determination differs from that of Englyst et al. (1982) with respect to the method of reduction of particle size. One portion of the sample (A) is minced once and the other portion (B) is either milled or homogenized, as appropriate, to obtain particles b 0.2 mm in diameter. Then, using the protocol for the determination of RDS and SDS, glucose released after 120 min of enzyme hydrolysis (G120) is determined for each portion. RS1 is calculated as RS1 = [G120 (B) − G120(A)] × 0.9. Starch in raw foods and foods cooked with little water (preventing complete gelatinization of starch) contribute to RS2.

The method for the determination of RS2 compares the amount of glucose released enzymatically from a cooked sample to that from a raw sample (Englyst et al. 1992). One portion (C) of a raw food sample is heated in 0.1 M sodium acetate buffer, pH 5.2, in a boiling water bath for 30 min, while another portion (D) is used directly without a heat treatment. The samples are hydrolysed with amylase, amyloglucosidase, and invertase and the glucose released after 120 min (SDS). RS2 is calculated as RS2 = [G120(C) − G120(D)] × 0.9.

Determination of RS3 is limited to samples containing retrograded starch (Englyst et al. 1992), and the protocol is similar to that of Englyst et al. (1982) in many ways. In contrast to the protocol for the determination of RDS and SDS for the estimation of total RS, where α-amylase, amyloglucosidase, and invertase are used, the protocol for RS3 determination uses α-amylase and pullulanase at 40 °C. After removing the enzyme-hydrolyzable starch from the sample, retrograded starch is solubilized in 4 M potassium hydroxide at room temperature, whereas Englyst et al. (1982) used 2 M potassium hydroxide. However, this concentration is lower than that used (7 M) in the protocol for the determination of TG. The temperature at which retrograded starch is solubilized in potassium hydroxide also is different in the two procedures: 0 °C for TG and room temperature for RS3 determination (Englyst et al. 1992).

Goñi et al. (1996) cautioned about the possible effect of sample preparation in RS protocols as drying and cooling, and conditions of storage can alter the level of RS in foods. In this procedure, a 100 mg sample is milled or homogenized before use and by doing so RS1 is eliminated. Sample pH is decreased to 1.5 with HCl–KCl buffer to simulate gastric pH. As a major modification to the procedure of Englyst et al. (1992), hydrolysis with pepsin at 40 °C for 1 h is introduced. Sample pH is adjusted to 6.9 with tris–maleate buffer to simulate conditions in the small intestine. Instead of using a cocktail of enzymes as in Englyst et al. (1992), samples are hydrolysed with α-amylase only for 16 h at 37 °C. As this method is focused on the determination of RS (not SDS and RDS), products from enzyme hydrolysis are discarded at this point. As 40 °C is the highest temperature to which the sample is exposed, any RS2 in raw foods is included in the unhydrolyzed fraction. The pellet is dispersed in 4 M potassium hydroxide at room temperature for 30 min to solubilize RS3. Dextrin in the alkali digest is hydrolyzed to glucose with amyloglucosidase at 60 °C for 45 min. Glucose content is determined using a colorimetric assay. Based on the analytical procedure of Goñi et al. (1996), the presumed definition for RS is starch that is not hydrolyzed by α-amylase within 16 h at body temperature, which is in contrast to the Englyst et al. (1992) definition where RS is starch that is not hydrolyzed by pancreatin, amyloglucosidase, and invertase within 120 min.

Other modifications to the Englyst et al. (1992) method have been proposed, but the essence of the method (to remove hydrolyzable starch with enzymes) remained unchanged. Chung et al. (2006) modified the Englyst et al. (1992) protocol to eliminate redundancy and to introduce a reduction in sample size (to 0.5 g). Instead of adding guar gum separately to each sample tube with subsequent dissolution in acetate buffer, Chung et al. (2006) prepared a solution of guar gum in HCl (to increase its solubility) and then added aliquots to each sample. The volume of sodium acetate is decreased to 5 mL by increasing its molar concentration to 0.5 M (20 mL of 0.1 M sodium acetate is used in the Englyst et al. (1992) protocol). Chung et al. (2006) deleted invertase from the enzyme mix. The digests collected (0.5 mL) after 20 and 120 min were mixed with 4 mL of 80 % ethanol, which replaced the 20 mL of 66 % ethanol in the Englyst et al. (1992) protocol. RS was calculated as the difference between total starch and starch not hydrolysed at 120 min as in Englyst et al. (1992).

The Megazyme® kit for RS determination is widely used in analytical laboratories and is the basis of both AOAC method 2002.02 and AACC method 32–40 (Megazyme 2008; Perera et al. 2010). This method, in many ways, is similar to that of Englyst et al. (1982). Samples are ground to pass a 1 mm sieve which generates a coarse meal; thus RS1 is accounted for in this method. The Megazyme® protocol eliminates the initial boiling of samples in acetate buffer and the use of pullulanase. Instead, it employs a mixture of α-amylase (3 Ceralpha units/mg) and amyloglucosidase (3,300 U/mL) to hydrolyze starch in raw or processed food samples. Hydrolysis is carried out for 16 h at 37 °C as proposed by Englyst et al. (1982). As a result, RS2 in raw foods would not be hydrolyzed during this procedure. After inactivation of enzymes with 99 % ethanol, glucose in the sample is removed by two washings with 50 % ethanol. Similar to Englyst et al. (1982), the pellet collected from digestion is treated with 2 M potassium hydroxide to extract RS3 from the fiber-rich matrix. Dextrins thus produced are hydrolyzed to glucose with amyloglucosidase. The incubation time with amyloglucosidase is reduced to 30 min at 50 °C (Megazyme 2008) from 1 h at 65 °C (Englyst et al. 1982). The Megazyme® method uses the glucose oxidase–peroxidase colorimetric assay to determine the glucose concentration in the final hydrolysate, as did Englyst et al. (1992). The Megazyme® protocol for RS does not enable the determination of SDS and RDS.

Eerlingen et al. (1993) proposed enzymatic gravimetric isolation of RS from wheat starch pre-gelatinized at 121 °C and subsequently stored at 0 °C, 68 °C, and 100 °C. In this method, samples are placed in boiling phosphate buffer, pH 6, and hydrolyzed with Termamyl® (heat stable α-amylase) at 100 °C for 30 min. After cooling to room temperature, the pH is adjusted to 4.5 with 2 % phosphoric acid. Samples are hydrolyzed with amyloglucosidase at 60 °C for 30 min and then centrifuged. The pellet is washed with deionized water, dispersed in phosphate buffer at pH 7.5, and incubated with protease at 42 °C for 4 h. The residue after digestion is collected by filtration and oven-dried at 80 °C. The weight of the enzyme-insoluble residue represents the RS content of the starch sample. Faraj et al. (2004) also isolated RS gravimetrically but introduced a preliminary hydrolysis with lichinase and β-glucosidase. Then, samples are treated with a fungal protease at 42 °C, Termamyl® at 100 °C, and amyloglucosidase at 50 °C, respectively. RS is collected by drying the enzyme-resistant residue. It is important to note that the gravimetric separation of RS is acceptable only for analytes free of nonstarch polysaccharides.

Other modifications have been made to the sample preparation step of RS determination with the intent of more closely simulating in vivo digestion. Åkerberg et al. (1998a, b) employed chewing of food samples to initiate hydrolysis by salivary amylase. According to these authors, the number of times a food sample is chewed has a significant effect on the end result. Briefly, the chewed food sample (15 chews in 15 s) and subsequent mouth wash are expectorated into a beaker containing pepsin in Na/K phosphate buffer. Then, the pH is adjusted to 1.5 with HCl and the sample is incubated for 30 min at 37 °C to simulate gastric conditions. Sample pH then is adjusted to 5 with sodium acetate buffer (0.5 M, pH 5) and sodium hydroxide.

Subsequently, starch hydrolysis is carried out with a mixture containing $MgCl_2$, $CaCl_2$, 2-propanol, pancreatin, and amyloglucosidase for 16 h at 40 °C. Then, 95 % ethanol at 60 °C is added in excess, leading to the formation of a precipitate at room temperature. The precipitate, collected by filtration, is dehydrated with 95 % and 99 % ethanol and solubilized in 2 M potassium hydroxide. The total starch content of the alkali digest is determined and expressed as RS. Muir and O'Dea (1992) also employed chewing for sample preparation. Sample pH is then adjusted to 2 and the sample is hydrolysed with pepsin for 30 min at 37 °C. After neutralizing the pH with sodium hydroxide, pancreatin and amyloglucosidase are added to samples in acetate buffer at pH 5 and incubated for 6 h at 37 °C. According to the authors, this reduction in hydrolysis time (6 h instead of 16 h) is intended to represent better the movement of food through the small intestine. The Muir and O'Dea (1992) protocol significantly deviates from the approaches already discussed as the pellet collected after pancreatin hydrolysis (at 37 °C) is further treated with thermostable α-amylase (Termamyl) at 100 °C, presumably to hydrolyse RS2. The pellet recovered after Termamyl hydrolysis is boiled with dimethyl sulfoxide (DMSO) for 1 h. The supernatant from Termamyl hydrolysis (RS2) and the digest from DMSO treatment (RS3) are mixed, and the glucose content of the mixture is determined. Introduction of DMSO in lieu of potassium hydroxide for solubilization of RS3 is a novel feature of the Muir and O'Dea (1992) protocol. Saura-Calixto et al. (1993) determined RS in dietary fiber. Instead of using pancreatin as the source of α-amylase, they used heat stable α-amylase at 100 °C to eliminate traces of starch. The pellet from enzyme digestion is sequentially hydrolyzed using a protease and amyloglucosidase for 35 min at 60 °C. The residue is collected and dehydrated with ethanol and acetone and subsequently dispersed in 2 M potassium hydroxide at room temperature. After adjusting the pH to 4.75 with acetate buffer, dextrins in the solution are hydrolyzed with amyloglucosidase at 60 °C for 35 min. The glucose oxidase–peroxidase system is used to measure glucose in the supernatant collected after centrifugation.

It is important to note that very little information is available on the in vivo digestibility of foods for which in vitro RS data is available. From a nutritional point of view, this is a drawback. Grinding and homogenization would certainly produce food particles much smaller than those resulting from chewing. Englyst et al. (1992) compared chewing of samples against mincing and reported that differences in results were more related to the source of starch than to the method of mincing. Individuals differ in their chewing habits and may not chew the same number of times as in an experimental procedure. Therefore, available RS1 may vary from person to person as its level depends on particle size.

It is important to consider possible variability in RS data associated with the technique employed to sample the enzyme hydrolysate, because the colorimetric glucose assay is sensitive to inaccuracies in sample volume. Englyst et al. (1992) proposed drawing of 0.5-mL volumes of the hydrolyzate after 20 and 120 min to determine RDS and SDS, respectively. Then, RS is computed as the difference between total starch and enzyme-hydrolyzed starch. Chung et al. (2009) reduced the volume of sample drawn to 0.1 mL. It has been observed that undigested food particles interfere with pipetting (especially at the 20-min sampling point) more

frequently than one might expect. This would significantly influence the reproducibility of the colorimetric assay of glucose. The advantage of using gas–liquid chromatography to measure glucose is such errors can be corrected by using an internal standard, as did Englyst et al. (1982). In this connection, the Megazyme® protocol eliminated variability related to sample volume by employing the total volume of the digest after potassium hydroxide treatment for centrifugation and then drawing an aliquot for color development. The availability of RS controls from Megazyme® is useful for understanding experimental errors.

It is reasonable to expect that variations in analytical procedures, including differences in the enzymes used and in their concentration and sequence of application, and dissimilarities in the conditions of experiments, would significantly alter the levels of RS detected in similar foods. RS values of selected foods as reported by some authors (Szczodrak and Pomeranz 1991; Englyst et al. 1992; Muir and O'Dea 1992; Åkerberg et al. 1998a; Faraj et al. 2004; Chung et al. 2006; Tribess et al. 2009; Perera et al. 2010). Englyst and Hudson (1996) reported RS values of a large number of different food items, but the non-availability of information on handling and storage pre- and post-processing prevent any useful comparison. The analysis of resistant starch in Englyst et al. (1982) is limited to a few sources including white bread, whole wheat bread, and corn flakes (0.8 %, 0.8 %, and 2.9 % RS, respectively, on a dry weight basis) because the main focus of this study was to propose a method to determine nonstarch polysaccharides in foods, not RS.

4 Prebiotic Effects

The definition of "prebiotics" as a concept in nutrition was proposed in 1995, by semantic analogy with the term "probiotics" (Fuentes-Zaragoza et al. 2011). Even if slightly different definitions are proposed and discussed by several international instances, prebiotics always refer to the fact that food ingredients or nutrients escape the digestion in the upper part of the digestive tract and are selectively fermented by bacteria, thereby changing the composition and/or activity of the gastrointestinal microbiota. An important point of the definition is that it must confer benefits upon host health (Roberfroid 2007; Delzenne 2009; Fuentes-Zaragoza et al. 2010, 2011; Sánchez-Zapata et al. 2013; Viuda-Martos et al. 2010).

Prebiotics are utilized to promote the survival of probiotics. Prebiotics are nondigestible carbohydrates that are not absorbed in the intestine, such as RS (Coskun 2006; Fuentes-Zaragoza et al. 2010, 2011; Sánchez-Zapata et al. 2013). RS is not absorbed in the small intestine; it provides the colonic microbiota with a fermentable carbohydrate substrate. It has been suggested that RS promotes a higher proportion of butyric acid than other indigestible carbohydrates. Butyrate constitutes a major energy substrate for the colonocytes and is associated with benefits in relation to colonic health (Scourboutakos 2010; Leeman et al. 2006). They travel to the colon where they promote the growth of specific advantageous microbiota (probiotics) by supplying food/energy, while simultaneously influencing the microbiota's gene expression (Lee et al. 2009).

RS is currently attracting widespread interest for its potential health benefits leading to growing demand for robust, cost-effective RS assays for industrial, regulatory, and research use. Unlike other dietary fiber components, RS is neither intrinsically indigestible nor a fixed entity and is determined by an individual's upper gut digestive capacity as well as food processing and storage conditions so any analysis needs to accommodate physiological factors such as transit time (Fuentes-Zaragoza et al. 2011).

The possible applications of RS such as prebiotic constituents in functional food formulations could be summarized (Charalampopoulos et al. 2001):

(i) As fermentable substrates for growth of probiotic microbiota, especially lactobacilli and bifidobacteria
(ii) As dietary fiber promoting several beneficial physiological effects
(iii) As encapsulation materials for probiotic in order to enhance their stability

Short-chain fructooligosaccharides (FOS) and RS may act synergistically (by combining, and thus increasing, their prebiotic effects) (Rodríguez-Cabezas et al. 2010); the administration of the combination of FOS and RS induced changes in the intestinal microbiota, by increasing lactobacilli and bifidobacteria in cecum and colonic contents. Several types of prebiotic fibers can be distinguished considering their rate of fermentability. Such role depends on the carbohydrate chain length as it has been demonstrated in vitro in a fermentation system, showing that FOS are rapidly fermented whereas long chain prebiotic, like inulin, are steadily fermented. These observations have been confirmed in vivo once the different prebiotics reach the large intestine: FOS are rapidly fermented, whereas RS is slowly degraded. In consequence, the particular kinetics would determine the region of the intestine where the effects will be clearer. Thus, FOS would be more active in the first parts of the large bowel, whereas RS would reach the distal part of the colon. In fact, Le Blay et al. (2003) have reported that administration of FOS or raw potato starch induces different changes in bacterial populations and metabolites in the cecum, proximal, and distal colon, as well as in feces. As compared with RS FOS doubled the pool of cecal fermentation products, like lactate, while the situation was just the opposite distally. These observations confirm that each prebiotic shows particular properties, which should be considered before their application for intestinal diseases; thus, rapidly fermentable prebiotics are particularly useful in those affecting the proximal part of the large intestine, while slowly fermentable prebiotics should be chosen for more distal intestinal conditions. Moreover, an association with different prebiotics with complementary kinetics should be considered when a health-promoting effect throughout the entire colon is required. So, functional foods based on the combination of two different dietary fibers, with different rate of fermentability along the large intestine, may result in a synergistic effect and, thus, in a more evident prebiotic effect that may confer a greater health benefit to the host (Younes et al. 2001).

Also, RS and inulin combination showed synergistic effects on intestinal calcium and magnesium absorption and balance in rats. The fermentation of these

substrates in the large bowel to SCFA is the main reason for this increase in mineral absorption (Younes et al. 2001).

Functional foods have changed and increased the role of food in health. Probiotics are the fastest-growing sector of the functional food industry, through their role in increasing the number of beneficial bacteria in the gut. These living microorganisms are primarily derived from lactic acid bacteria, comprising multiple strains from the genera *Lactobacillus* and *Bifidobacterium*. Some foods combine several microorganisms (Scourboutakos 2010). Beneficial health effects attributed to probiotics are shortening of the duration of rotavirus diarrhea, relief of signs and symptoms of lactose intolerance, decreasing the risk of allergy in atopic individuals, cancer prevention, lowering of serum cholesterol levels, prevention of urogenital infections, and synthesis and enhancement of the bioavailability of nutrients. Probiotic bacteria may compete with pathogens for nutrients and mucosal adherence, produce antimicrobial substances, and modulate mucosal immune functions. The beneficial effects of probiotics are strain-specific; therefore, the definition of which probiotics (as a single strain or a combination) are most effective in specific diseases is needed (Coskun 2006).

The breakdown of prebiotic molecules by bacterial enzymes into SCFA (acetate, butyrate, and propionate) is crucial for gut integrity and function, modulation of the immune system, calcium and magnesium absorption, and maintenance of normal serum cholesterol levels. The end products of this fermentation are consumed by both these bacteria and intestinal epithelium as fuel. Symbiotic refers to nutritional supplements which contain probiotics and prebiotics in combination. Since it has been hypothesized that prebiotics ensure the survival of some beneficial bacteria, their effects might be additive or symbiotic (Coskun 2006). Prebiotics selectively can stimulate probiotic strains. Prebiotics may improve the survival of bacteria crossing the upper part of the gastrointestinal tract, thereby enhancing their effects in the large bowel (Iyer and Kailasapathy 2005).

The best model of prebiotic–probiotic symbiosis is the encapsulation (Fuentes-Zaragoza et al. 2011). Encapsulation is often mentioned as a way to protect bacteria against severe environmental factors. The physical protection of probiotics by microencapsulation is a new approach to improve the probiotic survival. Encapsulation helps to isolate the bacterial cells from the effects of the hostile environment and gastrointestinal tract, thus potentially preventing cell loss (Homayouni et al. 2008). The goal of encapsulation is to create a microenvironment in which the bacteria will survive during processing and storage and be released at appropriate sites (e.g., small intestine) in the digestive tract. The benefits of encapsulation to protect probiotics against low gastric pH have been shown in numerous reports and similarly for liquid-based products such as dairy products (Weinbreck et al. 2010).

Microcapsules consist of a liquid core surrounded by a semipermeable membrane which retains the cells inside, reduces mass transfer limitation, and minimizes phage contamination. For the encapsulation of viable cells, the materials and formulation conditions used should be gentle and nontoxic. In this respect, the antibacterial properties of chitosan limit its use as a core solution.

However, alginate and starch liquid core capsules offer the possibility to immobilize lactic acid bacteria without loss of viability and fermentation ability (Jankowski et al. 1997). Starch and its derivative (RS) are widely used in the encapsulation of various food components, as probiotics. Indeed the use of starch in many encapsulation processes has provided solutions to problems such as thermal stabilization, process-induced controlled release, and extended shelf-life of sensitive compounds (Shimoni 2008). The (micro)encapsulation of probiotics has received attention from food companies interested in producing probiotic-containing consumer products, but faced with the difficulty of maintaining cell viability over the shelf-life of the product (Weinbreck et al. 2010).

A symbiotic approach is often accomplished by co- encapsulation of RS in the form of high-AM maize starch together with the probiotic microorganisms within the microcapsule. Usually, 1–2 % insoluble starch grains are added to the probiotic–hydrocolloid precursor directly before the encapsulation process, with the aim to further maintain the viability of probiotics (Sultana et al. 2000; Iyer and Kailasapathy 2005).

RS had been used to improve encapsulation of viable bacteria in yogurt. Sultana et al. (2000) reported that the incorporation of Hi-Maize1 starch (commercial RS) improved encapsulation of viable bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* spp.) in yogurt, as compared to when the bacteria were encapsulated without the starch.

Iyer and Kailasapathy (2005) selected three different complementary prebiotics and were separately coencapsulated with *Lactobacillus acidophilus* and tested for their efficacy in yogurt. Addition of Hi-maize1 starch to capsules containing *Lactobacillus* spp. provided maximum protection to the encapsulated bacteria after 3 h of incubation at pH 2.0 compared with other two prebiotics, Raftiline1 and Raftilose1. Viable counts of *Lactobacillus* spp. increased significantly ($p < 0.05$) with Hi-maize concentration of up to 1.0 % w/v the viability of bacteria under in vitro acidic conditions. Addition of Hi-maize (1.0 % w/v) to capsules containing *Lactobacillus* spp. and further coating with chitosan significantly increased ($p < 0.05$) the survival of encapsulated bacteria under in vitro acidic and bile salt conditions and also in stored yogurt compared with alginate encapsulated cells.

Homayouni et al. (2008) have shown that encapsulation can significantly increase the survival rate of probiotic bacteria in ice cream over an extended shelf-life. They manufactured two types of symbiotic ice cream containing 1 % of RS with free and encapsulated *Lactobacillus casei* (Lc-01) and *Bifidobacterium lactis* (Bb-12). The addition of encapsulated probiotics had no significant effect on the sensory properties of non-fermented ice cream in which the RS was used as prebiotic compound and *L. casei* and *B. lactis* survive in high numbers in the encapsulated samples. Encapsulation thus may enhance the shelf-life of probiotic cultures in frozen dairy products.

Probiotic cell concentrates are often required to be stored over longer periods prior to food manufacture and ingestion. Hence, it is often required to dry the probiotic microcapsules after production. This is particular important for dry foods, such as cereal products or beverage powder where probiotics are added in dry form.

Nevertheless, the impact of microencapsulation on drying and storage of dried probiotic microorganisms prior to application in food systems is barely investigated, since efforts with respect to increase in survival are mainly focused on the application of protective substances. Heidebach et al. (2010) studied the viability of probiotic cells (*Bifidobacterium* Bb12 and *Lactobacillus* F19) with dried protein–hydrogel microcapsules during a freeze-drying step as well as during subsequent storage in the dried state and the influence of RS granules in the protein matrix. In the case of *Bifidobacterium* Bb12, no difference in survival was found between free and encapsulated samples with and without RS. In the case of *Lactobacillus* F19, survival rates of microencapsulated cells without RS were significantly higher compared to free cells as well as cells that were encapsulated together with RS corns.

5 Evaluation of the Fermentation Properties and Potential Prebiotic Activity

The prebiotic effect of a substrate can be measured as a selective effect upon growth of major bacterial groups commonly found in human gut, in particular a selection for increased numbers of bifidobacteria and lactobacilli in comparison with "undesirable" microorganisms, such as certain clostridia and bacteroides (Gómez et al. 2010). There are a number of methods currently in use to determine the prebiotic properties of a substrate, from pure culture studies to human trials (Vulevic et al. 2004; Mandalari et al. 2007; Gómez et al. 2010; Rodríguez-Cabezas et al. 2010; Parkar et al. 2010). However, a defining characteristic of prebiotic is the selective nature of certain groups of colonic bacteria seen as beneficial toward human health. This can only be determined in studies using mixed microbial culture which mimic the microbial ecology of the human intestinal tract. For a rapid comparative evaluation, anaerobic batch fermentations inoculated with fecal slurries are used. Because they represent the diverse gut microbiota, but are completed rapidly with several sets running simultaneously, these anaerobic batch fermentations present an excellent mode for small-scale screening of novel substrates. Until recently, growth of specific bacteria in such fermentations was measured through colony counting on selective agars. This approach, however, suffers from several drawbacks (time-consuming, labor-intensive, and non-recovery of uncultivable organisms). As a result, molecular techniques such as fluorescence in situ hybridization (FISH) have been developed to study microbial communities (Parkar et al. 2010).

6 The Rat as a Model for Starch Digestion in Humans

Animal models have been used in some RS research studies, and thus it is important to discuss briefly the dependability and/or applicability of the results obtained (Perera et al. 2010). Animal models used in biomedical research should be either analogous or homologous to humans considering the anatomy and physiology of the organ or system under investigation. Other criteria include, but are not limited to,

the selected animal should be easily available in substantial numbers, should be easy to handle, and should be manageable with the resources available, and the life span should be sufficiently long to carry out the research. In keeping with these criteria, omnivores such as the rat and the pig are used to simulate the gastrointestinal tract and liver functions of the human (Chow 2008; Perera et al. 2010). In a comprehensive review, DeSesso and Jacobson (2001) compared the similarities and differences between the gastrointestinal tracts of the human and the rat. Some significant differences are listed here. The human pharynx is a single tube that is the passageway for both food and air, whereas the rat pharynx is separated into two compartments for respiratory and digestive purposes. The human stomach is a single chamber where food is mixed with enzymes and acid. The rat stomach, although a single chamber, is functionally divided into two areas. The stomach is the site of bacterial digestion, whereas the glandular stomach secretes enzymes and acid. Bacterial digestion is absent in the human stomach. Although the rat is used to imitate digestion in the human, there is the likelihood of species differences in enzyme activity. The small intestine has three functionally different regions, the duodenum, the jejunum, and the ileum. Compared to the human, where the gallbladder secretes bile, the rat liver secretes twice as much as bile into the duodenum per kg of body weight per day.

Nutrient absorption takes place in the duodenum and the jejunum of both rats and humans through diffusion, active transport, and solvent drag mechanisms. Compared to the total length of the digestive tract, the jejunum of the rat is proportionally longer than that of the human. The jejunum of the rat represents 90 % of the length of the small intestine and that of the human, 38 %. The transit time of food through the rat small intestine is slower than that in the human.

Despite the human small intestine being 5.5 times longer than that of the rat, the transit time of food through the small intestine of the human and the rat is similar, 3–4 h. The surface area of the human small intestine is 200 times greater than that of the rat, which is significant given the difference in their lengths. Water and nutrients formed through bacterial metabolism are absorbed in the large intestine. The human large intestine is divided into the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anus. The sigmoid colon is absent in the rat. The cecum is responsible for most of the microbial digestion of food in the rat and represents up to 26 % of the length of the large intestine. In contrast, the human cecum is only 5 % of the length of the large intestine. In addition, the types of microorganisms naturally occurring in the digestive systems of the human and the rat may be different (DeSesso and Jacobson 2001). The differences described above indicate that the rat model may not provide reliable data on RS digestion in humans, but it may be useful in understanding the fate of RS within the digestive tract.

7 Other Biological Activities

Scientific interest upon RS has increased significantly during the last decades, mostly due to its capacity to produce a large amount of butyrate all along the colon. Butyrate has been observed to have a range of effects on cell metabolism,

differentiation, and cell growth as well as inhibition of a variety of factors that underlie the initiation, progression, and growth of colon tumors (Champ et al. 2003).

Starch processing can increase its prebiotic properties. The digestibility of starch in cooked potatoes changes when the potatoes are cooled after cooking, and these changes may be of considerable nutritional significance. When potato is cooled after cooking, the starch undergoes profound changes in digestibility: the content of rapidly digested starch (RDS) decreases; the content of slowly digested starch (SDS), defined as starch digested between 20 and 120 min in vitro, increases markedly and the proportion of RS increases. The increase in RS means that the loading of non-digested polysaccharide ("dietary fiber") into the colon is greatly increased, making the cooled potato a valuable source of dietary fiber which, moreover, is enriched in a type (RS) that has demonstrated benefits as a prebiotic (Mishra et al. 2008).

Without heat gelatinization the starch remains in granular form, highly resistant to enzymic attack (RS2) (Fuentes-Zaragoza et al. 2010). In such a state the potato would have little glycemic impact but may contribute to prebiotic effects, although its use would be limited to products of low water content in which the granular form of the starch would remain intact. As a result, this type of RS (RS2) is almost completely transformed into RDS upon cooking, but upon cooling, the content of RS again increases. Because this form of RS must have been regenerated from the almost completely digestible form (RDS) present in the potatoes immediately after cooking, it is RS type III (RS3), or retrograded starch Mishra et al. 2008).

Shimoni (2008) studied a fraction of RS, from high-AM cornstarch, called RS type III (RS III). RS III is fermented by the microbiota in the colon, and it indicates that it may have health benefits such as modifying lipid metabolism and reducing the risk of colon cancer. RS III can be produced by heat-induced gelatinization of starch followed by recrystallization. The amount of RS III produced is affected by its composition, heat treatment, and recrystallization conditions. Understanding the relation between RS III polymorphism and its resistance is critical for the development of RS with improved prebiotic properties. The crystallite polymorphism and the lamella structure of RS III affect its enzymatic resistance, thus changing its prebiotic activities (e.g., its properties as an enzymatic substrate). This hypothesis was formulated based on studies that found correlation between starch crystallinity and its resistance to enzymatic digestion and on other studies showing the effect of crystallization conditions on RS polymorph type.

8 Conclusion

Functional foods have revolutionized and augmented the role of food in health. Probiotics are the fastest-growing component of the functional food industry, through their role in increasing the number of beneficial bacteria in the gut. Resistant starch (RS) is a recently recognized source of fiber and is classified as a fiber component with partial or complete fermentation in the colon, producing

various beneficial effects on health. RS also offers an exciting new potential as a food ingredient. As a functional fiber, its fine particles and bland taste make the formulation of a number of food products possible with better consumer acceptability and greater palatability than those made with traditional fibers. Technically, it is possible to increase the RS content in foods by modifying the processing conditions such as pH, heating temperature and time, the number of heating and cooling cycles, freezing, and drying. RS shows improved crispness and expansion in certain products, which have better mouthfeel, color, and flavor than products produced with traditional insoluble fibers. RS can be fermented by human gut microbiota and provides the colonic microbiota with a fermentable carbohydrate substrate. Scientific interest upon RS has increased significantly during the last decades, mostly due to its capacity to produce a large amount of butyrate all along the colon. Short-chain FOS and RS may act synergistically by combining, and thus increasing, their prebiotic effects. The best model of prebiotic–probiotic symbiosis is the encapsulation. Starch and its derivative (RS) are widely used in the encapsulation of various food components, as probiotics. A symbiotic approach is often accomplished by co-encapsulation of RS in the form of high-amylose maize starch together with the probiotic microorganisms within the microcapsule.

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Pharmaceutical Applications of Various Natural Gums

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Abstract

Gums are the important ingredients in many pharmaceutical preparations owing to their biodegradability, abundant availability, non-toxicity, and low cost. In today's scenario, these are competing with many synthetic polymers for use as various pharmaceutical products and have made a remarkable journey from just being an excipient to novel drug delivery carriers. Particularly, the better understanding of their physiochemical properties and establishment of safety profile data has attracted researchers and pharmaceutical industries worldwide to unveil the hidden potential of natural gums. Although modification of natural gums has generated the entirely new class of polymeric materials useful in

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advanced drug delivery applications, exploration of natural gums in the design for development of nanomedicine and gene therapy has widened their applications as novel delivery materials. In this chapter, we have highlighted both basic and novel pharmaceutical applications of natural gums along with their future potential.

Keywords
Natural gums • Pharmaceutical applications • Drug delivery • Nanomedicine

1 Introduction

Polysaccharide hydrocolloids including mucilages, gums, and glucans are abundant in nature and are commonly found in many higher plants. These polysaccharides constitute a structurally diverse class of biological macromolecules with a broad range of physicochemical properties, which are widely used for various applications in pharmacy and medicine. In the last few decades, these have gained the reputation of being pharmaceutically acceptable excipients in a variety of dosage forms such as dietary fiber, diluents, binders, disintegrants in tablets, thickeners in liquid orals, protective colloids in suspensions, gelling agents in gels, and bases in suppository (Zatz and Kushla 1989). The niche, however, lies in their untapped potential in the design and development of novel drug delivery systems. Therefore, attention has been paid by the researchers worldwide to explore the potential of plant-based polymers in the design and development of various pharmaceutical dosage forms such as matrix controlled systems, film-coating agents, buccal films, microspheres, nanoparticles, viscous liquid formulations like ophthalmic solutions, suspensions, and implants (Pandey and Khuller 2004; Chamarthy and Pinal 2008; Alonso-Sande et al. 2009). This has proved their worth (applicability and efficacy) in drug delivery.

Even though polysaccharides offer distinct advantages like hydrophilicity, non-toxicity, biodegradability, low cost, and abundant natural availability over those of synthetic materials, their use in pharmaceutical industries is quite limited, and therefore, the natural gums are expected to play a very basic role in pharmaceutical preparations such as excipient in the preparation of cosmetics and conventional drug-loaded formulations. For any polysaccharide to be used as an excipient, they must meet the criteria of "safety for human use" as laid down by the health governing regulatory authorities of various countries. From the regulatory perspective, a thorough investigation of toxicity profile of any new material is necessary before its approval as an excipient for human use. Pharmaceutical industries are well aware of this (regulatory) directive, and therefore, before selecting any potential polysaccharide as an excipient, a detailed investigation of its toxicity profile is established by the well-designed preclinical studies. The data generated by these studies (acute, short-term, and chronic toxicity) are then to be submitted to the respective food and drug administrative bodies of the country for approval. Many gums and mucilages are however obtained from edible sources such as mango

(*Mangifera indica* L., family Anacardiaceae), papaya (*Carica papaya* L., family Caricaceae), banana (*Musa acuminata*, family Musaceae), carambola (*Averrhoa carambola* L., family Oxalidaceae), and guava (*Psidium guajava* L., family Myrtaceae) (Chin et al. 1999), and this makes them a suitable candidate for use in oral drug delivery applications where the toxicity-related regulatory issues can be easily addressed on the ground of their generally recognized as safe (GRAS) status.

2 Gums

Chemically, natural gums and mucilage are polysaccharides or complex carbohydrates, containing one or more types of monosaccharide units or their derivatives linked together in bewildering way and in a variety of linkages and structures to create the macromolecular structure. Upon hydrolysis, they yield simple sugar units such as arabinose, galactose, glucose, mannose, xylose or uronic acids, etc. Natural gums are exudates or pathological products often produced by higher plants as a result of their protection mechanisms following an injury which do not form part of the cell wall. The mucilages, on the other hand, are a part of the cell and are the physiological products (Kulkarni et al. 2002a, b).

The word "gum" often used as an adjective seems to acquire a different meaning. Ideally, there is no chemical category of gums or mucilages, but was once used to describe any substance that produces slimy or tacky aqueous dispersions, whose properties are manifested by many types of water-soluble plant, animal, and microbial polysaccharides, including starches and modified starches. The term "gum" is therefore an umbrella term used commonly to different areas with different definitions in each field. For example, when applied to water-soluble substances, it refers to all water-soluble, non-starch polysaccharides (NSP), whether natural or those that are made from natural NSP by structural modification. Generally, gums are thought to have the ability to produce highly viscous aqueous solutions at low concentrations, but again there are exceptions to this principle. Gum arabic and "low viscosity grades" of certain gums are the most notable exceptions; they require rather high concentrations to produce highly viscous aqueous solutions.

Other exception of the term gum is resinous products; the gum obtained from pine pitch (produced by the parenchyma cells of softwoods) is conventionally known as "gum turpentine" and "gum rosin." The "gum reins" as gum benzoin, gum camphor, and others are also few such examples. The alleged "ester gum" is a semisynthetic reaction product of rosin and a polyhydric alcohol. All these are actually resinous products having properties greatly different from those of natural gums. Further complicating, the word "gum" is also used to address such plant lattices (chicle and natural rubber), which are quite different from both carbohydrate gums and resins.

The starch polysaccharides (amylose and amylopectin) and their derivatives are water soluble and are capable of forming viscous gel, but they are not considered to be gums, and rather they are put in a separate category, whereas water-soluble

polysaccharides (other than those from starches) that are used industrially are referred as industrial gums (BeMiller 2012). Therefore, it is necessary for the reader to understand the term "gum" in its relative field of application.

3 Classification of Natural Gums

Gums constitute a structurally diverse class of biological macromolecules with a broad range of physicochemical properties, and for this reason, they are widely used for various applications in pharmacy and medicine. Like any other chemical substances, it would be helpful to discuss the properties of gum to classify them. But it is not so simple and there is no single useful way to classify gums (BeMiller 2001). Different authors have classified gums in different ways; one such way to classify them is on the basis of their source. The sources of some pharmaceutically useful gums are seaweeds, higher plants, microorganisms, and derivatives of cellulose as shown in Table 1.

Another way to classify gums is on the basis of their charge, shape, gelation behavior, and chemical structures (as shown in Table 2). However, one has to keep this fact in mind that none of these classifications are effective when used alone due to multiple properties of gums. For example, it is suitable to classify the gums on the basis of their structures (Table 2), but here again it is complicated due to two reasons: first, majority of gums have similar structures, and second, in most cases, the structure of a given gum is sufficiently unique that it needs to be placed in a category by itself (BeMiller 2012).

Because of better understanding of structural chemistry, various gums have now received a tremendous attention in the design and development of advanced drug delivery systems. But from the viewpoint of pharmaceutical industries, the use of natural gums in formulations is mainly governed by the regulatory norms of the state or country they are working in. Therefore, detailed knowledge not only of the physical and chemical properties but also of the safety, handling, and regulatory status of these materials is essential for formulation scientists. This requirement creates a new way of classification of gums on the basis of their safety status for human use (Table 3). Regulatory status of gums changes from one country to another, and therefore, for detailed information and further clarification, websites of regulatory bodies of referred country should be consulted for guidance.

4 Advantages and Limitations of Natural Gum

Natural polysaccharide gums are renewable biopolymers found abundantly in nature. They are non-toxic, biodegradable, economic, and independent of fossil sources, which make them an ideal candidate for use in non-food industries over the synthetic molecules (Chang et al. 2009). Furthermore, most of the natural gums are safe enough for oral consumption in the form of food additives or drug carriers. Gums are metabolized by intestinal microflora and ultimately degraded to their individual component sugars. The enzymes available in the intestine can cleave the

Table 1 Classification of gum on the basis of source (Taken from BeMiller (2012) http://www.springerreference.com/index/chapterdbid/135002)

Origin/source	Gums
Marine algae	Agars, alginates, carrageenans, and furcellaran
Higher plants	
Extract	Larch arabinogalactan, pectins
Seeds	Guar gum, locust bean (carob) gum, tara gum
Tubers and root	Inulin, konjac glucomannans
Exudates	Gum arabics
Microorganism	Curdlan, dextrans, gellans, xanthans
Cellulosics (by chemical modification)	Carboxymethylhydroxyethylcelluloses, cetylhydroxyethylcelluloses, ethylhydroxyethylcelluloses, hydroxyethylcelluloses, hydroxyethylmethylcelluloses, hydroxypropylcelluloses, hydroxypropylmethylcelluloses, methylcelluloses

Table 2 Classification of gums (Taken from Prajapati et al. (2013) Pharmaceutical applications of various natural gums, mucilages and their modified forms. Carbohydrate polymers 92: 1685–1699. Reprinted with permission from Elsevier copyright clearance center)

Basis	Class	Examples
Charge	Nonionic gums	Guar gum, locust bean gum, tamarind gum, xanthan gum
	Anionic gums	Gum arabic, karaya gum, gellan gum, carrageenans
Shape	Short branch	Xanthan gum, guar gum
	Branch on branch	Gum arabic, tragacanth gum
Origin	Seed gums	Guar gum, karaya gum, ipomoea, fenugreek, locust bean gum, premcem gum, Lesquerella fendleri gum
	Plant exudates	Chicle gum, konjac, gum arabic, gum ghatti, gum karaya, acacia gum, tragacanth
	Microbial exudates	Dextran, gellan gum, xanthan gum, tara gum, spruce gum
	Seaweed	Sodium alginate, alginic acid, carrageenans, agar–agar
Gelation behavior	Cold set gels	Gellan gum, flaxseed gum, gelatin
	Heat set gels	Konjac
	Re-entrant gels	Xyloglucan
Chemical structure	Galactomannans	Fenugreek gum, guar gum, locust bean gum, tara gum, dhaincha gum, cassia gum
	Glucomannans	Konjac
	Uronic acid containing gums	Xanthan gum
	Tri-heteroglycans	Gellan gum
	Tetra-hetroglycans	Gum arabic, psyllium seed gum
	Penta-hetroglycans	Gum ghatti, tragacanth

Table 3 Classification based on regulatory status

Class/category	Regulatory status	Gums
Food/food additives	GRAS[a], CLNAI[b]	Acacia, gaur gum, gum ghatti, carob bean gum
Licensed pharmaceuticals	FDA inactive ingredients, excipient in nonparenteral medicine	Sodium alginate, acacia, gaur gum, xanthan gum

[a]Generally recognized as safe
[b]Canadian list of acceptable non-medicinal ingredients

gums at specific sites. For example, α-galactosidase can hydrolyze terminal non-reducing galactose residues to produce free α-D-galactose (Rana et al. 2011). These properties make gums to stand tall over the synthetic polymers. In spite of these advantages, the use of polysaccharides as industrially useful raw material at a large scale is impeded by some drawbacks associated with gums like uncontrolled rate of hydration, microbial contamination, reduced viscosity on storage, pH-dependent solubility, and batch-to-batch variation (Jani et al. 2009; Rana et al. 2011). Therefore, researchers are now prompted to work on the chemical modification of natural gums to overcome these limitations without the loss of their inherent characteristics. Modification of natural polysaccharides gives tailor-made materials suiting the various needs of drug delivery systems and thus can compete with the available synthetic excipients.

5 Pharmaceutical Applications

Due to intricate molecular structure, gums show diverse physicochemical properties, and such properties are highly useful in the design of various pharmaceutical preparations such as in suspensions and emulsions as suspending and emulsifying agents, respectively, in liquid orals as thickening and stabilizing agent, in conventional tablet dosage form as binder and disintegrating agent, in gels as gelling agent, and in cosmetics as emollient and demulcent. The list of commonly used gums for pharmaceutical applications is shown in Table 4.

From application viewpoint, pharmaceutically gums can be categorized into two major classes: (a) as excipient for conventional dosage form and (b) as polymeric material for novel drug delivery application. Initially, gums were explored as excipient in pharmaceutical industries for their cohesive and adhesive properties, and till today, many commercial formulations include gums as one of their important ingredients. Literature review revealed several applications of gum in pharmaceutical industries.

5.1 As Excipient for Conventional Dosage Form

Excipients are the materials used to: (a) increase the bulk of formulation, (b) improve the physicochemical characteristics of the product, and (c) increase

Table 4 Pharmaceutical applications of natural gum (Taken from Prajapati et al. (2013) Pharmaceutical applications of various natural gums, mucilages and their modified forms. Carbohydrate polymers 92: 1685–1699. Reprinted with permission from Elsevier copyright clearance center)

S. no	Common name	Botanical name	Family	Pharmaceutical applications	References
1	Agar	*Gelidium amansii*	Gelidaceae	Suspending agent, emulsifying agent, gelling agent in suppositories, surgical lubricant, tablet disintegrants, medium for bacterial culture, laxative	John et al. (2006)
2	Albizia gum	*Albizia zygia*	Leguminosae	Tablet binder	Oluwatoyin (2005)
3	Carrageenan	*Chondrus crispus*	Gigarginaceae	Gelling agent, stabilizer in emulsions and suspensions, in toothpaste, demulcent, and laxative	Ahmed and Mutasim (2005), Bonferoni et al. (1993, 1994)
4	Cashew gum	*Anacardium occidentale*	Anacardiaceae	Suspending agent	Pontes (1971), Zakaria and Zainiah (1996)
5	*Cassia tora*	*Cassia tora* Linn.	Leguminosae	Binding agent	Pawar and D'mello (2004)
6	Guar gum	*Cyamopsis tetragonolobus*	Leguminosae	Binder, disintegrant, thickening agent, emulsifier, laxative, sustained release agent, colon targeted drug delivery, cross-linked microspheres	Krishnaiah (2001, 2003), Chourasia and Jain (2004), Saleh et al. (2005)
7	Gum acacia	*Acacia arabica* and *Acacia senegal*	Leguminosae	Suspending agent, emulsifying agent, binder in tablets, demulcent and emollient in cosmetics, osmotic drug delivery	Lu et al. (2003), Beneke et al. (2009)
8	Gum ghatti	*Anogeissus latifolia*	Combretaceae	Binder, emulsifier, suspending agent	Jain and Dixit (1988)
9	Gum tragacanth	*Astragalus gummifer*	Leguminosae	Suspending agent, emulsifying agent, demulcent, emollient in cosmetics, and sustained release agent	Owen (2003)

(*continued*)

Table 4 (continued)

S. no	Common name	Botanical name	Family	Pharmaceutical applications	References
10	Karaya gum	*Sterculia urens*	Sterculiaceae	Suspending agent, emulsifying agent, dental adhesive, sustaining agent in tablets, bulk laxative, mucoadhesive	Sreenivasa et al. (2000), Munday and Philip (2000), Park and Munday (2004)
11	Khaya gum	*Khaya grandifoliola*	Meliaceae	Binding agent	Odeku and Itiola (2003)
12	Leucaena seed gum	*Leucaena leucocephala*	Fabaceae	Emulsifying agent, suspending agent, binder in tablets, disintegrating agent in tablets	Verma and Razdan (2001, 2002a, b, 2003a, b)
13	Pectin	*Citrus aurantium*	Rutaceae	Thickening agent, suspending agent, protective agent, beads, floating beads, colon drug delivery, microparticulate drug delivery, transdermal drug delivery, iontophoresis, hydrogels	Pornsak (1998), Giunchedi et al. (1999), Sungthongjeen et al. (1999), Vandamme et al. (2002), Tho et al. (2002), Musabayane et al. (2003), Pornsak et al. (2007)
14	Sodium alginate	*Macrocystis pyrifera*	Lessoniaceae	Suspending agent, gelation for dental films, stabilizer, sustained release agent, tablet coating, mucoadhesive microspheres	Howard and Timmins (1988), Viernstein (1988), Seiyaku (1989), Alison et al. (1995)
15	Tamarind seed polysaccharide	*Tamarindus indica*	Leguminosae	Binding agent, emulsifier, suspending agent, sustaining agent, hydrogels, mucoadhesive agent, and nasal drug delivery	Kulkarni et al. (1997, 2005), Datta and Bandyopadhyay (2006)
16	Xanthan gum	*Xanthomonas campestris*	Xanthomonadaceae	Suspending agent, emulsifier, stabilizer in toothpaste and ointments, sustained release agent, buccal drug delivery system	Dhopeshwarkar and Zatz (1993), Santos et al. (2005), Vendruscolo et al. (2005), Ganesh et al. (2011)

17	Gellan gum	*Pseudomonas elodea*	–	Disintegrating agent, floating drug delivery system, ophthalmic drug delivery, sustaining agent, hydrogels	Rozier et al. (1997), Coviello et al. (1998), Kedzierewicz et al. (1999), Miyazaki et al. (2001b), Agnihotri et al. (2006), Rajinikanth and Mishra (2007), Rajnikanth et al. (2007)
18	Locust bean gum	*Ceratonia siliqua*	Fabaceae	Thickener, stabilizer, and controlled release agent	Xiaohong et al. (2003), Deshmukh et al. (2009)
19	Neem gum	*Azadirachta indica* A. Juss.	Meliaceae	Suspending agent, binder, and transdermal film-forming agent	Kulkarni et al. (2002a, b)
20	Badam gum	*Prunus amygdalus*	Rosaceae	Binding, sustaining, and transdermal film-forming agent	Kulkarni et al. (2002a, b)
21	–	*Caesalpinia pulcherrima*	Fabaceae	Mucoadhesive agent	Sudarshan et al. (2010)
22	–	*Leucaena leucocephala*	Fabaceae	Mucoadhesive agent, emulsifier and binder	Deodhar et al. (1998), Verma and Razdan (2003b), Sudarshan et al. (2010)
23	–	*Cissus populnea*	Amplidaceae	Binding agent	Eichie and Amalime (2001)
24	–	*Acassia senegal*	Leguminosae	Binder, disintegrant	Eichie and Amalime (2001)
25	Okra gum	*Hibiscus esculentus*	Malvaceae	Binder and hydrophilic matrix for controlled release drug delivery	Kalu et al. (2007), Amelia et al. (2011)
26	–	*Sterculia foetida*	Malvaceae	Controlled release preparation	Amelia et al. (2011)
27	Honey locust gum	*Gleditsia triacanthos*	Fabaceae	Matrix tablet, sustained release formulation	Amelia et al. (2011)
28	Tara gum	*Caesalpinia spinosa*	Leguminosae	Thickener, stabilizer	Amelia et al. (2011)
29	Hakea gum	*Hakea gibbosa*	Proteaceae	Sustained release formulation, mucoadhesive agent	Hemant et al. (1999), Alur et al. (1999, 2000), Amelia et al. (2011)

(*continued*)

Table 4 (continued)

S. no	Common name	Botanical name	Family	Pharmaceutical applications	References
30	Konjac	*Amorphophallus konjac*	Araceae	Controlled release formulation, gelling agent	Amelia et al. (2011)
31	–	*Mimosa scabrella*	Mimosaceae	Release controlling agent	Amelia et al. (2011)
32	–	*Mimosa pudica*	Mimosaceae	Sustained release material	Amelia et al. (2011)
33	Hupu gum (gum kondagogu)	*Cochlospermum gossypium*	Cochlospermaceae	Gastric floating drug delivery	Amelia et al. (2011)
34	–	*Lepidium sativum*	Cruciferae	Controlled release formulation	Amelia et al. (2011)
35	Gum copal	*Bursera bipinnata*	Burseraceae	Film-forming agent, coating material for sustained release, and colon targeted drug delivery	Amelia et al. (2011)
36	Gum damar	*Shorea wiesneri*	Dipterocarpaceae	Water-resistant coating material, sustained release formulation	Amelia et al. (2011)
37	Moi gum	*Lannea coromandelica* (Houtt.) Merrill.	Anacardiaceae	Microencapsulating agent, release rate controlling material	Amelia et al. (2011)
38	*Moringa oleifera* gum	*Moringa oleifera*	Moringaceae	Mucoadhesive agent, disintegrant, and binder	Mitul et al. (2012)
39	–	*Abelmoschus esculentus*	Malvaceae	Sustained release formulation and binder	Ofoefule and Chukwu (2001), Ofoefule et al. (2001)
40	Mucuna gum	*Mucuna flagillepes*	Papillionaceae	Microencapsulating agent	Anthony and Obichukwu (2007)
41	Grewia gum	*Grewia mollis*	Malvaceae	Suspending agent, binder	Elijah and Barbara (2010)
42	*Myrrh oleo* gum	*Commiphora myrrha*	Burseraceae	Mucoadhesive agent	Gurpreet et al. (2011)

43	Gum cordia	*Cordia obliqua*	Boraginaceae	Enteric-resistant and sustained release material	Subas and Biswajit (2009); Mukherjee et al. (2008)
44	Sesbania gum	*Sesbania grandiflora*	Leguminosae	Gelling agent	Gayatri and Madhabhai (2009)
45	Katira gum	*Cochlospermum religiosum*	Bixaceae	Colon drug delivery	Bharanirajaa et al. (2011)
46	Malva nut gum	*Scaphium scaphigerum*	Sterculiaceae	Stabilizer, thickening agent	Somboonpanyakul et al. (2006)
47	Welan gum	Produced by fermentation using *Alcaligenes* species CGMCC2428	Alcaligenaceae	Thickening agent	Hui et al. (2012)
48	Bhara gum	*Terminalia bellerica*	Combretaceae	Microencapsulation	Nayak et al. (2008)

the stability of formulation without affecting the drug or active ingredient adversely. Pharmaceutically, gums have received a special attention as promising excipients in various dosage forms.

5.1.1 Binder and Disintegrating Agent in Tablet Dosage Form

The role of gums as a binder is attributed to its adhesive property when mixed with water. Gums are used for binding the powder blend into a cohesive mass for the preparation of the granules, which is then compressed to form a tablet. A large number of gums are reported to be used as a binder in tablet formulations (see Table 4). Due to hydrophilic nature, gums imbibe a large quantity of water and swell enormously before they solubilize. This feature makes them an ideal disintegrating agent. When gums are added into tablet formulation as disintegrating agent, they increase the internal pressure (by absorbing the dissolution fluid) to an extent that the structural integrity of tablet disrupts, leading to disintegration and disaggregation of tablet into fine particles, resulting in a rapid solubility and, hence, rapid dissolution of admixed drug(s).

5.1.2 Hydrophilic Gelling Agent

In recent times, the potential of natural gums as gelling agent has been realized. They can form hydrophilic gels all alone or in combination with other natural gums and synthetic polymers. Gellan gum, carrageenan, pectin, sodium alginate, and gelatin are the widely used gelling agents in pharmaceutical industries. The multiple sugar units in gums, apart from hydroxyl groups, may also contain carboxylic groups (uronic acid derivatives), and these hydrophilic groups can enter into both H-bonding (inter and intra) and weak van der Waal forces with water molecule to make three-dimensional network aggregates capable of holding a large quantity of water. Researchers have also reported the physical (like pH and temperature) and chemical (ionic solutes and cross-linkers) methods to transform natural gums into gels. For example, the gelation of gellan gum with citrate ions is reported to produce soft gels for the delivery of paracetamol (Gohel et al. 2009). Most of the natural gums, due to their branched structure, need higher concentrations to reach a satisfactory gelling strength. In addition, the ratio of gelling component versus non-gelling component in gum structure decides its gel strength. A study on gum ghatti by Pass and Phillips (1977) has demonstrated the effect of change in gelling component on the overall gelling capability of gum ghatti. Xanthan gum, on the other hand, is an example of natural gum, which forms a strong gel at much lower concentrations as compared to many natural gums and synthetic polymers. Various examples of natural gums as gelling agent are given in Table 4.

5.1.3 Emulsifier and Suspending Agent

As can be seen from Table 4, numbers of gums are reported to be used as emulsifier and suspending agents in pharmaceutical preparations. Principally, gums stabilize the emulsions by slowing down the thermodynamically favored breakdown and suspensions by discouraging the crystallization by means of increasing the viscosity

of the medium. The other probable factors behind stabilizing property of gums in emulsion includes: (a) the structural heterogeneity of gums, which may lead to self-aggregation (as in gum ghatti and gum acacia) at higher concentrations, and (b) the presence of protein impurity, which may undergo electrostatic interaction with the gum (polyelectrolyte). Both these factors lead to the formation of colloidal system capable of increasing the miscibility of oils and water by reducing the interfacial tension between them (http://www1.lsbu.ac.uk/water/hydro.html). However, in case of suspensions, it is believed that natural gum increases the tensile strength of the hydration layer formed around the suspended particles by H-bonding and molecular interactions. Since these agents do not reduce the surface and interfacial tension, they function best in the presence of wetting agents (Prajapati et al. 2013). But increase in viscosity of the system is the most accepted reason for suspension stability.

5.1.4 Coating Agent

Gums are the good coating agents used in pharmaceutical preparation for two purposes: (a) to protect the drug from degradation in specific part of gastrointestinal tract (GIT) and (b) to sustain the drug release. Both granular coating (Mitul et al. 2012) and tablet coating (Joshi et al. 2010) are reported in the literature (see Table 4). In a study carried out by Ogaji and Okafor (2011), the potential of grewia gum extract as aqueous film-coating material for pharmaceutical application was demonstrated, wherein the aqueous dispersion of gum was used to coat the praziquantel tablet. The study showed that coating of grewia gum extract remained intact, durable and resistant to chipping and other coating issues when challenged to catastrophic fall or rubbed on a white paper (Ogaji and Okafor 2011; Ogaji et al. 2013). In another study, compression coating of tablet using guar gum and xanthan gum for colon targeted drug delivery is reported (Niranjan et al. 2013). Both these gums have provided the satisfactory delivery of drug in the colonic region by protecting the premature release of drug in the upper part of GIT. One of the major advantages of employing natural gum in oral drug delivery is its biodegradability. As gums are composed of repeating sugar units, they get easily digested by microflora of colonic region giving biocompatible metabolites and thus ensuring complete drug release and biocompatibility together.

5.2 Gums in Drug Delivery

With the advancement of technology and better understanding in fields like molecular biology, pharmacokinetics, and drug discovery, in the last 5 years, drug market has witnessed the availability of better therapeutic alternatives over the conventional therapies. These advanced drug delivery systems not only ensured improved efficacy and safety of pharmaceutical preparations but also improved the overall therapeutic experience of the patient. Interestingly, natural gums have captured their share in this technological pursuit and were evaluated for the development of a variety of novel dosage forms.

5.2.1 Matrix Tablets and Patches

The formulation of matrix-based delivery systems such as tablet and patches is widely accepted pharmaceutically, due to better patient compliance and ease of formulation procedures. Matrix-type delivery system is the specific type of release system, which prolongs and controls the release of a drug that is either dissolved or dispersed into it. Many natural gums have been investigated as matrix forming materials for the design and development of oral tablet to achieve sustained and controlled drug delivery. Both matrix dispersion type (Demiröz et al. 2004) and encapsulated or coated type (Joshi et al. 2010) matrix tablet are studied for the delivery of drugs in GIT. The drug transport mechanisms from this type of matrix system are governed by mathematical models like diffusion controlled, swelling controlled, and erosion controlled, either alone or in combination (Ritger and Peppas 1997). The gel layer formation at the interface between dry polymer and surrounding dissolution fluid by hydration of polymeric chains is assumed to be responsible for retarding the drug release. Tables 4 and 5 represent the examples of various natural gums used as matrix tablet formulation.

Patches are thin-film-type formulation applied either on the skin or mucous membrane for prolonged drug delivery. The mechanism of drug release is similar as discussed for the matrix tablet. A number of reports are available mentioning the utility of natural gums for the design of mucoadhesive and dermal patches (refer to Tables 4 and 5). Being hydrophilic, viscous solution of gums can be easily prepared in water and cast to prepare films or patches of different dimensions. Furthermore, based on the features of retarding gum, they are used alone or in combination with other polymers to meet the desired specifications. The preparation of mucoadhesive patches by polyelectrolyte cross-linking called inter-polymer complex (IPC) (Kaur and Kaur 2012) is relatively new and is a niche area for active researchers. One such a study performed by Singh et al. (2010) reported the novel IPC formulation formed between xanthan gum and cationic gaur gum to develop novel mucoadhesive films for sustained delivery of domperidone. The developed IPC bio-adhesive films released the drug for 8 h as well as enhanced the bioavailability of the drug compared to the orally administered conventional tablet.

In another study, the bilayer nicotine mucoadhesive transdermal patches were prepared using xanthan gum and Carbopol 934 as mucoadhesive polymers and ethylcellulose as the backing membrane. The results have shown significant improvement in mucoadhesive strength, drug–polymer compatibility, and controlled biphasic drug release for up to 10 h using xanthan gum compared to Carbopol (Abu-Huwaij et al. 2011).

5.2.2 Microparticulate Systems

Microparticles or microbeads are multi-unit drug delivery systems ranging in size between 10 and 2,000 μm. These are widely studied for oral, parenteral, and ocular drug delivery applications. A number of synthetic, semisynthetic, and natural polymers have been used to prepare microparticles using various techniques. For oral drug delivery, microparticles have received much popularity because of more uniform distribution of drug in the GIT, more uniform drug absorption, reduced

Table 5 Pharmaceutical applications of natural gum in drug delivery

Sr. No	Natural gum	Drug	Dosage form	References
1	Acacia	Gentamycin	Hydrogel wound dressings	Singh et al. (2013)
2	Acacia	Nicotine	Chewing gum	Aslani and Rafiei (2012)
3	Acacia	Pheromone	Microcapsules	Gu et al. (2010)
4	Acacia	5-Fluorouracil	Dry emulsion	Wang et al. (2010)
5	Acacia	Flurbiprofen	Sustained release matrix tablets	Shah et al. (2009)
6	Acacia	Vinpocetine	SMEDDS versus solid dispersion	Chen et al. (2009)
7	Acacia	Microbicides	Vaginal rings	Saxena et al. (2009)
8	Acacia	Tamoxifen	Microcapsules	Ma et al. (2009)
9	Acacia	Nonhormonal contraceptives and anti-HIV agents	Vaginal rings	Han et al. (2007)
10	Acacia	Extract containing shikonin	Microcapsules	Huang et al. (2007)
11	Acacia	Low molecular weight heparin	Microparticles	Lamprecht et al. (2007)
12	Acacia	Puerarin	Solid self-microemulsion	Yu et al. (2006)
13	Acacia	Theophylline	Core-in-cup tablet	Danckwerts et al. (1998)
14	Acacia	Rhbmp-2	Minipellet	Maeda et al. (2004)
15	Acacia	Eicosapentaenoic acid	Microcapsules	Jouzel et al. (2003)
17	Acacia	Nifedipine	Tablets	Varshosaz and Dehghan (2002)
18	Acacia	Hyaluronic acid and chitosan	Microspheres	Lim et al. (2000)
19	Acacia	Rifampicin	Multiple w/o/w emulsion	Nakhare and Vyas (1995)
20	Acacia	Diclofenac sodium	Microcapsules	Bhatnagar et al. (1995)
21	Acacia	Acetylsalicylic acid	Liposomes	Dong and Rogers (1993)
22	Acacia	Triterpene saponins		Pakrashi et al. (1991)
23	Acacia and tragacanth	Verapamil HCl	1- and 3-Layer matrices tablet	Siahi et al. (2005)
24	Acacia and tragacanth	Alkannin	Microcapsules	Assimopoulou and Papageorgiou (2004)
25		Glipizide		

(*continued*)

Table 5 (continued)

Sr. No	Natural gum	Drug	Dosage form	References
	Acacia and ghatti gum		Microwave-generated	Kushare and Gattani (2013)
26	Guar gum	Dexamethasone	Tablets	Kenyon et al. (1997)
27	Guar gum	Indomethacin	Matrix tablets	Prasad et al. (1998)
28	Guar gum	Albendazole	Matrix tablets	Krishnaiah (2003)
29	Guar gum	Mebendazole	Matrix tablets	Krishnaiah et al. (2001)
30	Guar gum	Diltiazem	Matrix tablets	Ravi et al. (2008)
31	Guar gum	Ornidazole	Matrix tablets	Krishnaiah et al. (2003b)
32	Guar gum	Tinidazole	Tablets	Krishnaiah et al. (2003a)
33	Guar gum	Calcium sennosides	Matrix tablets	Momin (2004)
34	Guar gum	Mesalazine	Tablets	Demiröz et al. (2004)
35	Guar gum	Rofecoxib	Matrix tablets	Al-Saidan et al. (2005)
36	Guar gum	Albendazole	Matrix tablets	Shyale et al. (2006)
37	Guar gum	Ondansetron	Matrix tablets	Demiroz and Takka (2006)
38	Guar gum	Indomethacin	Pellets	Ji et al. (2007)
39	Guar gum	Metronidazole	Tablets	Mundargi et al. (2007)
40	Guar gum	BSA	Hydrogels	George and Abraham (2007)
41	Guar gum	5-Fluorouracil	Tablets	Krishnaiah et al. (2003c)
42	Guar gum	Itraconazole	Mucoadhesive tablet	Shaikh et al. (2012)
43	Gaur gum	Ephedrine HCl and sulfadimidine	Tablets	Elsabbagh et al. (1978)
44	Gum ghatti	Domperidone	Sustained release mucoadhesive matrix tablets	Arora et al. (2012)
45	Gum ghatti	Antibacterial	Silver nanoparticles	Kora et al. (2012)
46	Gum ghatti	Gum ghatti and Fe_3O_4	Magnetic nanoparticle-based nanocomposites	Mittal and Mishra (2014)
47	Gum ghatti	Diclofenac sodium	Interpenetrating polymer matrix network microparticles	Reddy et al. (2014)

(*continued*)

Table 5 (continued)

Sr. No	Natural gum	Drug	Dosage form	References
48	Gum ghatti	Ofloxacin	Polyelectrolyte nanoparticles	Shelly et al. (2013)
49	Tragacanth	Amoxicillin	pH-responsive hydrogels	Singh and Sharma (2014)
50	Tragacanth	Diltiazem	Sustained release matrices	Adibkia et al. (2013)
51	Tragacanth	Diclofenac sodium	Mucoadhesive microspheres	Amin et al. (2013)
52	Tragacanth	Propranolol	Sustained release matrix tablet	Saeedi et al. (2013)
53	Tragacanth	Ibuprofen and theophylline	Pellets	Akhgari et al. (2011)
54	Tragacanth	Metoprolol tartrate	Tablets	Rasul et al. (2010)
55	Tragacanth	Albendazole	Tablet	Gohel et al. (1996)
56	Tragacanth	Diclofenac sodium tablets	Sustained release matrix tablet	Iqbal et al. (2011)
57	Tragacanth	Ascorbic acid and phenobarbitone	Tablet	Asker et al. (1975)
58	Tragacanth	5-Aminosalicylic acid	Suspension	Montgomery et al. (1986)
59	Xanthan gum	Labetalol HCl	Mucoadhesive buccal tablets	Ganesh et al. (2011)
60	Xanthan gum	Caffeine, indomethacin, sodium indomethacin	Matrix tablets	Talukdar and Kinget (1995)
61	Xanthan gum	Cellulose–polymer–Ag nanocomposite	Nanocomposite fibers scaffolds	Raghavendra et al. (2013)
62	Xanthan gum	5-FU	Compressed coated tablets	Sinha et al. (2007)
63	Xanthan gum and guar gum	Dipyridamole	Floating matrix tablets	Patel and Patel (2007)
64	Xanthan gum:guar gum	5-FU	Matrix tablets	Sinha et al. (2004)

local irritation, and elimination of unwanted intestinal retention of polymeric material compared to non-disintegrating single-unit dosage form such as tablets. The gums are mostly polyanionic in nature (except few neutral gums) and thus offer a number of suitable sites for reaction with a variety of chemical agents to develop microparticles. Furthermore, their aqueous solubility reduces the concern of residual solvents in the final formulation and improves the biosafety of formulations. Locust bean gum (Deshmukh et al. 2009), Mucuna gum (Anthony and Obichukwu 2007), and xanthan gum (Ray et al. 2010) are some of the commonly used gums for the design of microparticles based oral controlled drug delivery systems and have gained a worldwide interest, mainly because of their biocompatibility.

Gellan gum is one of the extensively studied biopolymers used for parenteral applications. A number of studies have demonstrated its utility in parenteral preparation. Mahajan and Gattani (2009) reported microparticles of metoclopramide hydrochloride prepared by spray drying technique using gellan gum as a biocompatible material for intranasal drug delivery. The results showed that the process was simple, reproducible, and easy to scale up. The microencapsulation of drug was particularly less dependent on the solubility characteristics of polymer, and gum-based microparticles effectively delivered the drug through the nasal mucosa.

In another study, gellan gum was used to prepare novel microparticles/hydrogel matrices for use in intervertebral disc regeneration that improved mechanical properties. However, their cell culture studies (in L929 cells) demonstrated noncytotoxicity of the formulated discs that makes them a strong candidate for nucleus pulposus regeneration (Pereira et al. 2011). Odeku et al. (2013) recently investigated four natural gums, viz., albizia, cissus, irvingia, and khaya gums, to develop microbeads for the controlled delivery of diclofenac sodium. Gel blends of natural gums and sodium alginate at different ratios were tested. Drug release from the beads containing polymer blends of four gums and sodium alginate fitted the Korsmeyer–Peppas model and sustained the release of drug for 5 h. However, overall characteristics of the microbeads depended on the material property of individual gums.

5.2.3 In Situ Gels

In situ gels are relatively new type of pharmaceutical preparations, wherein drug-loaded viscous polymeric solution called sol, when comes into contact with stimulus (such as pH, temperature, enzyme, and ionic concentration.), present at the various locations of human body, get transformed into a gelled mass instantly (in situ) to prolong the drug release. In situ gels are administered by various routes, viz., oral, ocular, rectal, vaginal, subcutaneous, and intraperitoneal. Natural gums, being water soluble, biocompatible (except few), and polyionic in nature, find obvious advantages over those of the synthetic polymers. Gellan gum and xyloglucan are the widely studied gums for the preparation of in situ gels, and when xyloglucan is partially degraded by β-galactosidase, the resultant product exhibits thermally reversible gelation by the lateral stacking of rodlike chains (Miyazaki et al. 2001a). The sol–gel transition temperature varies with the degree of galactose elimination. Xyloglucan-based in situ gels have been used for oral, intraperitoneal, ocular, and rectal drug delivery (Miyazaki et al. 1998; Suisha et al. 1998; Kawasaki et al. 1999; Miyazaki et al. 2001a). The thermoreversible gelation behavior of xyloglucan is quite similar to poloxamer, but the major advantage of using xyloglucan is its capacity to gel at much lower concentrations as compared to poloxamer (Miyazaki et al. 1998).

Gellan gum (also known as Gelrite^{TM} or Kelogel^{TM}) is an anionic polysaccharide having both thermal- and ionic-induced gelling capacity (Crescenzi et al. 1990). The in situ gel of gellan gum prepared by calcium ion-induced gelling for the oral delivery of theophylline has been investigated that showed better

bioavailability and prolonged release in rabbits compared to the marketed sustained release liquid formulation (Miyazaki et al. 1999). In another study, Cao et al. (2009) developed in situ gel using gellan gum as a carrier for nasal administration of mometasone furoate. The histopathology of rat nasal cavity showed that in situ formulation of gellan gum was non-toxic to the nasal mucosa. The drug release was equally effective in controlling the nasal symptoms of rhinitis compared to the marketed formulation Nasonex^{TM}.

5.2.4 Nanoparticles

In the past decade, rapid development of nanotechnology has brought many fascinating ideas and opportunities to the diagnosis and treatment of a disease. Nanotechnology has revolutionized the whole concept of drug delivery from nonspecific drug delivery to highly target-specific drug delivery. The utilization of natural gums in the design of nanostructures is still in its infancy and relatively few studies are reported where natural gums are evaluated for the development of nanomedicine. One such a study was carried out by Yadav and Ahuja (2010) where novel polymer-surfactant nanoparticles of gum cordia (as the polymeric carrier) were evaluated and optimized for ophthalmic delivery of fluconazole using a response surface model. A w/o/w emulsion containing fluconazole and cordia gum in aqueous phase, methylene chloride as the oily phase, and di-octyl sodium sulfosuccinate and polyvinyl alcohol as the primary and secondary emulsifiers, respectively, was cross-linked by ionic gelation technique to produce fluconazole-loaded nanoreservoir system. Comparison of in vitro release profiles of the optimized nanosuspension formulation (containing gum cordia 0.85 %) with commercial formulation provided a comparable corneal permeability of fluconazole across the isolated goat cornea. These results have demonstrated the importance of gum cordia in the design and development of nanosuspensions.

In another study, Kumar and Ahuja (2013) have studied the interaction between carboxymethyl gum kondagogu (also known as hupu gum) and chitosan to prepare polyelectrolyte complex-based nanoparticles for sustained delivery of ofloxacin as model drug. The optimized batch of nanoparticles (285.9 nm) successfully delivered the drug in a sustained manner for 24 h, following the Higuchi's diffusion-controlled kinetic model. This study opened new possibilities for further investigation on this gum for many other potential pharmaceutical applications. On the other hand, the possibility of using guar gum for the synthesis of nanoparticles as drug carrier has been recently explored by Gupta and Verma (2014), wherein carboxymethyl guar gum nanoparticles were synthesized by using nanoprecipitation and sonication method. The size of the nanoparticles was 12–30 nm and these nanoparticles may have utility in medical, cosmetics, biosensors, pharmaceuticals, and food supplements areas.

5.2.5 Hydrogels

Hydrogel is a three-dimensional cross-linked network with high water retention capacity. It neither disintegrates nor dissolves in excess amount of water. Nowadays, natural gum-based hydrogels as drug delivery systems have gained a much

wider attention. Although natural gums are the potential candidates in the design of hydrogels, but due to their high water solubility and drop in viscosity upon storage, the functionalization/modification is needed to overcome these problems. Further modification helps to control the interaction of the polymer with drugs, to enhance the load capability, and to tailor the release profiles of the drug (Nishimura et al. 1993). In view of the pharmacological importance of sterculia gum and drug delivery devices based on hydrogels, Singh and Sharma (2008) synthesized sterculia gum- and polyacrylamide-based hydrogels. The release dynamics of model antiulcer drug (ranitidine hydrochloride) from these hydrogels was studied to evaluate the release mechanism and diffusion coefficients in different media. The results showed that the release of drug from the sterculia gum-based hydrogels was of Fickian nature in distilled water and in pH 2.2 buffer and was non-Fickian in pH 7.4 buffer.

D'Arrigo et al. (2012) have demonstrated the usefulness of gellan gum in the preparation of nanohydrogels. They developed and characterized self-assembling nanohydrogels based on sonicated gellan gum chains. Prednisolone, a poorly water-soluble anti-inflammatory drug, was chemically conjugated to carboxylic groups of gellan gum that served as the hydrophobic moiety responsible for self-assembling process. From the results, it was concluded that these nanohydrogels represent an interesting and innovative carrier system that improved the bioavailability of poorly water-soluble prednisolone.

In another study, D'Arrigo et al. (2014) have developed gellan gum nanohydrogels to deliver simultaneously anti-inflammatory and anticancer drugs (as a combination therapy) for the treatment of cancer. However, the anti-inflammatory drug prednisolone was chemically linked to carboxylic groups of gellan gum to serve as a hydrophobic moiety promoting nanohydrogel formation, whereas paclitaxel was physically entrapped in it by the hydrophobic core–shell mechanism. These nanohydrogels improved the drug performances by acting as solubility enhancer, thus favoring the drug uptake into the cells. Moreover, nanohydrogels allowed increased cytotoxic effect in vitro on several types of cancer cells due to synergistic effect of the combination of anti-inflammatory and anticancer drugs.

6 Modified Gums in Pharmaceuticals

As discussed in Sect. 4, natural gum suffers from various limitations, and hence, it is to be chemically modified (to improve the functionality) for their use in specific drug delivery purposes (Rana et al. 2011). A list of some chemically modified gums used in pharmaceuticals is presented in Table 6. The science of chemical modification is very exhaustive, and hence, we restrict our discussion to some of the commonly used chemical modification techniques and their relevant pharmaceutical applications in drug delivery area. Gums can be chemically modified by three techniques: (a) chemical cross-linking, (b) substitution of functional group, and (c) grafting modification.

Table 6 Examples of modified gum for pharmaceutical applications

Gum	Type of chemical modification	Pharmaceutical applications	References
Gaur gum	Carboxymethylation	Microbeads for sustained drug delivery	Thimma and Tammishetti (2001)
Konjac glucomannan	Carboxymethylation	Microbeads; polymeric carrier for site-specific bioactive drug delivery	Jiangyang et al. (2008)
Cashew gum	Carboxymethylation	Carrier for controlled drug delivery	Silva et al. (2004)
Gaur gum	Phosphate cross-linked	Colon targeted drug delivery	Kabir et al. (2000)
Gaur gum	Cross-linked with glutaraldehyde	Microbeads for controlled drug delivery	Soppimath et al. (2000)
Tamarind gum	Cross-linked with epichlorohydrin	Rectal drug delivery	Sumathi and Ray (2002)
Cashew gum	Cross-linked with epichlorohydrin	Controlled drug delivery	Silva et al. (2006)
Gellan gum	Acetalated gellan gum cross-linked with aluminum ions	Hydrogel network beads for prolonged drug release	Maiti et al. (2011)
Gaur gum	Grafting of polyacrylamide chains by microwave-assisted method	Matrix for colon targeted drug delivery	Sen et al. (2010)
Gum kondagogu	Grafting of polyacrylamide chains by microwave-assisted method	Controlled release matrix tablet	Malik and Ahuja (2011)
Locust bean gum	Grafting of polyacrylamide chains by microwave-assisted method	Controlled release matrix tablet	Kaity et al. (2013)
Tamarind gum	Grafting of polyacrylamide chains by microwave-assisted method using carboxymethyl derivative of tamarind gum	Microbeads; polymeric carrier for pH-responsive site-specific drug delivery	Setty et al. (2014)

Chemical modification by substitution of functional groups such as carboxymethylation and carbamoylethylation is the more commonly employed technique to increase both hydrophilicity and solution clarity of gums and make them more soluble in aqueous systems. Even though the process of carboxymethylation/carbamoylethylation of natural gums can be accomplished quite easily, the degree of substitution is usually low. Furthermore, this method is expected to be more suitable for gums containing (1, 4)-linked units because carboxymethylation/carbamoylethylation occurs primarily at the free $–CH_2OH$ groups (C6 position) due to steric reasons. The steric hindrance by –OH groups present in gum needs to be considered while attempting such modifications in order to achieve significant degree of substitution. Konjac gum (Jiangyang et al. 2008)

and gaur gum (Thimma and Tammishetti 2001) are modified in this way for their applications in the development of ionically cross-linked microbeads.

Cross-linking of gum by chemical agent is an easy technique and mostly used in the design of microbeads and hydrogels and for colon-specific drug delivery. A number of cross-linking agents based on charge, size, and chemical nature are used to cross-link the gums. Cross-linking decreases the solubility and increases the water retention capacity of gums and this feature serves to be useful for drug delivery applications. However, cross-linking of gums requires the availability of active functional groups in their basic structure. Therefore, guar gum, cashew gum, or sterculia gums that possess free alcoholic and/or carboxylic units are the good choice for cross-linking modification. Moreover, it is essential to investigate the vulnerability of cross-linking in different pH conditions to ensure the modified molecule for site-specific delivery.

Glutaraldehyde has been used extensively for cross-linking of hydroxyl groups in gums. It was observed that with an increase in the concentration of glutaraldehyde, there is an increase in cross-linking density, and as a result, there is a decrease in swelling of polymer. In an investigation carried out by Soppimath et al. (2000), interpenetrating network microspheres of poly(vinyl alcohol) and guar gum were prepared by cross-linking with glutaraldehyde, wherein aldehyde groups of glutaraldehyde reacted with hydroxyl groups of gum to form acetal cross-links. The in vitro release of nifedipine from these microspheres was observed to be dependent on the extent of cross-link density. The solvent uptake by these microspheres decreased with increased cross-linking and drug release continued for several hours. Similarly, in a study by Kabir et al. (2000), phosphate ion cross-linked gaur gum matrix was developed for colon targeted delivery.

The grafted gums (or copolymers) are an entirely different class of modified gums, wherein the chains of synthetic polymers are grafted onto the backbone of gums using free radical initiators like chemicals, enzymes, radiation, or their combinations. Most of the graft copolymers are prepared through graft polymerization of vinyl or acryl monomers onto biopolymer backbone as they can be grafted under mild reaction conditions. Among all types of vinyl monomers, grafting of polyacrylamide chains is quite simple and economic. Furthermore, the use of radiation such as microwave as compared to conventional chemical method for grafting of polyacrylamide chains on natural gum is cleaner, simpler, straightforward, highly reproducible, and eco-friendly (Sen et al. 2010).

Much of the research has now been carried out on polyacrylamide grafted natural gums for various types of drug delivery systems. The work reported by Sen et al. (2010) demonstrated the application of microwave for grafting of polyacrylamide chains onto gaur gum. The degree of grafting was precisely controlled by the time of exposure to microwave radiation and by virtue of which different matrix compositions (% grafting) were synthesized and evaluated for their potential in colon-specific drug delivery. Malik and Ahuja (2011) have reported the microwave-assisted grafting of polyacrylamide chains onto gum kondagogu by employing two-level, four-factor full factorial experimental design. Microwave power, microwave exposure time, and concentration of ammonium persulfate

showed significant synergistic effect on grafting efficiency. Comparative evaluation of in vitro release study of diclofenac sodium from matrix composed of either gum kondagogu or gum kondagogu-*g*-polyacrylamide revealed the faster release of drug from tablet prepared from gum kondagogu-g-polyacrylamide. The drug release from commercial tablet and matrix tablet composed of gum kondagogu was found to fit into zero-order release kinetics, while matrix tablet composed of gum kondagogu-*g*-polyacrylamide followed the Higuchi's square-root kinetics. A study by Soppimath et al. (2001) demonstrated the application of hydrolyzed polyacrylamide-*g*-gaur gum as an anionic microgel for pH-specific drug delivery. The pH-sensitive microgels were loaded with two different anti-hypertensive agents, viz., diltiazem hydrochloride (water soluble) and nifedipine (water insoluble), and studied for their release in both simulated gastric and simulated intestinal pH media. Results showed that irrespective of drug solubility, the release of drugs from microgels was pH specific, i.e., higher in alkaline medium compared to acidic medium.

7 Miscellaneous Pharmaceutical Application

7.1 As a Stabilizing Agent in Nanopharmaceuticals

Metal nanoparticles, especially those of gold and silver covered with appropriate stabilizing agents, are actively considered for drug delivery applications. The stabilizing agents are expected to provide stability to the nanoparticles against aggregation and help them to survive in both alkaline and acid media. In addition, if the stabilizing agent provides the functionality necessary for drug to be efficiently loaded onto the nanoparticle surface, it would be an added benefit.

A natural gum acts as a good stabilizing agent since they stabilize inorganic nanoparticles by two mechanisms: first, by adsorbing to the surface of nanoparticles, which creates steric repulsion among the particles, and second, they increase the viscosity of nanoparticle suspension and, therefore, slow down the aggregation process (Tiraferri et al. 2008; Comba and Sethi 2009; Xue and Sethi 2012). Furthermore, natural gums are used as a template for the synthesis and stabilization of metal nanoparticles due to: (i) natural availability and low cost, (ii) non-toxicity, (iii) abundant availability of hydroxyl, acetyl, carbonyl, and carboxylic functional groups, and (iv) metal-biosorption properties.

It was demonstrated that plant-based exudate gum such as gum acacia can be utilized as a reducing and stabilizing agent for silver nanoparticle biosynthesis (Mohan et al. 2007). In a study, a facile and eco-friendly method was developed for the synthesis of silver nanoparticles from silver nitrate using gum kondagogu (*Cochlospermum gossypium*), a natural biopolymer as a reducing and stabilizing agent. The influence of different parameters such as gum particle size, concentration of gum, concentration of silver nitrate, and reaction time on the synthesis of nanoparticles was studied. The formed silver nanoparticles were highly stable and had significant antibacterial action on both the Gram classes of bacteria (Kora et al. 2010).

Gum gellan (Dhar et al. 2008) and xanthan gum (Pooja et al. 2014) were also employed similarly for the synthesis of gold nanoparticles.

The potential of xanthan gum in the synthesis of gold nanoparticle as both reducing and stabilizing agent was studied by Pooja et al. (2014), wherein gold nanoparticles were synthesized using natural and biocompatible xanthan gum as a reducing and capping agent. Xanthan gum at very low concentration of 1.5 mg/mL converted the ionic gold to neutral gold particles with a particle size of 15–20 nm. These nanoparticles were found to be non-toxic and biocompatible in hemolysis study. Doxorubicin as a model drug was loaded into these nanoparticles through non-covalent interaction. Finally, the prepared carbohydrate-rich gold nanoparticles showed high drug loading, good colloidal stability, and enhanced cytotoxicity in A549 lung cancer cells. This has shown that natural gum-based approach is a better method for the synthesis of gold nanoparticles.

7.2 As a Therapeutic Agent

It is rare to see natural gum as therapeutic agent, but sterculia gum is one such example, which is used as a therapeutic agent in various medical conditions like diarrhea (Huttel 1983), ulcers (Love-Mignogna and Wind 1978; Zide and Bevin 1980; May 1982), irritable bowel syndrome (Capron et al. 1981), and chronic colonic diseases (Guerre and Neuman 1979; Hunold 1979), and in reducing cholesterol and improving glucose metabolism without adversely affecting most mineral balances (Behall et al. 1987; Behall 1990). Sterculia gum is also an effective bulk laxative. Similar to isabgol husk, the coarse particles of gum absorb a large quantity of water (about 100 times of its original volume) and start swelling enormously, forming a discontinuous type of mucilage that is very effective as a laxative (Meier et al. 1990; Verbeken et al. 2003).

7.3 As a Gene Delivery Agent

Among the non-viral vectors designed for the safe delivery of therapeutic genes to target sites, polymeric vectors are a leading class of gene carriers due to several advantages including safety, low immunogenicity, and capacity to deliver larger genes and cost-effectiveness (Curiel et al. 1991; Lehrman 1999; Felgner 2007). Polyethylenimine, 25 kDa (PEI), is particularly a promising candidate as a vector for its relatively high level of transfection in a number of target organs by various routes of delivery (Boussif et al. 1995; Abdallah et al. 1996; Erbacher et al. 2004). But very high positive charge density on PEI appears to be the primary cause of its marked toxicity, consequently limiting its use as a gene delivery vector in vivo. To circumvent PEI toxicity, natural gums containing anionic groups are used for partial neutralization of its excess positive charge. It helps to increase the transfection efficacy by mitigating its positive charge. Goyal et al. (2011) reported PEI blended with gellan gum (an anionic heteropolysaccharide) to form gellan

gum–polyethylenimine nanocomposites for efficient intracellular delivery of plasmid. The results showed that gellan gum blended PEI nanocomposites have improved transfection efficiency in comparison with PEI alone and the standard commercial transfection reagents in all the cell lines and also in primary mouse keratinocytes with negligible toxicity. This has shown that natural gum blended PEI approach holds great promise for future applications in gene delivery.

8 Conclusion

Natural gums, due to their biodegradability, plenty availability, non-toxicity, and easy processing conditions, are the materials of choice for various pharmaceutical applications. Initially, gums were utilized in formulations as excipient for improving the physiochemical property and stability. Hitherto many pharmaceutical preparations use natural gums as one of their key ingredients. The role of gum in novel drug delivery systems has changed the entire perception of gums and earned them an identity as potential matrix/carrier material for a wide variety of novel drug delivery systems. The modified gums are a new type of polymeric materials explored for pharmaceutical applications, and these widened the scope of gums in formulation development. Recently, we have witnessed some niche applications of gum in the area of nanotechnology and gene delivery, which opens up a new avenues for further investigations and applications of natural gums in the design and development of advanced drug delivery systems.

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Cationic Polymers for the Delivery of Therapeutic Nucleotides

64

Anjali Jain, Hossein Hosseinkhani, Abraham J. Domb, and Wahid Khan

Contents

Abstract

In the last decade, insertion of a therapeutic gene and nucleotide into cells termed as gene therapy has shown tremendous potential to cure life-threatening severe diseases. This therapy involves carrying the therapeutic gene to the nucleus of an affected cell through a vector. Initially viral vectors were employed for this purpose, but low production yield, limited carrying capacity, and long-term safety concerns associated with viral vectors led to the development of nonviral vectors as gene carriers. Currently, used nonviral carriers include polymers and lipids with cationic charge. Cationic polymers provide

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an excellent alternative for gene delivery due to their water solubility, biodegradability, ease of modification, and excellent compatibility with body systems, but challenges still persist to optimize them as ideal vectors. This chapter provides an overview of present status of cationic polymers and challenges associated with their use for gene delivery.

Keywords

Cationic polymers • siRNA • Gene delivery • Toxicity • Chitosan • Dextran • Cyclodextrin • Polyethyleneimine • Poly-L-lysine • SPG • Pullulan • Dendrimers

Abbreviations

PEI	Polyethyleneimine
PLL	Poly-L-lysine
ROS	Reactive oxygen species
siRNA	Small interfering RNA
FITC	Fluorescein isothiocyanate
DNA	Deoxyribonucleic acid
PEG	Polyethylene glycol
CD	Cyclodextrin
SPG	Schizophyllan
PAMAM	Poly(amidoamine)

1 Introduction

Gene therapy has been considered to be a promising treatment for many diseases ranging from acquired disease (such as AIDS) and cancer to inherited illnesses. The basic concept of gene therapy revolves around the transfer of genetic material into specific cells of a patient to replace or supplement defective genes responsible for disease development (Khan et al. 2013; Swami et al. 2013). This therapy, which began with the successful treatment of an X-linked severe combined immunodeficiency in the year 2000, is now gaining attention (Horino et al. 2013). This statement can be supported by the approval of Glybera® by European Union Marketing Authorization to treat lipoprotein lipase deficiency and the existence of more than 1800 clinical reports in more than 31 countries for the treatment of incurable diseases (Yla Herttuala 2012).

The main purpose of gene delivery is to deliver the genetic material to the target cell while surviving an array of biological defenses present in the body. This process requires the development of an efficient delivery vector. Viral vectors such as retroviruses, adenoviruses, and adeno-associated viruses are very effective in terms of transfection efficiency, but immunogenicity, inflammatory effects, and safety concerns restrict their usefulness (Nishikawa and Huang 2001; Lim et al. 2006; Mao et al. 2010). Subsequently, researchers moved towards the development of nonviral vectors, and cationic polymers and lipids gained a

significant place as nonviral vectors. Cationic polymers interact with DNA to form a polyplex (particulate complex) due to charged interactions between negatively charged DNA and positively charged polymers (El Aneed 2004).

Water solubility, ease of manipulation of polymer properties such as molecular weight (MW), geometry (linear vs. branched), and ligand attachment have been explored regarding the extensive structure/function relationships of cationic polymers. In addition, the small size of polyplex compared with cationic lipid complex (lipoplex) also helps them reach otherwise difficult to reach sites (Ruponen et al. 1999; Elouahabi and Ruysschaert 2005).

Gene delivery with cationic polymers provides a number of research opportunities, but at the same time challenges also exist. These challenges include strong interaction of cationic delivery vehicles with blood components, uptake by the reticuloendothelial system (RES), toxicity, managing bioactivity, targeting ability of the carriers with respect to cells of interest, and the metabolic fate of these systems in the body (Guo and Huang 2011). Cationic polymers for gene delivery can be divided into two main categories: polysaccharide cationic polymers and non-polysaccharide cationic polymers. Cationic polysaccharides include chitosan, cyclodextrins, dextran, dextran–spermine, schizophyllan, and pullulan, while non-polysaccharide cationic polymers include poly-L-lysine, polyethyleneimine, and cationic dendrimers (Fig. 1).

2 Mechanism of DNA Polyplex Formation

Due to the surface charge of cationic polymers, they rapidly form stable charged complex (polyplex) with oppositely charged DNA molecules. Multiple binding sites on DNA provide an integrated stabilization to polyplexes. These polyplexes can be administered into the body through different routes (intravenous and oral have been most widely explored so far). Apart from that polyplex can also be adsorbed on the surface of polymeric films or can be designed into layer-by-layer assembly carrying alternate layers of DNA and cationic polymers (De Smedt et al. 2000). After administration, polyplexes were taken up by cells through endocytosis (Luo and Saltzman 2000). Cellular entry of these polyplexes is followed by endosomal escape and dissociation, which release nucleic acid into the nucleus for gene expression (Smyth Templeton 2002). Figure 2 gives an overview of polyplex formation and release of DNA followed by expression.

With viral vectors, endosomal escape proceeds easily due to the inherent property of a virus to undergo conformational change in the coat proteins, but nonviral vectors require the addition of lysosomotropic agents like chloroquine, membrane-destabilizing peptides such as synthetic N-terminal, and peptides of rhinovirus VP-1/influenza virus HA-2 to mediate endosomal release. Alternatively, some macromolecules that have amine groups with low p*K*a values display the phenomena of "proton sponge effect" and thus mediate endosomal escape (Bron et al. 1993; Behr 1997).

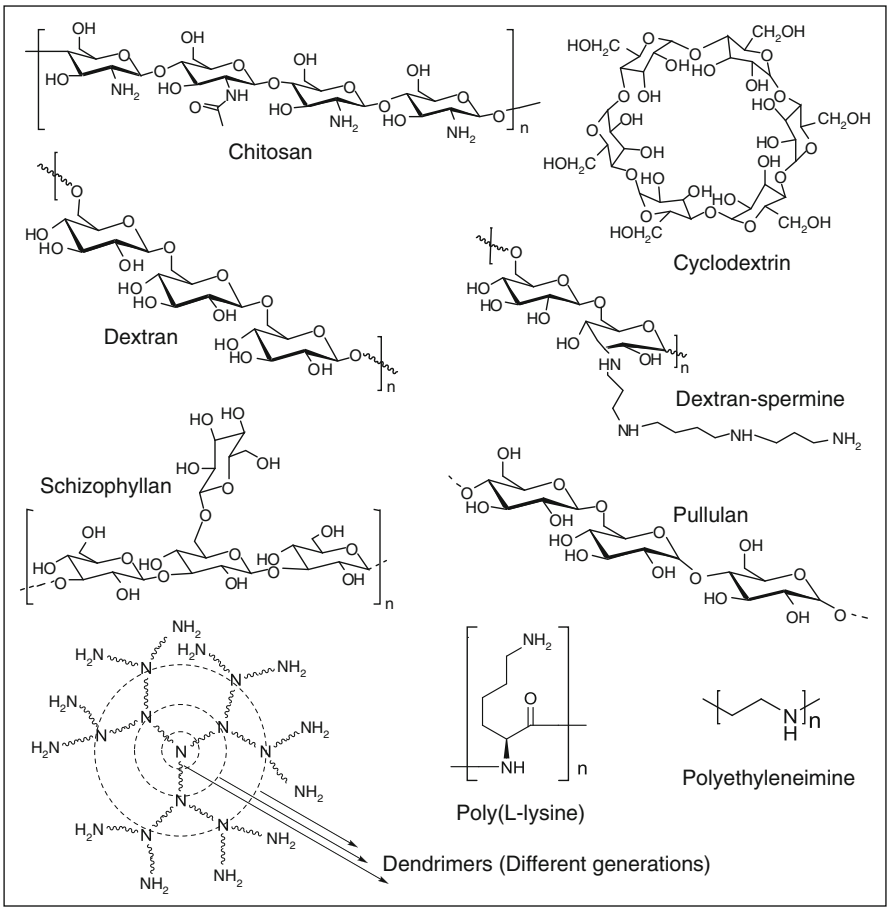

Fig. 1 Chemical structures of polysaccharides used in gene delivery

3 Bioactivity of Cationic Polymers

Bioactivity can be defined as the effect of a given agent upon a living organism or on living tissue. For the activity of cationic polymers as gene delivery vector, cationic charge is the basic requirement which facilitates the formation of a charged complex due to electrostatic interactions. However, the positive charge of polymer allows these complexes to interact with anionic serum proteins, resulting in the formation of undesirable aggregations or premature release of nucleic acids. PEGylation has been suggested as a solution for this problem due to its ability to protect the complex from RES system by increasing circulation time and by neutralizing surface charge, which minimizes the undesired interaction of polyplex with plasma components. On the other hand, PEGylation has been found to prevent

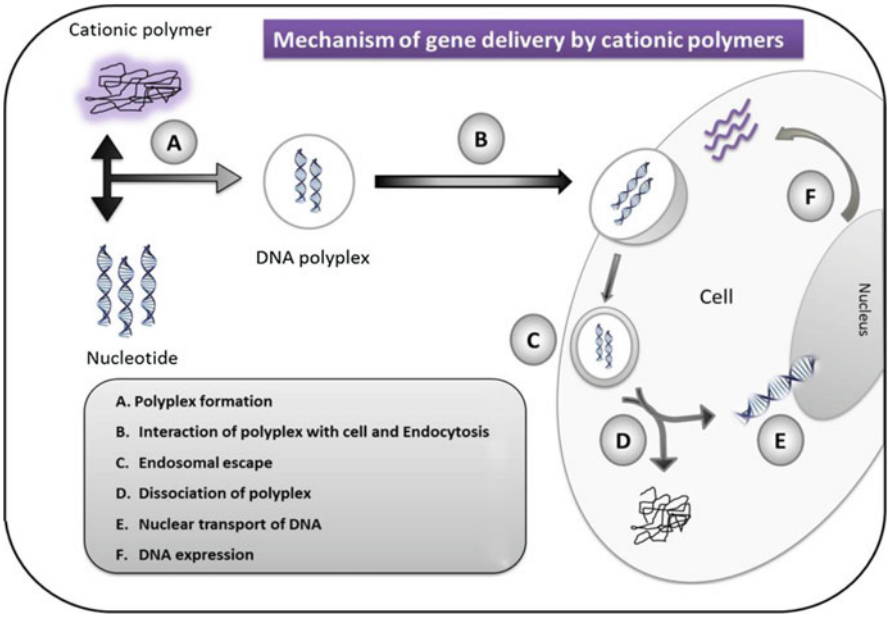

Fig. 2 Mechanism of polyplex formation and gene delivery by cationic polymers

gene vectors from interacting efficiently with cellular membranes and/or endosomal membranes due to steric hindrance and neutral charge. This results in lower transfection efficiency of a gene vector (Wang et al. 2012).

Efforts are being made to offer the advantage of both cationic charge and PEGylation by designing a pH-responsive PEG conjugate (Romberg et al. 2008), enzyme-sensitive PEG conjugate (Coussens et al. 2002), etc. Cationic polyplexes protect DNA from degradation by forming stable complexes. It is necessary to prevent premature release of nucleic acids in the extracellular environment and in maintaining the integrity of the complex for targeting and intracellular delivery of nucleic acids. However, the strong affinity of polymers towards nucleic acid can be a problem for release of nucleic acid at its site of action; this results in low transfection efficiency (Grigsby and Leong 2010).

4 Cationic Polymers for Delivery of Nucleotides

Gene delivery has a potential to cure many heretofore incurable diseases, but the lack of a suitable vector has hampered its clinical progress. An ideal vector for gene delivery must be stable in its systemic circulation, able to escape the reticuloendothelial system and to extravasate tissues, escape lysosomal degradation, and transport DNA to the nucleus to be transcribed. The advantages of a cationic polymer lie in the ability to accept structural modification. Numerous studies are underway to

explore the application of rational design into their structure. The goal is to overcome barriers in gene delivery. This section gives a brief introduction to cationic polymers, their properties, toxicity, and degradation behavior.

4.1 Polysaccharide Cationic Polymer

4.1.1 Chitosan

Chitosan is a chitin-derived cationic linear polymer composed of β(1–4)-glucosamine and randomly located *N*-acetyl-D-glucosamine. It is obtained by deacetylation of chitin. The degree of deacetylation for commercial chitosan can range from 70 % to 95 % with MW ranges between 10 and 1,000 kDa (Khan et al. 2013). Chitosan received approval to be used as a dietary supplement in Japan, Italy, and Finland (Ilium 1998). Also, bandages impregnated with chitosan and chitosan granules have been approved by the FDA to control blood loss in emergency situations (Wedmore et al. 2006).

When used for gene delivery, the toxicity of chitosan was found to be directly proportional to the charge density of the molecule. Chitosan in small amounts was found to be relatively less toxic; also, chitosan with a degree of deacetylation between 40 % and 60 % has been reported to have significantly less toxicity. A general trend of increasing toxicity with an increasing degree of trimethylation has been reported. However, higher toxicity has been seen in polymeric chitosan derivatives than oligomeric chitosan derivatives at similar degrees of trimethylation (Kean et al. 2005). Various drug delivery applications of chitosan have been reported with or without noticeable toxicities. Hirano et al. report no detrimental effects of chitosan oligosaccharides when injected (dose of 7.1–8.6 mg/kg over 5 days) for about 65 days (Hirano et al. 1991). When implanted subcutaneously, no toxic effect has been observed with 200 μl of 30 mg/ml photocrosslinked azide–chitosan–lactose gel (Ono et al. 2000). Upon oral administration of trimethyl chitosan/pDNA nanoparticles, slight toxicity has been observed (diarrhea); this was relieved by stopping administration (Zheng et al. 2007). Also, no oral toxicity was found in mice treated with 100 mg/kg chitosan nanoparticles (80 kDa, 80 % DD) (Sonaje et al. 2009). Biodistribution studies of ultrafine chitosan particles after intravenous injections of a dose 3.3–4 mg/kg in mice report no adverse effects (Banerjee et al. 2002). A phase II clinical trial involving percutaneous injection of chitosan–166 holmium complex for the treatment of hepatocellular carcinoma also reports safe and efficacious results (Kim et al. 2006). Also, nasal administration of chitosan solutions (0.5 % w/v) over 1 h produces no significant changes in mucosal cell morphology (Illum et al. 1994).

The metabolic fate of any formulation and its constituents is an important aspect of drug delivery. In general, the degree of deacetylation affects both the rate and extent of chitosan biodegradation with a higher degree of deacetylation; it also decreases the degradation rate in living organisms (Yang et al. 2007b). For oral delivery, in vivo studies show gut-mediated digestion of chitosan, which has been found to be species dependent. Hens and broilers are more efficient digesters

(67–98 % degradation) than rabbits (39–83 % degradation) (Hirano et al. 1990). FITC-labeled chitosan was administered to mice by i.p. route, and the low MW products were detected mostly in urine (Onishi and Machida 1999). Reports on degradation after i.v. administration are rare, and reports on human beings are not available due to lack of approval for human use. In vitro, both chemical as well as enzymatic degradation has been suggested for chitosan. Enzymes such as lysozyme (Onishi and Machida 1999), proteases (Rao and Sharma 1997), pectinase, (Kittur et al. 2003), rat cecal and colonic bacterial enzymes (Zhang and Neau 2002), porcine pancreatic enzymes (Verheul et al. 2009), and enzymes present in human fecal preparations (McConnell et al. 2008) have been reported for degradation of chitosan.

4.1.2 Cyclodextrin

Cyclodextrins (CDs) are cone-shaped natural cyclic oligosaccharides found as α-, β-, or γ-CD. They are composed of 6, 7, or 8 D(+)-glucose units, respectively, joined by α-1,4 linkages (Davis and Brewster 2004). The emergence of CDs in gene therapy has been considered since 1999 (Davis et al. 2004). For gene delivery purposes CDs have been explored either as part of the backbone of polymer (CD-embedding polymers) or grafted with polymer (CD-pendant polymers). Polymers containing CDs in their backbone are advantageous, since they are able to be further modified by forming an inclusion complex and adopting versatile properties (Reineke and Davis 2003; Davis et al. 2004; Liu et al. 2007; Muntimadugu et al. 2013). Cytotoxicity of these complexes has been found to be dependent on the alkyl chain length between CD units; it generally decreases with longer chain lengths (Hwang et al. 2001). Further, polymers containing 4–10 carbon alkyl chains between CD units have been found to display less toxicity, higher transfection efficiency, but less water solubility (Popielarski et al. 2003).

CDs are nephrotoxic at a reported dose of 0.7–1 g/kg (Frank et al. 1976). With this, few in vitro studies also report slight hemolytic effect of CDs. In vitro hemolytic activity of CDs follows the order β-CD > α-CD > HP-β-CD (TDS 4.2) > γ-CD > > HP-γ-CD ≥ HP-α-CD in erythrocytes freshly collected from human and P388 murine leukemic cells (Leroy-Lechat et al. 1994). Kiss et al. studied the cytotoxic and hemolytic properties of various beta-CDs in correlation with their cholesterol-solubilizing capacities to expose the mechanism of toxicity. Introduction of both ionic and methyl substituent shows less cytotoxicity than the parent compounds, but cell toxicity of methylated β-CDs is higher than ionic derivatives. Further, they also report significant cholesterol extraction for CD-mediated cell toxicity. However, the in vivo toxicological implications are considered negligible (Kiss et al. 2010).

Oligoethyleneimine (OEI)-based cationic star polymers for gene delivery in which OEI is conjugated with α-CD core also result in a lower in vitro cytotoxicity and excellent gene transfection efficiency in HEK293 and Cos7 cells (Davis et al. 2004; Yang et al. 2007a). Similarly, OEI-grafted β-CDs display a superior delivery system for gene delivery when combined with pluronic, polycaprolactone, and PEI (Shuai et al. 2005).

Polyrotaxane-based CD-containing gene carriers have attracted significant attention in the past few years. Cytocleavable necklace-like structures with many α-CDs containing cationic groups were threaded onto a succinimidyl succinate (SS)-terminated PEG chain. Under reducible conditions, the SS bond cleavage results in dissociation of the non-covalent linkages between α-CDs and PEG, which finally triggers pDNA release. The main advantage with these carriers is reduced cytotoxity compared with high MW polymers. This might be due to dissociation behavior and relatively fewer cationic groups in these carriers (Ooya et al. 2006; Yui et al. 2009; Mellet et al. 2011).

4.1.3 Dextran and Dextran–Spermine (D-SPM)

Dextran is a highly water-soluble biodegradable and biocompatible polysaccharide composed predominantly of α-1,6-linked glucopyranose units with a low degree of 1,3-branching (Varshosaz 2012; Raemdonck et al. 2013). Low MW dextrans have achieved significant importance in the biomedical field due to flexibility in chemical modification and the network-forming ability of dextran (van Dijk-Wolthuis et al. 1995; Heinze et al. 2006; Van Tomme and Hennink 2007).

Intravenous administration of dextran has been reported with mild hemotoxicity and thrombocytopenia (Flexner et al. 1991). For gene delivery, dextran-induced toxicity has been reported due to undesired interactions with blood components. Chemical modification of dextran with spermine improves the stability and transfection efficiency of polyplex. However, it does not overcome the problem of undesired interaction with blood components. PEG/oleate residues are, therefore, attached to polymers, which results in better transfection efficiency in serum-rich medium (Azzam et al. 2004). Dextran–spermine for the delivery of pDNA to mouse lungs by intranasal route exhibits mild toxicity (Yeo et al. 2014). Polyelectrolyte complexes (PECs) with dextran sulfate have also been developed to minimize interaction with blood components. These complexes were made from the interaction of cationic polymers with negatively charged dextran sulfate in which the outer coat of dextran sulfate minimizes electrostatic interactions, while the inner positively charged polymer provides stability to nucleic acid (Tiyaboonchai et al. 2003; Zorzi et al. 2011; Cho et al. 2012). Dextranated bioreducible cationic polyamide was developed for systemic gene delivery. Polyplex formed between this polymer and nuclear material displayed near-neutral surface charge which is advantageous to avoid interaction with blood components. Moreover, the reducible nature of polymers allows sufficient gene release from the polyplexes in response to an intracellular reducing environment. The copolymer reveals low cytotoxicity in vitro and no death of mice upon intravenous administration (Lin et al. 2014).

4.1.4 Schizophyllan

Schizophyllan (SPG) is a natural β-(1–3)-D-glucan with one β-(1–6)-glycosyl side chain per three glucose residues. It is produced by the fungus *Schizophyllum* and shows potential antitumor effects (Kitamura et al. 1994; Khan et al. 2012). Binding of SPG with polynucleotide is driven by nonionic hydrogen bond interactions

(Sakurai and Shinkai 2000). SPG possesses several biological and immunopharmacological properties. These properties vary with MW, degree of branching, and confirmation. Systemic administration of soluble glucan over a wide dose range does not induce any significant toxicity (Williams et al. 1988). In vivo studies with i.p. injections of SPG/CpG-DNA complexes show an increase in the immune responses in mice twofold to ninefold that of uncomplexed CpG-DNA. Chemically modified SPG results in stable complexes with antisense oligonucleotides and negligible in vitro cell line toxicity (Takedatsu et al. 2012; Matsumoto et al. 2004).

SPG cannot bind to the heteronucleotide sequence of DNA, and hence homonucleotide sequences were usually attached to DNA which can form a stable complex with SPG. Takeda et al. suggested an alternative method to stabilize the complex of SPG with DNA. They prepared the complex of DNA with PEI, and this complex was further incorporated into SPG to form a ternary complex which exhibited better stability than SPG–DNA complex and less aggregation behavior than unencapsulated PEI–DNA complexes (Takeda et al. 2007). In fact, PEGylated cationic SPG also resulted in greater transfection efficiency for pDNA and long-term gene expression than PEI complexes (Nagasaki et al. 2004). Karinaga et al. developed galactose–PEG dual conjugation of β-(1 → 3)-D-glucan SPG for delivery of antisense oligonucleotides. This dual conjugation facilitates enhancement in cellular uptake along with endocytosis escape (Karinaga et al. 2006). Blood clearance of SPG was found to be confirmation dependent. Clinically used SPG was mostly found in triple-helix confirmation. Comparison of blood clearance of triple-helix SPG with single-helix SPG upon i.v. and i.p. administration resulted in faster clearance of single-helix form (Miura et al. 1995).

4.1.5 Pullulan

Pullulan is a water-soluble polysaccharide with repeated units of maltotriose condensed through the α-1, 6 linkage. It is nontoxic, non-immunogenic, non-mutagenic, and noncarcinogenic in nature (Hosseinkhani et al. 2002). Pullulan, due to its inherent affinity towards liver, is also used for gene transfection into liver. For this purpose pullulan is conjugated with spermine. This cationized pullulan derivative is complexed with a plasmid DNA and intravenously injected for in vivo gene transfection. This polyplex enhances survival time of mice and reduces the number of tumor cells compared with free plasmid DNA injection (Jo et al. 2006). Cellular uptake of pullulan–spermine DNA polyplex is mediated by clathrin- and raft/caveolae-dependent endocytotic pathways (Kanatani et al. 2006). Priya et al. developed a pullulan–protamine conjugate gene delivery system which is nontoxic and hemocompatible (Priya et al. 2014). Pullulan nanoparticles encapsulating pBUDLacZ plasmid have been prepared by w/o microemulsion method. Cytotoxicity studies demonstrated that cells incubated with nanoparticles remain more than 100 % viable even at a nanoparticle concentration of 1,000 μg/ml. Further, β-galactosidase expression in COS-7 cells resulting from pullulan nanoparticles is comparable to commercially available Lipofectamine 2000 (Gupta and Gupta 2004). Pullulan in combination with spermine has been used for transfection of pDNA to rat brain endothelial cells and human brain

microvascular endothelial cells (Thomsen et al. 2011), rat sensory neurons (Thakor et al. 2009), and mesenchymal stem cells (Jo et al. 2010).

4.2 Non-polysaccharide Cationic Polymers

4.2.1 *Polyethyleneimine*

Polyethyleneimine (PEI) is one of the most widely used polymers for gene delivery due to its high transfection efficiency, but some cytotoxicity has also been reported (Godbey et al. 1999a). It was first introduced by Boussif and coworkers for gene delivery application (Boussif et al. 1995). The most extensively used PEIs are linear and branched PEI polymers with MW ranging from 22 to 25 kDa. PEI–DNA polyplexes form due to electrostatic interaction. This process seems to be essentially entropy driven together with the contribution of other interactions such as hydrogen bonding and van der Waals forces (Bronich et al. 2001). Complete condensation of DNA with PEI has been reported at nitrogen to phosphate (N/P) ratios above 2. This complexation results in the formation of small nanoparticles with size ranging from 50 to 100 nm in optimized conditions (Tang and Szoka 1997).

PEI-associated cytotoxicity during gene transfection can be of two types: (1) immediate toxicity associated with free PEI and (2) delayed toxicity associated with cellular processing of PEI–DNA complexes (Godbey et al. 1999b, 2001). Free PEI exerts its toxic effect by interacting with negatively charged serum proteins (such as albumin) and red blood cells followed by clumping with other cells. The delayed toxicity by the PEI–DNA complex is associated with the release of DNA from PEI. PEI tends to restore inside the cell after release of DNA, and it thus interacts with various cellular components and inhibits normal cellular process (Godbey et al. 2001). Structurally branched PEI has been found to exert more cytotoxicity than deacetylated PEI (Thomas et al. 2005). Also, low MW (10 kDa), moderately branched polymers provide efficient delivery with low toxicity in comparison with commercial high MW PEI (Godbey et al. 1999c). Regional delivery of plasmid DNA through intrapericardial administration of DNA–PEI polyplex yields significant toxicity towards the myocardium, which hampers efficiency evaluation (Jeong et al. 2007).

To overcome these toxicity issues, many efforts have been made such as PEGylation (Kircheis et al. 1999), modification of PEI with acid-labile imine linkers (Kim et al. 2005), lipopolymers (Wang et al. 2002), etc. Adsorption of PEI–DNA complexes to silica nanoparticles results in comparable transgene expression in vitro with reduced toxicity (Kneuer et al. 2000). CD- and PEI-functionalized mesoporous silica nanoparticles were also developed for the delivery of siRNA. Here, PEI forms an electrostatic bond with siRNA, while cyclodextrin eliminates the charge-induced toxicity of PEI (Shen et al. 2014).

More attention must be given to develop peptide-grafted PEIs such as mannosylated cell-penetrating peptide-graft-polyethyleneimine (Hu et al. 2014) and TAT peptide-modified PEI (Yamano et al. 2014). The genotoxicity of

endocytosed PEI NPs is associated with the production of reactive oxygen species (ROS), which causes DNA damage without apparently affecting cell viability. This genotoxicity of PEI-based nanoparticles is reduced by the acetylation of PEI (Calarco et al. 2013).

PEI is nondegradable in nature and can accumulate in the body and induce long-term undesirable effects. Efforts have, therefore, been made to develop biodegradable and less toxic polymers with high transfection efficiency (Park et al. 2005). Some modifications for this purpose include hydroxyethyl starch–PEI (Noga et al. 2014), poly(vinyl alcohol)–PEI (Goyal et al. 2011), and poly(ethyleneimine-co-L-lactamide-co-succinamide) (Petersen et al. 2002).

4.2.2 Poly-L-Lysine

Poly-L-lysine (PLL) is one of the first polymers to be used for DNA condensation. PLL has a sufficient number of primary amines with positive charges to interact with the negatively charged phosphate groups of DNA. PLLs are biodegradable linear polypeptides of amino acid lysine. After entering into systemic circulation, rapid binding to plasma proteins causes their rapid clearance and results in lower transfection efficiency (Ward et al. 2001; Khan et al. 2011). Although use of high MW PLL increases the transfection efficiency, it also causes undesirable toxicity (Wolfert et al. 1999). PEGylation and palmitoyl group attachment result in the reduction of toxicity without compromising gene delivery efficiency (Brown et al. 2000). PLL-modified silica nanoparticles have shown their potential towards oral gene therapy. Efficient reporter gene expression was detected in mucous membrane cells of stomach and intestine with no obvious sign of toxicity (Li et al. 2005). In fact, Poly-L-lysine–Fe_2O_3/silica nanoparticles have been designed very recently for the delivery of siRNA. This system results in remarkable encapsulation efficiency and targeted gene silencing with negligible cytotoxicity (Cui et al. 2014). Dendritic poly-L-lysine of the sixth generation has shown high transfection efficiency without significant toxicity or cell specificity (Ohsaki et al. 2002).

Recent research has focused on the co-delivery of drug and gene. To co-deliver docetaxel (DOC) and MMP-9 siRNA plasmid for nasopharyngeal cancer therapy, a star-shaped porphyrin–arginine-functionalized PLL copolymer has been designed. This system induces a more significant apoptosis than DOC or MMP-9 alone, better blood compatibility, and lower cytotoxicity compared with PEI-25k (Ma et al. 2014). Similarly, a star-shaped cyclodextrin–poly-L-lysine derivative has also been used to co-deliver docetaxel and MMP-9 siRNA plasmid for the treatment of cancer (Liu et al. 2014b).

Degradation of PLL is an enzymatic process. Leclercq et al. report degradation behavior of PLL when complexed with polyanion. For this study polyanions poly (acrylic acid) and poly-L-lysine citramide were taken as a representative model of DNA in polyplexes. The researchers found that trypsin (an endopeptidase) degraded PLL into trilysin and tetralysin. However, exopeptidase like aminopeptidase does not cause degradation of PLL (Leclercq et al. 2010). Further, Ren et al. report PLL degradative behavior in the presence of α-chymotrypsin.

They prepared a layer-by-layer self-assembly of PLL and DNA and found that 90 % of DNA within the films is released almost linearly under α-chymotrypsin in PBS at 37 °C in 35 h (Ren et al. 2006).

4.2.3 Cationic Dendrimers

Dendrimers have gained significant importance in gene delivery due to their well-defined nanoscale polymeric scaffold with low polydispersity index and controlled surface functionalities. A complex formed between DNA and dendrimer is known as a dendriplex. PAMAM dendrimer is most widely used for gene delivery applications. Regardless of the extensive pharmaceutical and biomedical applications, toxicity associated with dendrimers due to terminal NH_2 groups and multiple cationic charge limits their candidatures for clinical applications (Jain et al. 2010). Dendrimers are reported with slight cytotoxicity (Jevprasesphant et al. 2003), hemolytic toxicity (Agrawal et al. 2007) and weak immunogenicity (Roberts et al. 1996). They also display concentration and generation-dependent toxicity of free amine groups present at their periphery (Chen et al. 2004).

Most commonly used dendrimers for nonviral gene delivery are synthesized via the divergent strategy and represent sixth generation Starburst™ PAMAM dendrimers either in intact (PolyFect®) or fractured (SuperFect®) form. A comparative in vitro cytotoxicity study with different water-soluble, cationic macromolecules used for gene delivery application was performed. Results were evaluated based on MTT assay and release of the cytosolic enzyme lactate dehydrogenase (LDH), which indicate the highest toxicity with poly(ethylenimine) comparable to poly-L-lysine followed by poly(diallyl-dimethyl-ammonium chloride), diethylaminoethyl-dextran, poly(vinyl pyridinium bromide), Starburst™ dendrimer, cationized albumin, and native albumin in descending order. According to this study Starburst dendrimers are the least toxic among cationic polymers (Fischer et al. 2003).

Liu et al. use fluorination to reduce the cytotoxicity of poly(propylenimine) (PPI) dendrimers. They also claim comparable or superior transfection efficacies with fluorinated G3, G4, and G5 PPI dendrimers as compared with Lipofectamine 2000, branched poly(ethyleneimine), SuperFect®, PolyFect®, as well as arginine-modified dendrimer on both HEK293 and HeLa cells (Liu et al. 2014a). The ternary complex of plasmid DNA polyamidoamine dendrimer and chondroitin sulfate results in no agglutination activity and no cytotoxicity against B16-F10 cells (Imamura et al. 2014). PEGylation is the well-known method to improve biocompatibility and transfection efficiency (Sun et al. 2014). Shen et al. tried a charge-reversal approach to convert PAMAM primary amines to negatively charged acid-labile amides in order to inhibit nonspecific interaction with cells; this again regenerates the active PAMAM in acidic environments (Shen et al. 2010). Cationic water-stable carbosilane dendrimers containing ammonium groups at the periphery are reported to be nontoxic for gene therapy against HIV and hepatocarcinoma (de Las Cuevas et al. 2012). AuPAMAM conjugates are reported to exhibit high yield, high transfection efficiency, and low cytotoxicity (Figueroa et al. 2014).

Table 1 Recent patents on gene delivery using cationic polymers

S. no.	Title	Patent no.	Remarks
Chitosan in gene delivery			
1	Nanoparticles for delivery of bioactive agents	US8461316 (B1)	Describes nanoparticles composed of chitosan, polyglutamic acid, and at least one DNA, RNA, or bioactive agent characterized with a positive surface charge and their enhanced efficiency for gene delivery and bioactive agent delivery
2	Preparation method of controllable arginine coupled chitosan	CN103539866	Arginine-modified chitosan carriers were used for gene delivery
3	Dually derivatized chitosan nanoparticles and methods of making and using the same for gene transfer in vivo	WO2013138930 (A1)	Chitosan was dually derivatized with arginine and gluconic acid which produced synergistic effect for gene delivery. This synergistic effect was found to be maximum for arginine and gluconic acid at a final functionalization degree of 26 % and 5 %, respectively
4	Mucosa M-cell targeted viral myocarditis gene vaccine and preparation method thereof	CN103341179 (A)	Vaccine was prepared by compounding a mucosa delivery system made of chitosan and b3-type Coxsackie virus antigen encoding plasmids through cross-linking copolymerization. This vaccine was administered by intranasal route and found to effectively induce specific immune response
5	Chitosan derivative used as gene vector, and preparation method and application thereof	CN102260356 (A)	Chitosan derivative was synthesized by introducing *N*, *N*, *N*-trimethyl-(1,2,3-triazol-4)-methyl ammonium bromide groups into the 6-site of chitosan. This derivative was found to be nontoxic and biocompatible with great improvement of water solubility, efficacy, and transfection efficiency

(*continued*)

Table 1 (continued)

S. no.	Title	Patent no.	Remarks
Cyclodextrin in gene delivery			
6	Nano non-viral gene delivery system and application thereof	CN102417914 (A)	Polyethyleneimine and cyclodextrin complex was used as gene transfection agent
			High transfection efficiency and low-toxicity profile for gene transfection
7	Polyamides for nucleic acid delivery	US2011183417 (A1)	Polymers comprising an oligoamine shell, a cyclodextrin core, and carbohydrate-containing degradable polyesters were used as gene delivery vectors
8	RNA delivery reagent specific to hepatocyte	JP2011103811 (A)	Hepatocyte-specific gene transfer agent comprising a conjugate of cyclodextrin with a dendrimer and lactose was synthesized
9	Cyclodextrin-polyethyleneimine-mediated supramolecular delivery system and preparation method	CN101991862 (A)	Cyclodextrin–PEI-based system for delivery of gene and adriamycin simultaneously
			Solubility and toxicity problems associated with adriamycin were resolved
10	Biodegradable copolymer and nucleic acid delivery system	US2004185564 (A1)	PEI–cyclodextrin copolymer formed through ester linkage
Dextran in gene delivery			
11	Dextran–maleic acid monoesters and hydrogels based thereon	US6476204 (B1)	Hydrogel made by photocrosslinking of dextran–maleic acid monoesters. It was used for encapsulating viral vector in gene therapy
12	Nucleic acid condensing agents with reduced immunogenicity	WO9621036 (A2)	Dextran and other polysaccharides were conjugated with polyethylene glycol to reduce immunogenicity and used to deliver the gene into cells
13	Biocompatible cancer cell-specific delivery system	KR20110013681 (A)	System contained a viral gene delivery vector along with soluble natural polymer (chitosan, heparin, hyaluronic acid, or alginate) connected to cancer cell-targeting moiety and PEG

(*continued*)

Table 1 (continued)

S. no.	Title	Patent no.	Remarks
Polyethyleneimine in gene delivery			
14	Applications of diamino carbamate cross-linked polyethyleneimine in gene therapy	CN103421846 (A)	Patent describes the method for preparation of polyethyleneimine for gene delivery applications
15	Folate-chitosan-graft-PEI copolymer and gene delivery carrier by using the same	KR20110068033 (A)	Folate–chitosan-graft-PEI copolymer was synthesized which exhibited excellent composite forming ability and a platform for an aerosol gene delivery system
16	Degradable imine polycation and synthetic method thereof, and nanoparticle	CN102516535 (A)	Invention describes the method of preparation of degradable derivatives of PEI for gene delivery application
17	Method for introducing siRNA into cells by photochemical internalization	RU2008150847 (A)	PEI–siRNA polyplex combined with photosensitizing agent to induce stimuli-responsive gene delivery
Poly-L-lysine in gene delivery			
18	PEG-PLGA-PLL polymer and method for preparing and using the same as the drug and gene carrier	US2013052134 (A1)	PEG–PLGA–PLL copolymer was reported for gene and drug delivery application
			The carrier was capable of passive targeting through control of the carrier particle size
19	Hepatocyte targeting polyethylene glyco-grafted poly-L-lysine polymeric gene carrier	US6177274 (B1)	PEG–PLL–lactose/galactose was synthesized for gene delivery application. PEG provided better solubility, improved transfection efficiency, and reduced cytotoxicity, while lactose or galactose acted as targeting moiety for hepatoma cell
20	Gene transfer system comprising cationic macromolecule and lipoprotein	KR20000023602 (A)	Poly-L-lysine was used with a lipoprotein for gene delivery application
21	Carrier system for specific artery wall gene delivery	US7264969 (B1)	An artery wall-binding peptide (AWBP) was conjugated to a cationic backbone of PEG–PLL. This composition enhanced gene transfer to artery wall cells

(*continued*)

Table 1 (continued)

S. no.	Title	Patent no.	Remarks
Pullulan in gene delivery			
22	Bioactive agent delivery system comprising modified pullulan-polyethyleneimine conjugate and method for producing it	KR20120001214 (A)	Bioactive delivery system was designed which contained pullulan derivatives and polyethyleneimine and capable of delivering gene efficiently to its target site
Dendrimers in gene delivery			
23	Poly(ether imine) dendrimers and uses thereof	WO2012090223 (A1)	Nonviral poly(ether imine) dendrimer-based gene delivery systems were designed which significantly reduced toxicity, over a broad concentration range
24	RNA delivery reagent specific to hepatocyte	JP2011103811 (A)	Patent described an RNA delivery reagent in which cyclodextrin was attached to dendrimer followed by surface modification with lactose for gene delivery to hepatic cells

5 Clinical Trials

Even though many clinical trials are ongoing, experiments with gene therapy using viral vectors and experiments using nonviral vectors such as cationic polymers are still limited. To the best knowledge of the authors, one clinical trial application using PEI as vector has thus far been filed (NCT No. NCT01274455). The purpose of the study is to evaluate the feasibility, tolerance, and antitumor effect of repeated intra-tumoral injection of a gene therapy product in combination with gemcitabine for the treatment of unresectable pancreatic carcinoma. The gene therapy product used for this purpose is CYL-02 plasmid DNA pre-complexed to linear PEI. This phase I study has been completed, but study results have yet to be published. Few recent patents on gene delivery using cationic polymers are given in table 1.

6 Conclusion

The progress in molecular biology has prepared the way for the introduction or replacement of missing as well as malfunctioning genes. Previously viral vectors have been used for this purpose, but immunogenicity associated with viral vectors has inspired researchers to find new alternatives. The emergence of cationic polymers as gene delivery vectors offers advantages of negligible immunogenicity and

biodegradability and proves their enormous potential for this purpose. Yet problems such as toxicity and low transfection efficiency require further efforts to bring these cationic polymeric vectors to a commercial level.

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Dietary Polysaccharides and Immune Modulation

65

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Contents

Abstract

Polysaccharides from fungi and plants are of great economic and clinical interest and exhibit a wide variety of biological activities. They have emerged as an important class of bioactive natural products with nutraceutical and chemopreventive properties and are relatively nontoxic. The activity of polysaccharides is

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determined by their conformation, composition, and size. Among polysaccharides with higher biological activity, β-glucans are the most important, and they vary in conformational complexity, molecular weight, and number of branches. These characteristics can alter the biochemical and solubility properties and also influence their biological properties, such as immunomodulation and antitumor activities; metastasis inhibition; increased host resistance to bacterial, viral, parasitic, and some microbial infections; and have antimutagenic, antiallergic, regenerative, antithrombogenic, anticoagulative, antioxidant, hypolipidemic, radioprotective, and antidiabetic effects. This chapter summarizes the immune mechanisms, biological properties, pharmacokinetics, and toxicity of dietary polysaccharides primarily from fungi.

Keywords

Dietary polysaccharides • β-glucans • Macrophages • NK • Cytokines • Innate immunity • Cellular immunity • Infection • Inflammation • Cancer

Abbreviations

AbM	*Agaricus blazei* Murill water extract
ABP-AW1	Low molecular weight polysaccharides isolated from *Agaricus blazei* Murill
AdvAMB	Advanced ambrotose
AMB	Ambrotose complex
APCs	Antigen presenting cells
BG	β-1,3/1,6-glucans derived from *Saccharomyces cerevisiae*
CD	Crohn's disease
CR3	Complement receptor 3
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA-PK	DNA-dependent protein kinase
DSS	Dextran sulphate sodium
GALT	Gut-associated lymphoid tissue
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IL	Interleukin
MAPK	p38 mitogen-activated protein kinase
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NO	Nitric oxide
NOAEL	No observed adverse effect level
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
SG	Soluble glucans
SP1	Sulfated polysaccharides isolated from *Caulerpa lentillifera*

TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
UC	Ulcerative colitis

1 Introduction

Polysaccharides from fungi and plants are of great economic and clinical interest and exhibit a wide variety of biological activities, including antitumor, antioxidant, and immunostimulant activity. Thus, they have emerged as an important class of bioactive natural products with nutraceutical or chemopreventive properties, being relatively nontoxic. Worldwide, they have attracted attention as a functional food and a source for the development of new immunomodulator compounds due to their dietary and medicinal benefits (Ooi and Liu 2000; Bertozzi and Kiessling 2001; Wasser 2002). The polysaccharide is localized in the intermediate layer of the cell wall, adjacent to the plasma membrane, with the function of maintaining the rigidity and shape of the cell (Angeli et al. 2006). Carbohydrate is a major component in mushrooms, and its total content ranges from 35 % to 70 % dry weight, with variations in different species (Manzi et al. 1999; Cheung 2010). In addition, fungal polysaccharides can be found in the fruiting bodies, mycelium, and fermentation broth of large edible and medicinal fungi (Wasser 2002). Fungal polysaccharides are long carbohydrate molecules of repeating units joined together by glycosidic bonds. They are often linear but may also be highly branched (Wasser 2002). Most of these polysaccharides are homoglycans (polysaccharides that contain residues of only one type of monosaccharide molecule) or heteroglycans (polysaccharides that contain residues of two or more types of monosaccharide molecules) and are able to combine with other proteins to make peptidoglycan or polysaccharide–protein complexes. This chapter summarizes immune mechanisms, biological properties, pharmacokinetics, and toxicity of dietary polysaccharides mainly from fungi.

2 Brief Overview of the Immune Activation

Cells from the immune system have the ability to discriminate nonself antigens, their released products, and other compounds present in the environment and dietary substances. The initial interaction between environment and host is mediated by innate immune nonspecific sensors called pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) (Beutler 2004; Joffre et al. 2009), which are present in innate immune response cells, which recognize the expression of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The activation triggered by these receptors leads to secretion of cytokines, chemokines, and microbicidal molecules and to the development

of adaptive immunity. This occurs via the uptake of dendritic (DC) and others antigen-presenting (APCs) cells and the process of antigen presentation to T lymphocytes (Kapsenberg 2003; Martin and Frevert 2005; Murray and Wynn 2011). Furthermore, DCs are essential for differentiation of T helper (Th) 1, Th2, Th17, and T-regulatory cells, which secrete different cytokines that modulate the immune response and the outcome of disease. For instance, Th1 cells secrete interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-12 (IL-12) and are involved in the control of intracellular pathogens. On the other hand, Th2 activation is determined by production of IL-4, IL-5, IL-9, and IL-13 (Zhu and Paul 2008; Jutel and Akdis 2011) and plays a role in hypersensitivity type I reactions and helminthic infections. The response mediated by Th17 cells (source of IL-17A, IL-17F, IL-22, IL-21), in some cases associated with Th1 cells, have an important function in host protection against extracellular bacterial and fungal pathogens. Indeed, Th17 and Th1 cells can also mediate deleterious effects in patients with autoimmune and inflammatory diseases (Aarvak et al. 1999; Weaver et al. 2006; Bettelli et al. 2008; Jäger and Kuchroo 2010). Regulatory T cells regulate the immune response by inhibiting the activity of Th1, Th2, and Th17 cells, mainly by the secretion of transforming growth factor-beta (TGF-β) or IL-10. T-helper cells are essential for activation of naive $CD8^+$ T cells (Guidotti and Chisari 2001), B lymphocytes, and phagocytes (Bachmann and Zinkernagel 1997). The gut-associated lymphoid tissue (GALT) is the largest immune compartment in the body and consists of both organized lymphoid tissues, such as mesenteric lymph node and Peyer's patches, and diffuse scattered lymphocytes in the intestinal lamina propria and epithelium. The immune system is regulated not only by its symbiotic relationship with microbiota, but is very sensitive to diet. In this context, the roles of dietary polysaccharides in modulating immune responses are herein analyzed.

3 Effects of Dietary Polysaccharides on Immune System

We are always asking how and which kind of food impacts the immune system. Recently, Wichers (2009) indicated that maybe the better question is which food components do not have an impact on immune function? Both APCs, such as macrophages and DCs, and intestinal epithelial cells express receptors involved in the recognition of carbohydrate (glycan) present in microorganisms, including bacteria, dietary components, or glycosylated proteins in many cells. Thus, migratory and resident DCs within the intestine have a crucial role in the regulation of immune response, considering that they can induce Th1, Th2, Th17, and T regulatory – cell subpopulations following activation (Coombes and Powrie 2008; Rescigno and Di Sabatino 2009). C-type lectins expressed in APCs, including DCs, have specificity for mannose-, fucose-, galactose-, and N-acetylgalactosamine-terminating glycans. In general, activation of DCs by the recognition of glycans leads to the uptake of antigen and T-cell activation (for review, see de Kivit et al. 2011). In addition, immunomodulatory mechanisms of polysaccharides involve natural killer cells (NK), T cells, B cells, neutrophils, and macrophages.

Table 1 Effects of β-glucans on innate and adaptive immune cells

Receptor involved	Effects on immunity
Dectin-1	Enhances:
	Phagocytosis
	Production of proinflammatory cytokines
	Pathogens elimination
	Human peripheral blood mononuclear cells proliferation
	Dendritic cells maturation and T cell-stimulation
Scavenger receptor	Triggers phosphatidylinositol-3 kinase (PI3K), Akt kinase and p38 mitogen-activated protein kinase (MAPK) signaling pathways in myeloid cells
Complement receptor 3	Induces lysis of iC3b-coated tumor cells by neutrophils
Lactosylceramide receptor	Induces proliferation of myeloid cells
	Stimulates the neutrophils activation and their microbicidal activity

In this regard, β-glucans, which are among the most abundant polysaccharides present in the cell wall of bacteria, fungi, and yeasts, have an immunostimulatory effect, which is probably associated with the activation of macrophages, T-helper, and NK cells; with differentiation of T lymphocytes; and with activation of alternative complement pathway (Agarwal et al. 2011; Municio et al. 2013). Moreover, β-glucans are modulators of both humoral and cellular immunity and are implicated in both the initial phase of immune activation and in the effector phase of the immune response (Falch et al. 2000; Tokunaka et al. 2000; Brown and Gordon 2003; Chan et al. 2009).

Most β-glucans are considered nondigestible carbohydrates, being fermented by the intestinal microbial flora, and are used as prebiotic supplements to augment innate immunity (Reynolds et al. 1980; Miura et al. 2003; Brown and Gordon 2003). Therefore, their immunomodulatory properties may be attributed to a microbial-dependent effect or to its binding to specific receptors on immune cells. Some dietary fibers such as β-glucan modulate the immune response by binding to PRRs present on cells of the innate immune system. The main receptors involved with the recognition of β-glucans are dectin-1, complement receptor 3 (CR3), lactosylceramide, scavenger receptors, and TLR-2, 4, and 6, as shown in Table 1 (Zimmerman et al. 1998; Brown et al. 2002, Rice et al. 2002; Macpherson and Harris 2004; Huang et al. 2009; Ren et al. 2012).

Moreover, some studies demonstrated that dietary polysaccharides can bind to PRRs on gut cells, such dendritic cells and intraepithelial lymphocytes (Abreu 2010; Abadie et al. 2012). Among the PRRs, dectin-1 receptor is the most important and is highly expressed in immunocompetent cells such as DC, neutrophils, eosinophils, macrophages, monocytes, and T lymphocytes (Brown and Gordon 2001; Taylor et al. 2002). Thus, after the binding of β-1,3/1,6 glucans to dectin-1 or CR3, a series of signaling events that modulate innate and subsequently adaptive immune responses are activated. This produces proinflammatory cytokines (IL-1α/β, IL-6, IL-8, IL-12, TNF-α) and reactive nitrogen and oxygen species, such as nitric oxide (NO)

and hydrogen peroxide (H_2O_2), which protect against pathogenic microbes, toxins, and carcinogens (Vetvicka and Yvin 2004; Brown and Gordon 2005; Goodridge et al. 2011).

Orally administrated β-glucans are taken up by macrophages via dectin-1 and transported to bone marrow, lymph nodes, and spleen (Hong et al. 2004a). Volman et al. (2010a) showed that β-glucans modulate the intestinal immune response. They also showed that oral administration of 3 mg dietary oat (1 → 3), (1 → 4)-β-D-glucans twice a day for 3.5 days increases intestinal nuclear factor kappa B (NF-κB) transactivation in leukocytes and enterocytes (dectin-1 negative cells) of the ileum. It also enhanced secretion of IL-12 protein in intestinal tissue lysates, and decreased the concentration of IFN-γ in the proximal small intestine of treated animals. These findings suggest that dietary oat β-glucans activate leukocytes from the intestine, which in turn activates enterocytes. In addition, macrophages are able to degraded large β-1,3-glucans into β-1,3-glucan soluble fragments, which can be taken up via CR3 by circulating granulocytes, monocytes, or macrophages (Chan et al. 2009). On the other hand, soluble β-glucans can be internalized directly by intestinal epithelial cells and cells from GALT (Rice et al. 2005).

Indeed, the receptor–glucan interaction triggers phagocytosis, respiratory burst, and secretion of cytokines such as TNF-α and IL-10 (Chen and Hasumi 1993; Kelly et al. 2010). Studies in vitro showed that sulfated polysaccharides purified from *Caulerpa lentillifera* (SP1) stimulated the NO production by murine macrophage RAW 264.7 cells, activates both NF-κB and p38 mitogen-activated protein kinase (MAPK) signaling pathways, increased the phagocytosis of latex beads, and induced the expression of proinflammatory cytokines IL-1β, TNF-α, and IL-6, which are important activities for the innate immune protection (Maeda et al. 2012). In addition, the nutritional supplement β-glucan in mice led to growth and improved nutrient retention and immune system function, stimulating cytotoxic T lymphocytes, B cells, and macrophages (Cross et al. 2001).

Carpenter et al. (2013) demonstrated that nutritional supplementation with defined β-1,3/1,6-glucans (BG) derived from *Saccharomyces cerevisiae* (BG: 250 mg/10 days) increased the total number of proinflammatory monocytes ($CD14^+/CD16^+$) and levels of plasmatic cytokines (IL-7, IL-4, IL-8, IFN-γ) induced by exercise (49 ± 6 min of a bout cycling, in a hot 38 ± 2 °C, 45 ± 2% humidity), altering the postexercise immunosuppressive condition induced by strenuous exercise. Furthermore, it was reported that supplementation with BG (VegeCap® capsules) for 28 days after completion of a marathon reduced the number of cold/flu symptom days postmarathon by 37 % during the supplementation period. On the other hand, a 10-day supplementation with BG prevented suppression of IgA secretion 2 h after strenuous exercise (cycling 49 ± 6 min, 45 ºC, 50 % relative humidity) by 32 % (McFarlin et al. 2013), reducing symptoms of upper respiratory tract infection and improving IgA-dependent mucosal immunity. Regarding the function of NK cells, oral supplementation with β-glucan from *Pleurotus ostreatus*, commercially available as Imunoglukan (100 mg β-glucan plus 100 mg of vitamin C) prevented the reduction of NK activity in elite athletes after 2 months of supplementation (Bobovčák et al. 2010).

Furthermore, low molecular weight polysaccharides isolated from *Agaricus blazei* Murill (ABP-AW1), used in folk medicine as antihypertensive and antitumor (Kim et al. 2009) remedies, present potential adjuvant activity. It was demonstrated that immunization of mice with ovalbumin plus ABP-AW1 (50, 100, and 200 μg) caused a significant antigen-specific $CD4^+$ T cell activation and IFN-γ production after challenge in vitro with ovalbumin (OVA), suggesting that ABP-AW1 significantly stimulates Th1 immunity (Cui et al. 2013). In addition, data corroborating its role in the induction of Th1 immune response were published. In this regard, *A. blazei* Murill water extract (AbM) presented significant antileishmanial activity in vivo. The daily oral administration of AbM at 100 mg/kg (day 0–20 days) after *Leishmania amazonensis* infection caused a 60 % reduction in lesion size and 60 % and 66 % reductions in parasite burden of the footpad and draining lymph nodes, respectively. In addition, the control of cutaneous leishmaniasis was correlated with higher levels of IFN-γ and NO production and lower levels of IL-4 and IL-10 in the spleen and lymph nodes (Valadares et al. 2012). Also, extracts from *A. bisporus*, a mushroom rich in β-glucan-rich polysaccharide, also induced NO production in bone-marrow-derived macrophages of mice (Volman et al. 2010b).

4 Bioactivity of Polysaccharides

The activity of polysaccharides is determined by their conformation, composition, and size (Zhang et al. 2007). Among the polysaccharides with higher biological activity, β-glucans that are homopolymers of D-glucose are the most abundant carbohydrates in the cellular walls of several microorganisms, such as mushrooms, yeast, algae, bacteria, lichens, and plants, and exhibit immunomodulatory, antitumor, and anti-infective activities. They are therefore used clinically for tumor immunotherapy in several countries (Wasser 2002; Falch et al. 2000; Barsanti et al. 2011). However, the fungal cell wall also contains other polysaccharides, such as chitin and cellulose, but glucans (glucose polymers) are the most important of this group of carbohydrates (Fig. 1). These carbohydrates display different types of glycosidic linkages, such as an α- or β-configuration, and at various positions, such as β-(1 → 3), β-(1 → 6), β-(1 → 4), or β-(1 → 2), and α-(1 → 3) glucans, or heteroglycans, and mostly bind to protein, forming a protein–glucan complex, such as glucomannan and galactomannan (Ooi and Liu 2000), and differences in their composition resulted in various biological activity. Table 2 summarizes some biological activity from mushrooms, besides antitumor and immunomodulatory activity, such antioxidant, antimicrobial, antiatherogenic, and hypoglycemic properties. The main molecule responsible for these activities are polysaccharides, mainly β-glucans, which normally are used in extracts; however, sometimes, they act together with other molecules, such phenolics, sterols, fibers, lectins, and others (Wasser 2011). Polysaccharides chitin and chitosan do not present antitumor activity (Mizuno et al. 1995); however α- or β-glucans present high antitumor activity. On the other hand, isolation of the protein from glucan resulted in reduced activity (Mizuno et al. 1999).

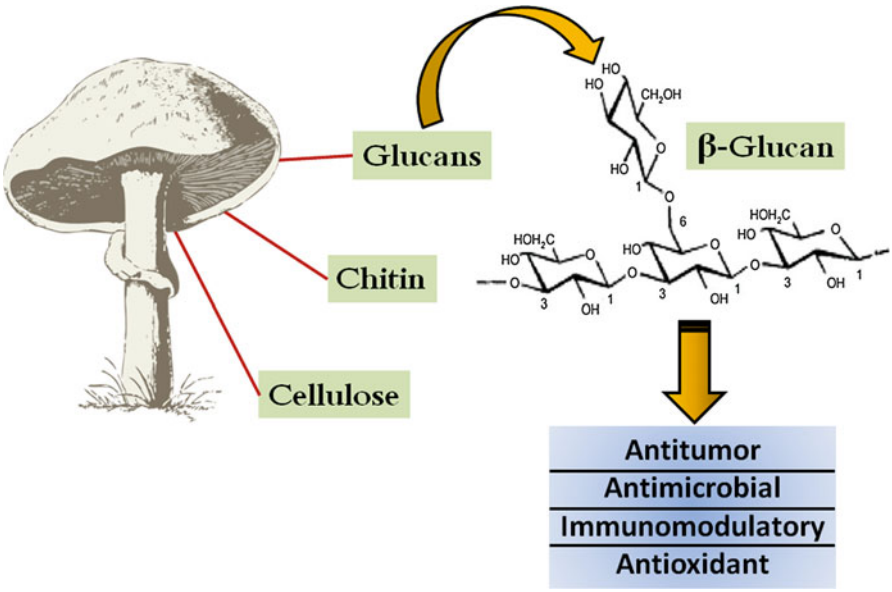

Fig. 1 Main cell wall polysaccharides of mushrooms and some biological activities of β-glucans

Variation in conformational complexity, molecular weight, and number of branches of β-glucan alters the biochemical and solubility properties and also influences their biological properties, such as immunomodulation both in vitro and in vivo (Brown and Gordon 2003; Miura et al. 2003; Volman et al. 2008; Huang et al. 2009). Regarding solubility, β-glucans can be classified according to their solubility properties: (a) alkali-insoluble, acetic-acid insoluble (1 → 3)-β-glucan; (b) alkali-soluble (1 → 3)-β-glucan; and (c) highly branched (1 → 6)-β-glucan. These properties may limit application and extrapolation of in vitro experimental data in humans (Zeković et al. 2005; Mantovani et al. 2008). Furthermore, other effects of β-glucans also are effective against cancer; inhibit metastasis; increase host resistance to bacterial, viral, and parasitic infections and some microbial infections; have antimutagenic, antiallergic, regenerative, antithrombogenic, anticoagulative, antioxidant, hypolipidemic, radioprotective, and antidiabetes properties (Reynolds et al. 1980; Meira et al. 1996; Wasser 2011; Chang and Wasser 2012).

Certain glucose polymers, such as (1 → 3), (1 → 6)-β-glucans, were recently proposed as potent immunomodulation agents (Ooi and Liu 2000; Falch et al. 2000; Barsanti et al. 2011). In this regards, glucans extracted from the mycelium of *A. subrufescens* are usually β-(1 → 6)-glucans, whereas the structure of those extracted from basidoma are β-(1 → 3)-glucans. Zong et al. (2012) notes that molecule-linked β-(1 → 6)-glucans are structurally important in the biological activity, whereas chains-linked β-(1 → 4) or β-(1 → 2) reinforce activity of the immunomodulatory body. Ahn et al. (2004) and Oba et al. (2009) report the

Table 2 Biological activity of polysaccharides from mushrooms

Activity	Mushroom	Reference
Antitumor	*Agaricus subrufescens*	Sugiura et al. (1980), Leifa et al. (2007), Jumes et al (2010)
	Agaricus bisporus	Grube et al. (2001)
	Agaricus sylvaticus	Fortes et al. (2009)
	Ganoderma lucidum	Wan et al. (2008)
	Pleurotus ferulae	Choi et al. (2004)
	Lentinula edodes	Chen et al. (2010)
	Grifola frondosa	Louie et al. (2010)
	Inonotus obliquus	Lee et al. (2009a)
	Cordyceps militaris	Lee et al. (2009b)
	Hypsizigus marmoreus	Saitoh et al. (1997)
	Phellinius linteus	Han et al. (2006)
Immunomodulatory	*Agaricus subrufescens*	Ahn et al. (2004)
	Pleurotus ostreatus	Bauerova et al. (2009)
	Lentinula edodes	Oba et al. (2009)
	Grifola frondosa	Sakamoto et al. (2006)
	Coriolus versicolor	Deng et al. (2009)
	Trametes versicolor	Ramberg et al. (2010)
Anti-inflammatory	*Ganoderma lucidum*	Hong et al. (2004b)
Antimicrobial action	*Agaricus subrufescens*	Bernardshaw et al. (2005)
	Pleurotus ostreatus	Hearst et al. (2009)
	Lentinula edodes	Hearst et al. (2009)
Antioxidant	*Pleurotus ostreatus*	Jayakumar et al. (2009)
Hypoglycemic activity	*Agaricus subrufescens*	Hsu et al. (2007)
	Ganoderma lucidum	Zhou et al. (2005)
Hypocholesterolemic	*Agaricus subrufescens*	Kwon et al. (2002)
	Lentinula edodes	Kwon et al. (2002)
	Pleurotus ostreatus	Khatun et al. (2009)
Antiviral	*Agaricus subrufescens*	Faccin et al. (2007)
	Ganoderma lucidum	El Dine et al. (2009)

increase activity of NK cells from extracts of *A. subrufescens* and *Lentinula edodes*, respectively, and showed that immunochemotherapy with lentinan was effective in increasing the survival rate of patients with gastric cancer with lymph node metastasis. Grube et al. (2001) reported a reduction in estrogen production related to a breast cancer cell line.

The antitumor effects of fungal β-glucans seem to be related to their structural features and conformation; the triple helical conformation plays an important role in enhancement of antitumor activities and is more effective than single flexible chains (Falch et al. 2000). Several soluble β-glucans (SG) showed antitumor activity, such as (1 → 3)-β-D-glucan, which can be obtained from *Sclerotinia sclerotiorum* (Kurachi et al. 1990), *Schizophyllum commune* (Tabata et al. 1981),

and *Grifola frondosa* (Kodama et al. 2003). Polysaccharides can inhibit tumor growth via several mechanisms, such as prevention of tumorigenesis by oral consumption of active preparations, direct anticancer activity by inducing tumor cell apoptosis, and inhibition of tumor metastasis and immunopotentiation activity in combination with chemotherapy (Zong et al. 2012). In this regard, glucans may inhibit tumor growth and metastasis in mice models via modulating the immune function, inducing expression of cell receptors, and inducing cell apoptosis via activation of the proapoptotic Bak-1 protein and DNA-dependent protein kinase (DNA-PK), which are responsible for inducing cancer cell arrest at the G1 cell-cycle checkpoint (Pyo et al. 2008; Louie et al. 2010).

Other antitumor activity was reported by Leifa et al. (2007), who reported that exopolysaccharide extract of *A. subrufescens* inhibited sarcoma 180 in mice by 72.2 % compared with the control group, and complete tumor regression was observed in 50 % of mice. Similar results were observed by Sugiura et al. (1980) who evaluated the antitumor action of exopolysaccharide and the basidioma of *A. subrufescens* on sarcoma 180 induced in mice. On the other hand, the administration of A. brasiliensis extracts and pure powdered basidiocarp by gavage (136 mg/kg × 14 days) in rats with Walker 256 tumor promoted a 92.2 % tumor reduction with pure extract and 72.2 % with purified extract (Jumes et al. 2010).

Peptidoglucan from *Ganoderma lucidum* led to an antiproliferation effect on and apoptosis of human breast cancer cell lines (Wan et al. 2008), and anti-inflammatory function in human carcinoma cells (Hong et al. 2004b). Bauerova et al. (2009) observed a positive immunomodulating effect of β-(1 → 3) and β-(1 → 6)-glucans of *Pleurotus ostreatus* on all cytokines present in rheumatoid arthritis, thereby preventing disease progression. Other mushrooms also presented immunostimulatory activity, such as *Grifola frondosa, Coriolus versicolor*, and *Trametes versicolor* (Sakamoto et al. 2006; Deng et al. 2009; Ramberg et al. 2010).

Studies have demonstrated the antimicrobial action of mushroom extracts against Gram-positive and Gram-negative bacteria in vitro. When shiitake (*L. edodes*) and oyster mushrooms (*P. ostreatus*) were tested for their antibacterial and antifungal properties, it was verified that shiitake extract presented more antimicrobial effect than ciprofloxacin (Hearst et al. 2009). In addition, the antimicrobial effects in vivo may be due to its effects on the immune system, as suggested, for example, by Bernardshaw et al. (2005) who reported the effect of *A. subrufescens* extract against systemic *Streptococcus pneumoniae* infection in mice. Some mushrooms promote anti-HIV action, one of which is *G. lucidum*, due to the presence of ganomycin. Another example is the polysaccharide of *A. subrufescens*, which acted in the initial phase during poliovirus infection (El Dine et al. 2009; Faccin et al. 2007).

Mushroom extracts induces alterations in the diabetic state and in parameters related to the disease. For example, *A. subrufescens* extract (in combination with metformin and gliclazide) improve insulin resistance, potentially by the mechanism that caused an increase in adiponectin concentration after taking the extract for 12 weeks (in vivo) (Hsu et al. 2007); *G. lucidum* extract resulted in hypoglycemic activity (in vivo) (Zhou et al. 2005).

Hypocholesterolemic properties of mushrooms are involved in the fatty acid pattern of edible mushrooms, which seems to contribute to reduce serum cholesterol. Several investigations have shown the influence of eating mushrooms in improving some cardiac metabolic markers, such as high-density lipoprotein (HDL) cholesterol, fasting triacylglycerol, homocysteine, very-low-density lipoproteins, and lipid metabolism, consequently preventing the development of atherosclerosis (Fukushima et al. 2000; Cheng et al. 2002; Hossain et al. 2003). Antioxidant and anti-inflammatory compounds in mushrooms may also contribute to reduce atherosclerosis risk. Moreover, their high-fiber and low-fat content, with low trans isomers of unsaturated fatty acids, low concentration of sodium, presence of eritadenine, phenolic compounds, sterols (such as ergosterol), chitosan, and triterpenes are properties responsible for reducing cardiovascular diseases (Hu et al. 2006; Guillamón et al. 2010).

Antioxidant properties of ethanolic extracts of oyster mushrooms were identified and were shown to be more powerful than the commercial antioxidant butylated hydroxyl toluene (BHT), indicating their possible use as food supplement or pharmaceutical agent (Jayakumar et al. 2009). The fungi *Hericium erinaceous* produces hericenones, which stimulate the synthesis of nerve growth factor, and studies demonstrate that this mushroom protects against neuronal-cell death caused by β-amyloid peptide toxicity, endoplasmic reticulum (ER) stress, and oxidative stress, and retards disease progression in patients with dementia (in vivo) (Kawagishi and Zhuang 2008).

Inflammatory bowel disease (IBD) is an inflammatory condition of gastrointestinal tract that is chronic, remitting, relapsing, and progressive. IBD comprises two major clinical entities: Crohn's disease (CD) and ulcerative colitis (UC), which may affect the entire gastrointestinal tract and the colonic mucosa, respectively. Ambrotose complex (AMB) and advanced Ambrotose (AdvAMB) powder are dietary supplement formulas that include polysaccharides derived from *Aloe barbadenis*, *Larix* species, *Anogeissus latifolia*, *Astragalus gummifer*, *Oryza sativa*, and *Undaria pinnatifida*, which are rich in fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid. Using a rat model of colitis induced by replacement of drinking water with a solution of 5 % dextran sulphate sodium (DSS), Koetzner et al. (2010) demonstrated that both AMB and AdvAMB decreased the activity of colitis. The authors showed that oral administration of AMB (377.1 mg/kg) or Adv-AMB (57.4 and 574 mg/kg) once a day for 14 days significantly inhibited shortening of the colon in DSS-treated animals, a characteristic finding in colitic animals and caused a significant decrease in the number of circulating monocytes.

5 Bioavailability and Metabolism

Pharmacokinetics for three different glucan polymers (glucan phosphate, laminarin, scleroglucan) were described after intravenous administration in rats, using estimates of compartment size and rate of change. The authors found significant

differences in pharmacokinetics due physicochemical properties such as molecular size and solution conformation, with glucan phosphate having a smaller volume of distribution and clearance than laminarin and scleroglucan, demonstrating higher blood levels are acquire with water-soluble glucans (Rice et al. 2004). Plasma levels after oral administration were measured and showed differences among polymers, such as bioavailability. Laminarin and scleroglucan had a bioavailability of 4.9 % and 4.0 %, respectively, whereas glucan phosphate bioavailability was tenfold lower (Rice et al. 2005).

Regarding molecular size, this feature can affect the biological activity of polysaccharides, and usually, those of low molecular weight are more effective. Thus, reduction in the particle size of drugs can lead to an increased rate of dissolution and higher oral bioavailability. Similar results were obtained with mushroom powder of the stipe of *Hericium erinaceus*, which was associated with an increase in disease resistance of shrimp: particle sizes of <74 mm were more effective than particle sizes of >74 mm, indicating that they modulate immune function (Yeh et al. 2011). The same occurs with soluble β-glucans, which appear to be stronger immunostimulators than insoluble ones (Xiao et al. 2004), although the reasons are not totally clear; large molecules and orally administered insoluble β-glucans may subsequently degrade into smaller bioactive oligomers after ingestion (Hong et al. 2004a).

Another important feature for the biological activity of polysaccharides is the administration route. Most polysaccharides are activated after oral (Suzuki et al. 1989, 1990; Nicoletti et al. 1992; Dritz et al. 1995; Cheung et al. 2002; Hong et al. 2004a), intraperitoneal, and subcutaneous (Yun et al. 1997, 1998, 2003; Volman et al. 2008) administration. To exert biological effects, glucans should be absorbed from the GI tract and enter the systemic circulation. This process varies according to certain parameters, such as molecular weight distribution, polydispersity, side-chain branching, root-mean-square radius, and solution conformation (Mueller et al. 2000). The organism kinetics is improved in macrophages due to internalization of polymer particulates, which transport glucan to various sites in the body and release a bioactive, soluble, glucan product (Hong et al. 2004a). Orally administered β-glucans arrive at the proximal small intestine and are taken up by macrophages for specific receptors (dectin-1) and carried to other organs, such as spleen, lymph nodes, and bone marrow, where macrophage glucans are degraded into small particles that then interact with complement receptors of marginated granulocytes, thus producing immunological activity (Hong et al. 2004a; Chan et al. 2009).

In a clinical trial of a new, soluble yeast product in healthy individuals, safety and tolerability of polysaccharides was evaluated for 4 days in order to assess clinical, hematological, biochemical, and mucosal tolerance after oral administration of doses ranging from 100 to 400 mg; it was found to be safe and well tolerated (Lehne et al. 2006). On the other hand, some studies reported that oral administration of purified fungal β-glucans have been used to treat human diseases, with no adverse effects; however, the interaction of some glucans with other drugs may be dangerous – drugs such as the nonsteroidal anti-inflammatory drug indomethacin (at doses 2.5–5 mg/kg), which has been reported to cause patient death due to maladjustment of cytokine production (Yoshioka et al. 1998; Takahashi et al. 2001).

6 Toxicity of Dietary Polysaccharides

Understanding the biological activities and adverse effects of natural products used in folk medicine are important to determine safety parameters, as there was once a popular belief that they present only minor adverse effects (Barbisan et al. 2002). Toxicity testing is necessary to provide the basis for substance regulation for clinical use to determine safety and biological effect of cosmetics, pharmaceuticals, food additives, pesticides, chemicals, and consumer products, the use of which toxicological effects can result from natural or manufactured substances and may manifest a variety of symptoms following both acute or chronic exposure (Gallagher 2003).

Dietary polysaccharides need to be evaluated for safety, due to their variability and high biological potential and to estimate the appropriate doses for human use and for clinical monitoring of adverse effects (Carbonero et al. 2006; Alavi et al. 2011; Velusami et al. 2013).

Some negative side effects have been observed in response to prolonged or large doses of polysaccharides, such as β-glucan preparations, as have the consequences of their modes of action and physical and chemical properties (Kwiatkowski and Kwiatkowski 2012). Fungal glucans are an important class of polysaccharides with positive biological activities, and most of them are used as dietary supplements and as food ingredients. Evaluating their safety is an important concern, especially in wild mushrooms due to their ability to accumulate radioactive substances in relatively high concentrations, i.e., heavy metals such as arsenic, lead, cadmium, and mercury (Borchers et al. 2004).

In addition, prolonged or large doses of β-glucan preparations may stimulate prolonged proinflammatory changes in cells, causing the development of autoimmune diseases. Indeed, since the human body is absent enzymes that hydrolyze β-glucans, their degradation occurs through the less efficient oxidative pathway, which prolongs their cellular half-life and thus may lead to the formation of granulomas in the liver, resulting in inflammation that may result liver cirrhosis. Therefore, careful clinical studies recommend caution in the use of glucans as a long-term food additive to prevent the accumulation of granulomas in these patients (Kwiatkowski and Kwiatkowski 2012).

Others studies suggest that polysaccharides are unlikely to cause severe adverse effects: based on acute- and chronic-toxicity testing in rodents, most polysaccharide products appear to be safe (Table 3), with some clinical studies suggesting reactions such as bloating or flatulence linked to a secondary effect of beneficially altering gut microflora (Kelly 1999).

7 Dietary Polysaccharides of Mushroom and New Trends

Mushrooms have long been used as dietary supplements and medicinal food in Asia, because they contain numerous biologically active compounds with antitumor and immune-stimulating action and hypocholesterolemic, antioxidant, and

Table 3 Toxicological studies involving polysaccharides from microorganisms and plants

Category	Source	Test	Animal	Dose	Result	Reference
Microorganism						
Glucans	*Ganoderma lucidum*	Subchronic toxicity, mutagenicity studies	Sprague Dawley rats	0, 500, 1,000 and 2,000 mg/kg body weight (bw)/ day for 90 days	NOAEL	Chen et al. 2011
Glucan (Curdlan)	*Alcaligenes faecalis* var. *myxogenes*	Acute toxicity	Male rats	10 g/kg body weight orally	Low acute toxicity, no deaths	Aomori and Tanida 1968
Glucan (Curdlan)	*Alcaligenes faecalis* var. *myxogenes*	Subchronic and chronic toxicity	Male rats	2.5, 8.5, 30 and 33.4 g/kg/ day, 1-month oral	No signs of toxicity	Yokotani 1969
Glucan (Curdlan)	*Alcaligenes faecalis* var. *myxogenes*	Month toxicity	Male and female Sprague Dawley rats	5 %, 10 % and 20 % for 12 weeks	No effects on hematology and no changes in histopathology	Nakaguchi et al. 1972
Glucan (lasiodiplodan)	*L. theobromae*	Sub-chronic toxicity (28 days)	Male and female Swiss albino mice	50 mg/kg/day	No signs of toxicity	Túrmina et al. 2012
Glucan	Agaricus blazei Murrill	90-day subchronic toxicity	Male and female F344 rats	2,654 and 2,965 mg/kg	No significant toxicity	Kuroiwa et al. 2005
Glucans	Ganoderma lucidum	Single dose toxicity	Sprague–Dawley rat	2,000 mg/kg i.p	No signs of toxicity	Kim et al. 2012
Chitin- glucans	Aspergillus niger	Subchronic toxicity (13 weeks)	Wistar rats	Diet 0; 1; 5; 10 % 6.6 and 7.0 g chitin-glucan/kg body weight/day)	No signs of toxicity	Jonker et al. 2010
Arabino-galactans	Argemone mexicana	Develomental toxicity	Pregnant rats	250, 500, or 1,00 mg/kg	No signs of toxicity	Tamboli et al. 2010

	A. blazei Murill (Aqueous extract)	Chronic carcinogenic bioassay	Male and female F344 rats	0, 6,250, 12,500, 25,000 ppm 1,176 mg/kg diet for 2 years	Not signs of carcinogenicity	Lee et al. 2008
	A. bisporus dry powder	Carcinogenic bioassay	Female Sprague–Dawley rats	30 % diet for 500 days	Not signs of carcinogenicity	Matsumoto et al. 1991
	Agaricus blazei aqueus extract	Hepatotoxicity	Male wistar rats	5,6 mg/mL for 2 weeks	Hepatoprotective effect	Barbisan et al. 2002
Plants						
Arabinogalactans	Larix sp. – plant	Acute toxicity	Mice and rats	5,000 mg/kg	No signs or symptoms of toxicity	Groman et al. 1994
Arabinogalactans	Larix sp. – plant	90 days toxicity	Mice and rats	500 mg/kg	No signs or symptoms of toxicity	Groman et al. 1994
Purslane crude polysaccharide	Purslane	Acute toxicity	Male and female mice	250, 500, 750 and 1,000 mg/kg	No symptom of toxicity or behavioral changes	Gong et al. 2009
Tea polysaccharide conjugate	Camellia sinensis	Acute toxicity	Kunming male mice	5,000 mg/kg	Low toxicity	Chen et al. 2007

hypoglycemic effects. Fungal β-glucan induces stimulation of the entire immune system, which could be an advantage in treating diseases. Furthermore, many of them can be administered orally and in combinational with other immune therapies, resulting in potentialization of results. For example, a synergistic effect is believed to occur when combining β-glucan with IFN-γ (Drandarska et al. 2005).

Several studies report that most orally ingested polysaccharide products (glucans, pectins, heteroglycans, glucomannans, fucoidans, galactomannans, Arabinogalactans, and mixed polysaccharides) in rodent models exerted significant stimulatory effects on the immune system. This led to increased interest in the use of mushrooms and mushroom extracts as dietary supplements and therapeutics. The main sources of mushroom polysaccharides are basidiomas and mycelia (Wasser 2002), which are used for cancer treatment together with surgery, chemotherapy, and radiotherapy (Mizuno et al. 1999; Wasser 2002; Gao et al. 2003; Zhang et al. 2007). These mushroom polysaccharides act as biological response modifiers primarily by stimulating the host immune system by activating dendritic cells, NK cells, T cells, macrophages, and cytokine production.

Among the main products developed from polysaccharides are Lentinan isolated from *Lentinula edodes*; schizophyllan (sonifilan, sizofiran, or SPG) from *Schizophyllum commune;* krestin (PSK) and polysaccharide peptide from *Trametes versicolor*; D-fraction from *Grifola frondosa*; Befungin from *Inonotus obliquus*; and *G. lucidum* polysaccharide fractions (Wasser 2011).

Mushrooms produce many other molecules of secondary metabolism, such as lectins, lactones, terpenoids, alkaloids, antibiotics, and metal-chelating agents, and together with the presence of polysaccharides produce >100 beneficial effects besides antitumoral mechanisms, such as immunomodulation, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemic, antiviral, antibacterial, antiparasitic, antifungal, detoxifying, hepatoprotective, and antidiabetic actions (Wasser 2011). Therefore, new products based on mushrooms are used as dietary supplements, mycopharmaceuticals, and – to use a new term – mushroom nutriceuticals; which should not be confused with nutraceuticals, functional foods, and pharmaceuticals (Chang 2006; Wasser and Akavia 2008). Attention should be given to the fact that nutriceutical mushrooms are not food but refined, or partially refined, extract or dried biomass from mycelium or basidioma, which is consumed in the form of capsules or tablets as a supplement and has potentially therapeutic applications. Regular intake may enhance immune response, thereby increasing resistance to disease and, in some cases, causing disease regression (Wasser 2011). Most polysaccharide products appear to be safe, with no adverse effect level (NOAEL) based acute and/or chronic toxicity tests done mostly in rodents; these products are commercialized in the form of powders, extracts, and capsules, although they have not been fully characterized, and the impact of polysaccharide intake on absorption of nutrients and medications is not known (Ramberg et al. 2010).

Prebiotics are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson et al. 2004).

Due their carbohydrate composition, such as chitin, hemicellulose, β-glucans, mannans, xylans, and galactans, mushrooms can be used as a prebiotic. *Pleurotus ostreatus* and *P. eryngii* extracts stimulate the growth of probiotic strains of *Lactobacillus*, *Bifidobacterium*, and *Enterococcus faecium*, benefic microorganism of the intestinal microbiota (Synytsya et al. 2008). Therefore, intake of mushroom extracts can be benefic due to their prebiotic activity and the medicinal benefits of immunomodulating properties of polysaccharides (Aida et al. 2009).

Another polysaccharide is sclerotium, a morphological form produced by some mushrooms, which has a compact mycelial structure under unfavorable conditions and remains dormant until the environment is suitable for its development of fruiting bodies for reproduction. The major cell wall components are chitin and β-glucan, with β-1,3 backbone and β-1,6-linked side branches (Cheung and Lee 2000). Their sclerotial cell wall components cannot be digested by human enzymes, and as it has ten or more monomeric units, it can be regarded as a novel source of dietary fiber (Codex Alimentarius 2010). This dietary polysaccharide is known to impact intestinal microbial ecology, and recent studies indicate its benefic effects on nutrition, immune modulation, pathogen resistance, intestinal epithelial development, and activity after regular intake (Ramberg et al. 2010).

Investigating new uses for and new products with biopharmacological and dietary benefits are ongoing. Some focus on simplifying and reducing the cost of production to provide access to a larger portion of the population or use in farm animals (Wasser 2011). Ingestion of myceliated wheat grains with *G. lucidum* and *A. subrufescens* or *A. brasiliensis* promote antitumor and hypocholesterolemic effects in sarcoma-180-bearing mice (Rubel et al. 2008; Dalla-Santa et al. 2012). Results of such trials led to the incorporation of many different myceliated grains, and later, using fruit and vegetable residues resulted in patents of processes and products. Some studies reported that extracted ergosterol from these alternative and complex substrates resulted in antitumor action in vitro (Takaku et al. 2001; Lv et al. 2012). It seems that this could be an economic and feasible tool to produce mushroom mycelia containing polysaccharides, ergosterol, and the other bioactive molecules.

In this regard, Wasser (2002) proposed the use of medicinal mushrooms as sources of active biological molecules, new types of food and dietary supplements, antibiotic replacement, and antiviral agents for farm-animal research. New technologies to improve the quality of such biologics resulted in the development in 2009 of a new method for nanoparticle extraction of water-soluble β-glucans from medicinal mushrooms by the Park group (Park et al. 2009) from South Korea for their use in the food, cosmetic, and pharmaceutical industries.

Another new use for mushroom extract, as a potential color stabilizer, showed doubled and tripled shelf life of beef and bigeye tuna (*Thunnus obesus*) by retarding browing: ergothioneine extracted from *Flammulina velutipes* prolonged storage of ground beef from 6 to 12 days and bigeye tuna from 2 to 7 days (Bao et al. 2008).

Consumption of edible mushrooms is beneficial due their nutritional value and because they are low in calories, sodium, fat, and cholesterol, while containing a high percentage of protein, carbohydrate, fiber, vitamins, and minerals.

Therefore, besides their innumerable medicinal properties, they can contribute to the formulation of a well-balanced diet (Manzi et al. 1999).

Fungal β-glucans and whole mushrooms in medicinal and dietary forms lead to significant health improvement and they are specially benefic for middle-aged people or those with impaired immune systems, those with active and stressful lifestyles, those with cancer and in chemotherapy and radiotherapy, and those with infectious diseases (Chen and Seviour 2007).

8 Conclusion

In conclusion, identifying foods that produce high levels of polysaccharides such as β-glucan may increase absorption and consequently efficacy of these beneficial polysaccharides by the production of functional foods with medicinal properties. Furthermore, foods and extracts containing β-glucans could support and enhance treatment of patients with certain pathologies, such cancer patients undergoing chemotherapy or radiotherapy by improving immunologic status and reducing untoward effects on normal tissues. However, careful clinical studies comparing the activity of polysaccharides remain necessary to determine whether they provide real clinical benefits. Also, negative side effects in response to prolonged or large doses of β-glucan preparations have been reported, and conclusive evidence is still lacking regarding the efficacy of medicinal mushrooms and their individual polysaccharides. However, preliminary evidence from studies of various dietary polysaccharides suggests that large, randomized controlled trials are needed, especially for determining toxicity with long-term use.

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Bacterial Polysaccharides: Production and Applications in Cosmetic Industry

66

Filomena Freitas, Vítor D. Alves, and Maria A. M. Reis

Contents

Abstract

Cosmetics are complex multiphase systems that include different components with distinct functions on the final product. Bacterial polysaccharides are biocompatible, biodegradable, and usually nontoxic natural biopolymers that possess physicochemical properties suitable for use as cosmetic ingredients. Some of them, namely, hyaluronic acid (HA), bacterial cellulose (BC), and levan, have biological properties (e.g., skin regeneration and protection) and are used as active agents in cosmetic formulations. Other bacterial polysaccharides, such as xanthan gum and gellan gum, are mostly used as viscosity controllers and psychosensorial agents and are applied in cosmetic vehicles (e.g., emulsions, gels, and suspensions). The nontoxic nature of these bacterial polysaccharides has been thoroughly assessed by innumerous studies and their safety as cosmetic ingredients has been established.

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1 Introduction

Cosmetics are substances or preparations intended to enhance the human body appearance, and they include products of several categories, such as lotions, creams, emulsions, liposomes, shampoos and hair conditioners, sunscreens, and color cosmetics (Milstein et al. 2001). Hence, cosmetics are placed in contact with various parts of the human body (epidermis, hair system, nails, lips, etc.) or with the teeth and the mucous membranes of the oral cavity. Cosmetics are required not to affect the body structure or functions. In contrast with drugs that have extensive regulatory requirements, cosmetic products are relatively easy to market since no premarket approval is required (Milstein et al. 2001). However, if the cosmetic products contain a pharmacologically active ingredient in therapeutically active concentrations, such information is taken in consideration for the determination of the product's regulatory status.

Cosmetics are complex multiphase systems that include different components: base substances, active agents that have specific biological effects and additives that provide physical and microbiological stability to the final product. Several bacterial polysaccharides possess properties that render them suitable for use in cosmetic applications (Table 1). The most relevant are xanthan gum and gellan gum that are mostly used as base substances in cosmetic vehicles (e.g., emulsions, gels, and suspensions) and hyaluronic acid, bacterial cellulose, and levan, which are bioactive ingredients. Besides its biological activity, hyaluronic acid has been also applied in nanoemulsions as encapsulating agent of other bioactive substances. In contrast with nonbiodegradable polymers that are not metabolized or degraded by the human body and can accumulate in the tissues, biodegradable polymers, including polysaccharides, can be naturally eliminated by normal metabolic pathways. This is advantageous for use in cosmetics applications. Moreover, biodegradable polymers are usually nonreactive with the human body and their degradation products are also biocompatible (Ammala 2013).

2 Bioactivity

2.1 Bioactive Bacterial Polysaccharides

The most relevant bioactive bacterial polysaccharide currently used in cosmetic formulations is hyaluronic acid (HA). Recently, the ability of bacterial cellulose and levan to preserve and/or improve the skin's natural condition has also been extensively studied and proposed for commercial applications. On the other hand, numerous reports about novel bacterial polysaccharides with new or improved biological activities are continuously being published.

Table 1 Bacterial polysaccharides used in cosmetic applications

Polysaccharide	Producing bacteria	Composition	Charge	Mw	Main properties	References
Hyaluronic acid	*Streptococcus* sp.	Glucuronic acid	Anionic	2.0×10^6	Water soluble	Allemann and Baumann (2008), Berkó et al. (2013), Chong et al. (2005), Liu et al. (2011), Marcellin et al. (2009), Vásquez et al. (2009, 2010, 2013), Widner et al. (2005), Yamada and Kawasaki (2005)
		Acetylglucosamine			Highly viscous hydrocolloid	
					Viscoelastic	
					High swelling capacity	
					Biological activity	
					Lubricant	
					Immunostimulant	
					Angiogenic	
					Antiaging affect	
Xanthan gum	*Xanthomonas* sp.	Glucose	Anionic	$(2.0–50) \times 10^6$	Highly viscous hydrocolloid	Becker and Vorholter (2009), García-Ochoa et al. (2000), Imeson (2010), Rottava et al. 2009; Ullrich (2009), Yang (2007)
		Mannose				
		Glucuronic acid				
		Acetyl				
		Pyruvyl				
Gellan gum	*Sphingomonas paucimobilis*	Glucose	Anionic	5.0×10^5	Water soluble	Bajaj et al., (2007), Coleman et al. (2008), Fialho et al. (2008), Imeson (2010), Ullrich (2009)
		Rhamnose			Hydrocolloid	
		Glucuronic acid			Gelling agent	
		Acetyl			Filmogenic	
		Glyceryl				

(*continued*)

Table 1 (continued)

Polysaccharide	Producing bacteria	Composition	Charge	Mw	Main properties	References
Bacterial cellulose	*Gluconacetobacter xylinum*	Glucose	Neutral	~10^6	Insoluble in most solvents	Amnuaikit et al. (2011), Bae et al. 2004; Chawla et al. (2009), Cheng et al. (2009), Czaja et al. (2006), Fu et al. (2013), Solway et al. (2010), Valla et al. (2009), Yang (2007)
					Highly crystalline	
					High tensile strength	
					Moldable membranes	
					High degree of hydration	
					Biological activity	
					Skin regeneration	
					Wound healing	
					Skin moisturizer	
Levan	*Zymomonas mobilis*	Fructose	Neutral	(1.0–3.0) × 10^6	Water soluble	Furukawa and Tsuboi (2006), Kang et al. (2009), Kim et al. (2003), Oliveira et al. (2007), Senthilkumar and Gunasekaran (2005), Shih and Yu (2005)
	Halomonas smyrnensis				Low viscosity	
	Bacillus subtilis				Adhesive strength	
					Filmogenic	
					Biological activity	
					Skin moisturizing	
					Skin whitening	
					Skin irritation-alleviation	
					Anti-inflammatory	

2.1.1 Hyaluronic Acid

Hyaluronic acid (HA) is a linear, high-molecular-weight glycosaminoglycan composed of alternating disaccharide units of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) linked by β-(1 → 3) and β-(1 → 4) glycosidic bonds (Fig. 1) (Chong et al. 2005; Liu et al. 2011). It is a major structural component of the intercellular matrix of mammalian connective tissues, where it controls tissue permeation and hydration, macromolecular transport between cells, and bacterial invasiveness. HA has a high swelling capacity (high hydrodynamic space in solution), being able to bind 1,000 times its volume in water (Allemann and Baumann 2008). These features, together with its chemical structure, confer a wide range of physicochemical and biological properties, such as lubricity, viscoelasticity, biocompatibility, and angiogenic and immunostimulatory effects (Chong et al. 2005; Vázquez et al. 2013) (Table 1). Although the activity of HA is dependent on the molecule's size, all ranges of molecular weights have specific applications. HA has great economical value with numerous applications, including the medical and cosmetic fields (e.g., antiaging agent) (Vázquez et al. 2013). Depending on the application, HA and its derivatives have market values within the range of US$ 2,000–60,000 per kg (Pires et al. 2010). Due to its biocompatibility, non-immunogenicity, biodegradability, and viscoelasticity, HA is a biomaterial widely used for cosmetic, medical, and pharmaceutical applications (Berkó et al. 2013).

Initially, commercial HA was obtained from mammalian tissues, such as rooster combs, synovial fluid, and umbilical cords. Due to public health concerns (e.g., animal-derived pathogens, interspecies viral, or prionic contaminations), microorganisms and marine organisms started to be investigated as sources of HA. Marine wastes (e.g., sponges, sea cucumbers, squids, mollusks, invertebrates, fish cartilage, and vitreous humor) have been explored, but the most important alternative in recent years has been the microbial production by pathogenic Lancefield group A and C streptococci (e.g., *Streptococcus equi* subsp. *zooepidemicus*), including wild-type and attenuated HA-high-producing mutants (Yamada and Kawasaki 2005).

Some efforts have been put on the development of alternative microbial systems, including the development of recombinant strains and the search for nonpathogenic microorganisms (especially Generally Recognized as Safe (GRAS) strains) for the production of HA to overcome the difficulty of using pathogenic strains (Kim et al. 1996). Izawa et al. (2010) reported that *Streptococcus thermophilus* YIT 2084, a GRAS strain isolated from a dairy food product, was able to produce HA from milk, although at a concentration lower than that achieved by *S. equi*. Recently, great effort has been put on the development of heterologous systems by transforming bacterial species that normally do not produce HA (e.g., *Escherichia coli*, *Lactococcus lactis*, or *Bacillus subtilis*) into HA producers (Mao et al. 2009; Prasad et al. 2010; Yamada and Kawasaki 2005; Yu and Stephanopoulos 2008; Widner et al. 2005). However, there are restrictions to the use of recombinant strains in some countries and the production achieved by heterologous HA-producer systems, as well as the polymer's molecular weight, was considerably lower than those obtained by wild-type streptococcal fermentation.

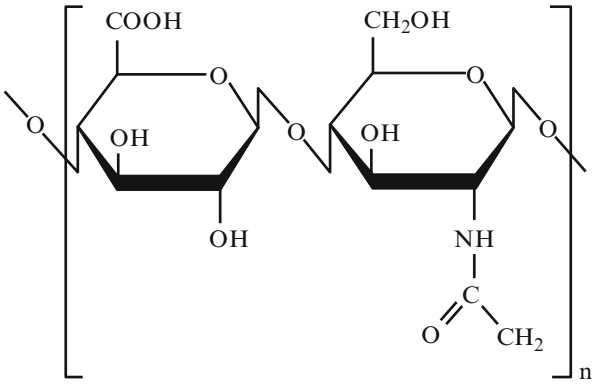

Fig. 1 Chemical structure of hyaluronic acid

Streptococci bacteria require complex nutrients, including organic nitrogen, which is also used by the microorganisms as carbon source for cell growth (Pires et al. 2010). The substrate costs are not a major concern in HA production due to the polymer's high market value. Considerably more focus has been put on strain and process development and on HA quality (molecular weight and purity) rather than on quantity (Chong et al. 2005). Cultivation is usually performed with complex growth media containing yeast or animal extract, peptone, and serum, but the use of chemically defined medium containing glucose, amino acids, nucleotides, trace elements, and vitamins has also been reported (Chong et al. 2005). Some agro-industrial wastes/by-products have been tested as carbon and/or nitrogen sources for HA production, including hydrolysate soy protein concentrate, whey protein concentrate, cashew apple juice, corn steep liquor, mussel processing wastewaters, and peptones obtained from fish viscera (Pires et al. 2010; Vázquez et al. 2009, 2010), but such substrates are still not used for commercial production of HA.

Although HA may be derived from several natural sources, only bacterial fermentation and rooster combs-derived HA is used in cosmetics, as skin-conditioning agents and viscosity-increasing agents. Due to its ability to provide skin water retention capacity and skin hydration, the main commercial application of HA is in cosmetic antiaging creams, which account for around 15 t/year (Marcellin et al. 2009). The use of HA and its salts has been reported in numerous cosmetic product categories (e.g., makeup, moisturizers, lotions, sprays, lipsticks), at concentrations up to 1 %, for HA, or up to 2 % for sodium hyaluronate (Becker et al. 2009). HA has been reported to have antiwrinkle effect by effectively moisturizing and enhancing the skin's elasticity (Allemann and Baumann 2008). In recent years, HA emerged as a dermal filler that rapidly replaced collagen in nonsurgical cosmetic procedures (Fakhar and Berkland 2013; Marcellin et al. 2009). Several dermal HA fillers have been approved by the FDA but only a few are available on the market, including Restylane® (Medicis, USA), Prevelle Silk® (Mentor Corp., USA), Anika® (Anika Therapeutics, Inc., MA), and Juvéderm™ (Allergan, USA) (Allemann and Baumann 2008; Ammala 2013).

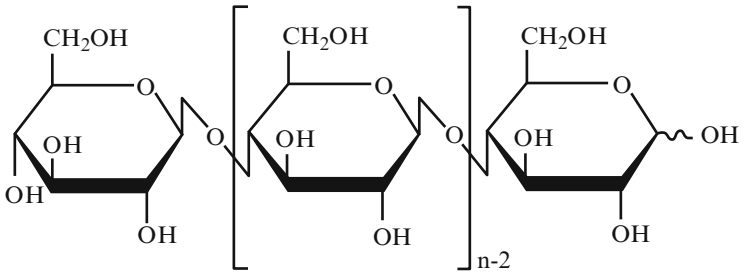

Fig. 2 Chemical structure of bacterial cellulose

The clinical characteristics of each filler is influenced by the characteristics of each product in terms of particle size, type of cross-linking agent used, degree of cross-linking, percentage of cross-linked HA, and amount of free HA and the elastic modulus. HA dermal filler competes with other fillers (e.g., collagen fillers and synthetic fillers) due to their longer clinical duration and no requirements for prior skin allergy testing (Allemann and Baumann 2008). Different cross-linking agents are used for preparation of HA gels to be used as dermal fillers, such as 1,4-butanediol diglycidyl ether (BDDE), divinyl sulfone (DVS), and 2,7,8-diepoxyoctane (DEO) (Allemann and Baumann 2008).

2.1.2 Bacterial Cellulose

Bacterial cellulose (BC) is a glucose homopolysaccharide, with β-(1,4) linkages (Fig. 2) (Chawla et al. 2009; Valla et al. 2009). It is a high-molecular-weight, water-insoluble biopolymer secreted by the bacterium *Gluconacetobacter xylinus* (formerly known as *Acetobacter xylinum*) (Table 1). There are other BC producing *Acetobacter* species (e.g., *G. hansenii*, *G. pasteurianus*) (Çoban and Biyik 2011; Mohite et al. 2013), as well as bacteria from several other genera (e.g., *Agrobacterium*, *Rhizobium*, *Pseudomonas*, *Alcaligenes*) (Barnhart et al. 2014; Chawla et al. 2009; Laus et al. 2005), but *G. xylinum* is the most extensively studied strain and considered for industrial production (Valla et al. 2009). BC exhibits a nanofibrous porous network structure with high strength and low density that makes it useful for the development of membranes for use in several applications, such a biomedicine, cosmetics, and electronics (Freitas et al. 2014).

BC production is performed using sugars as carbon sources, mostly under static conditions, which are not feasible for large-scale production due to the long culture periods. Stirred cultures may be used, but cellulose-negative mutants are generated due to rapid growth (Kim et al. 2007; Valla et al. 2009). Due to these process limitations, BC applications are currently limited to high-value products and specialty chemicals, where the polymer's superior performance justifies its high production costs (Chawla et al. 2009). Nevertheless, BC has valuable properties, which together with its GRAS status (Khan et al. 2007) render this biopolymer a huge potential for applications such as cosmetics, food, and biomedicine.

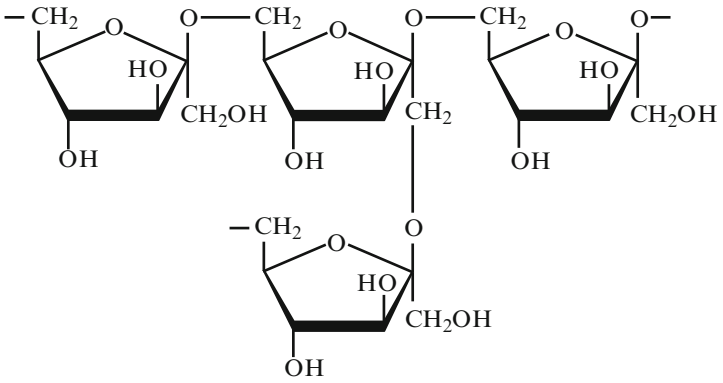

Fig. 3 Chemical structure of levan

The skin regeneration activity of BC has been described and the biopolymer has been proposed for the development of wound healing (Czaja et al. 2006; Solway et al. 2010), skin repair (Fu et al. 2013), and skin-moisturizing (Amnuaikit et al. 2011) materials. Due to its high degree of hydration, BC is used as an ingredient in moisturizing creams (Ioelovich 2008). BC dispersions are used as fingernail components and as the base of artificial nails (Gama et al. 2012). Due to its moldability, BC is also used for the preparation of masks for facial treatments, which can function also as carriers for skin care substance (Gama et al. 2012). Bacterial cellulose masks have been proposed for the treatment of dry skin, improving skin hydration (Amnuaikit et al. 2011). The *in vivo* efficacy of a BC mask after a single application has been evaluated by Amnuaikit et al. (2011) in a study involving 30 volunteers with ages between 21 and 40 years. Although the mask did not significantly change the skin's characteristics, the moisture uptake was increased by 7–28 %. BC has also been proposed for the formulation of a natural facial scrub, containing powdered BC, powdered glutinous rice, aloe vera extract, olive oil, and ascorbic acid. The facial scrub thus prepared was comparable to a commercial product (apricot facial scrub) in terms of viscosity profile, but it had no spreading capability (Hasan et al. 2012).

2.1.3 Levan

Levan is a homopolysaccharide composed of fructose residues joined by β-2,6 linkages with β-2,1 side branches (Fig. 3) that is found in many natural sources, including plants (e.g., grasses, wheat, barley), fungi (e.g., *Aspergillus sydowii*, *A. versicolor*), and bacteria (Kang et al. 2009). Microbial levan is synthesized from sucrose by the action of the enzyme levansucrase (Shih and Yu 2005), an extracellular enzyme synthesized by several bacteria, including species of the genera *Erwinia*, *Streptococcus*, *Pseudomonas*, and *Zymomonas* (Table 1) (Senthilkumar and Gunasekaran 2005). It is produced by Montana Polysaccharides Corp., in the USA (www.polysaccharides.us).

Levan has functional properties that render it suitable for use in a wide range of applications, such as food, feed, cosmetics, and pharmaceuticals. This polysaccharide does not swell in water and it has a very low intrinsic viscosity value (~0.14 dl/g) (Kasapis et al. 1994). It has been approved as a food additive in several countries, including the USA, Europe, Japan, Australia, and New Zealand. The safety of levan for use as a cosmetic ingredient has been evaluated (Kang et al. 2009).

The reported biological activity of levan include cell-proliferating, skin-moisturizing, and skin irritation-alleviating effects (Kim et al. 2003) and skin-whitening effects (Furukawa and Tsuboi 2006) (Table 1). The effect of levan as anti-inflammatory and protection agent against irritation was evaluated with a three-dimensional artificial skin model. Levan demonstrated good cell protective effects for irritation caused by sodium laureth sulfate (SLS). This outcome was probably related to the anti-inflammatory effect of levan, as shown by the reduced secretion of interleukin 1α (a proinflammatory mediator) compared to the control that was not treated with levan (Kim et al. 2005). The moisturizing effect of levan has been evaluated in a human volunteers test (ten 22–37-year-old female subjects). The polysaccharide has have a good moisturizing effect, decreasing significantly the water loss on the skin (Kang et al. 2009).

Despite its valuable biological properties, the use of levan in cosmetics is still limited, which is mainly due to its weak stability in aqueous solution. Levan is partially hydrolyzed to fructose and low-molecular-weight oligosaccharides at acidic conditions and/or high temperatures, making its processing and preservation considerably difficult (Kang et al. 2009).

2.2 Other Bacterial Polysaccharides Used in Cosmetics

Polysaccharides, such as xanthan gum and gellan gum, are commonly used in cosmetics due to their solution properties, being mainly used as base substances for viscosity control, emulsion stabilization, or moisturizing.

2.2.1 Xanthan Gum

Xanthan gum is a heteropolysaccharide composed of a glucose backbone linked through β-1,4 glycosidic linkages, with trisaccharide side chains on every alternate glucose residue (Fig. 4). The side chains contain a glucuronic acid residue between two mannose residues. About one half of the terminal mannose residues are linked to a pyruvil residue, while some of the nonterminal mannose is linked to acetyl residues (Becker and Vorholter; 2009; García-Ochoa et al. 2000; Imeson 2010). The level of acyl groups substituents in the molecule is highly dependent of the production conditions and has an impact on the polymer's rheological properties (García-Ochoa et al. 2000). It is the most widely accepted commercial microbial polysaccharide for both food and nonfood applications (Imeson 2010; Rottava et al. 2009). Xanthan gum is soluble in cold or hot water and forms highly viscous aqueous solutions, even at low concentration. Its aqueous solutions present a high

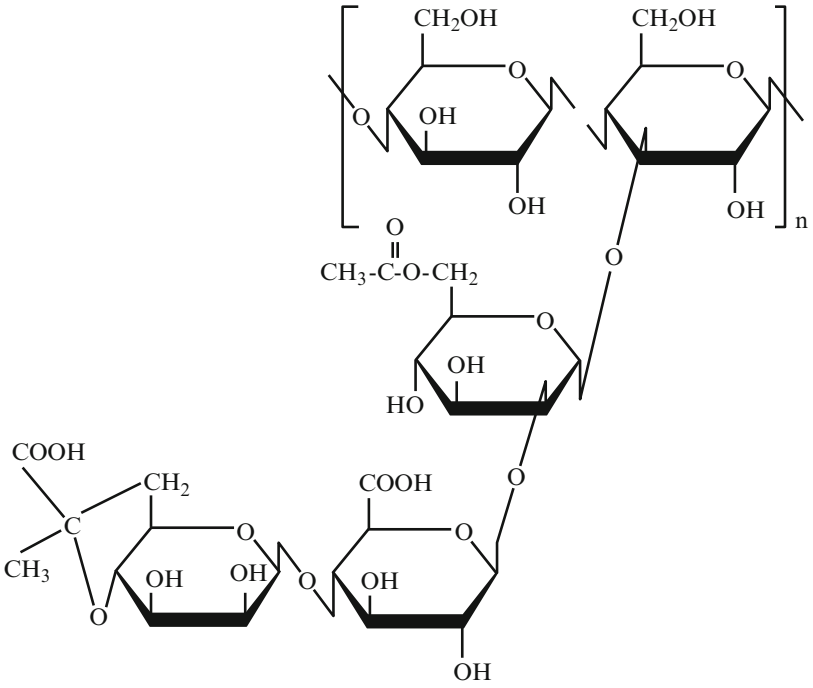

Fig. 4 Chemical structure of xanthan gum

low-shear viscosity, with a marked shear-thinning behavior. When compared to other hydrocolloids (e.g., guar gum, locust bean gum), it presents a higher low-shear viscosity and is more shear thinning at the shear rates typical of food processes (Imeson 2010).

Xanthan gum is synthesized by *Xanthomonas* sp. *X. campestris* is a very efficient polysaccharide producer because it is able to reach high productivities and substrate conversion into biopolymer. Sucrose or glucose are used as carbon sources in the industrial process. Nowadays, xanthan's major producers include CP Kelco, Merck, Pfizer, Rhône Poulenc, Sanofi-Elf, and Jungbunzlauer, and the polymer is marketed with different purity grades for applications ranging from food to personal care products and oil recovery (Freitas et al. 2013). Xanthan gum is the most used bacterial polysaccharide due to its high viscosity-enhancing ability at low concentrations, being the only significant bacterial biopolymer in the global hydrocolloids market (Imeson 2010).

Xanthan gum is used in almost every category of cosmetic products, including oral hygiene products, leave-on formulations, deodorants, baby products, etc. (Bergfeld et al. 2012). It is used for a wide range of functions, namely, as a binder, emulsion stabilizer, skin-conditioning agent, surfactant/emulsifier, and viscosity enhancer, in concentrations up to 4–6 % in dermal and mucous contact products,

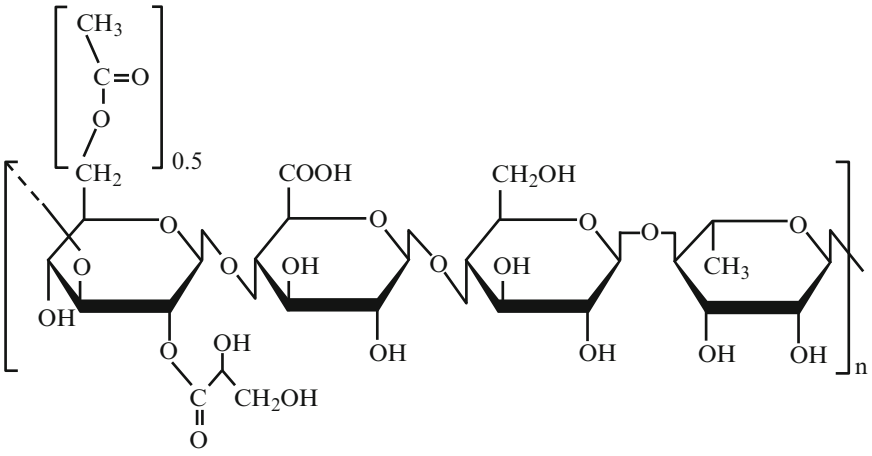

Fig. 5 Chemical structure of gellan gum

hair colorants, and nail products. Lower concentrations are used in deodorants (up to 0.6 %), baby products (0.2–0.6 %), and sprays (0.05 %) (Bergfeld et al. 2012).

2.2.2 Gellan Gum

Gellan is a heteropolysaccharide composed of a tetrasaccharide backbone with rhamnose, glucose, glucuronic acid, and glucose monomers and side chains of acetyl and glyceryl substituents (Fig. 5) (Coleman et al. 2008). Both substituents are located on the same glucose residue, and on average, there is one glycerate per repeat and one acetate per every two repeats. *Sphingomonas paucimobilis* ATCC 31461 is the bacterial strain used for industrial production of gellan gum (Bajaj et al. 2007). Simple sugars are the most common carbon sources. Gellan gum has received approval for use in the food industry and is marketed by CP Kelco under the trade name Kelcogel as a gelling agent (Bajaj et al. 2007).

Gellan gum forms gels upon cooling. Gel properties are dependent on gellan's acyl groups content. Generally, the high acyl form produces soft, elastic, non-brittle, thermo-reversible gels, whereas the low acyl form produces firm, nonelastic, brittle, and thermostable gels. The properties of the gels prepared with low acyl gellan are quite dependent on the pH and the presence of cations, while high acyl gellan gels are not as sensitive to the ionic environment (Freitas et al. 2013).

Gellan gum is used in cosmetic formulations mainly as an emulsion stabilizer and a viscosity-increasing agent. It is mostly used in leave-on and dermal contact products, in concentrations up to 0.3–0.5 %. Other uses include hair products, powders, and eye and mucous contact products, in which it is used in concentrations up to 0.0004 % (Bergfeld et al. 2012).

3 Mechanism of Action

3.1 The Human Skin

The human skin comprises three layers: the epidermis, the dermis, and the hypodermis (Fig. 6) (Elias 2001). The outer layer, the epidermis, consists of several cell layers that are continuously being regenerated from the basal cell layer to the stratum corneum. In the stratum corneum, the corneocytes (protein-rich cells) are embedded in a lipid-rich matrix composed of ceramides, cholesterol, and fatty acids. Corneocytes are linked together by corneodesmosomes that confer structural integrity to the multilamellar stratum corneum. The dermis is composed of collagen and glycosaminoglycans and contains blood vessels, nerves, glands, and hair follicles. The hypodermis or subcutaneous layer consists of adipose tissue and provides a thermal barrier (Ammala 2013; Elias 2001).

The stratum corneum is a highly impermeable lipophilic layer that protects the inner layers from environmental influence. It contains only around 13 % water, while the inner layers are significantly more hydrophilic (the water content in the viable epidermis is about 50 % and in the dermis it is higher than 70 %). The degree of hydration of the stratum corneum influences the skin's permeability. Moreover, the skin barrier function is influenced by age and external factors, and it also varies at different body locations (Ammala 2013). For example, after the age of 20 years, the amount of naturally occurring HA in the human skin continuously decreases, which results in decreased tissue elasticity and hydration, and contributes to the formation of wrinkles (Berkó et al. 2013).

When the water content of the skin is reduced below its normal moisture content (20–35 %), the skin becomes dry, rough, and scaly, often appearing reddish, with cracks and/or itching. In dry skin, the epidermis loses its moisture retention ability, and repair can only be achieved by specific treatments to enhance hydration and reduce water evaporation. The most common treatment is the topical application of a moisturizer. Occlusive masks from different sources are also commonly used.

The transport of molecules across the skin can follow different routes, namely, through the lipid bilayers between cells (the intercellular pathway), directly through the cells (the transcellular pathway), or through the hair follicles or gland ducts (Fig. 6). Only low-molecular-weight (<500 Da), moderately lipophilic molecules, with solubility in both the lipophilic stratum corneum and the hydrophilic inner epidermis layer, can be transported across the skin. Skin permeation can be enhanced by disruption of the structural organization of the intercellular lipid matrix of the stratum corneum by chemical enhancers (e.g., biodegradable polymers) or physical techniques (e.g., microneedling, electroporation) (Ammala 2013). Exposure of the skin to harmful environmental conditions, such as UV radiation, can lead to premature skin aging, mutation, or skin cancer. UV radiation causes peroxidation of the lipid matrix of the stratum corneum and leads to loss of this structure's barrier function (Trommer and Neubert 2005).

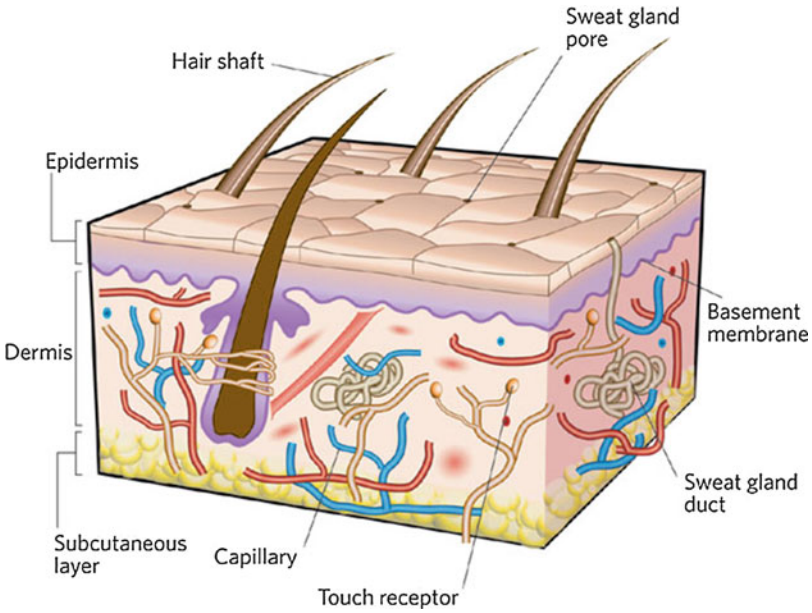

Fig. 6 Structure of the human skin (Reproduced with permission from MacNeil (2007))

3.2 Cosmetic Ingredients and Formulations

Cosmetic formulations include different types of substances, with distinct functions on the final product: base substances that are mainly natural skin components, such as oil-based substances (e.g., triglycerides, wax esters, fatty acids), barrier, and moisturizing substances; active agents that are substances that have specific effects, namely, protection, preservation, and/or improvement of the skin's natural condition; and additives that are used to provide physical and microbiological stability, protecting the product against microorganisms, temperature, oxygen, and light and improving shelf life (e.g., antioxidants, perfumes, emulsifiers) (Ekong et al. 2001; Lautenschlager 2002a, b, 2003; Siquet and Devleeschouwer 2001; Table 2). All additives, except UV filters and additives for consistency control, should be avoided because they may cause allergies under certain conditions. The microbiological stability of a cosmetic product may be assured by alternative processes (e.g., bottling under sterile conditions, additions of hydroxyl groups containing substances) that are seldom used due to their high costs.

By definition, active agents must not cause systemic effects on the whole body after its penetration into the skin. The effects of active agents on the skin include: smoothing and regeneration (e.g., antiwrinkle), influencing skin color (e.g., makeup), protection (e.g., sunscreens), and hygiene (e.g., cleansing) (Cunningham 2001; Lautenschlager 2003; Lodén 2001; Weber et al. 2001). The basic and minimal specifications for any cosmetic ingredient must include its chemical identity (chemical nature and structural formula; origin of the raw

Table 2 Type of substances in cosmetic formulations

Substances	Function	Ingredients
Base substances	Substances that every skin needs	Triglycerides (e.g., tristearin, tripalmitin)
		Fatty acids (e.g., caprylic acid, palmitic acid, linoleic acid)
		Wax esters (e.g., jojoba oil, shea butter, squalane)
		Glycols (e.g., glycerine, sorbitol, propylene glycol)
		Mineral oil products (e.g., vaseline, paraffin oil)
Active agents	Protection, preservation, and/or improvement of the skin's natural condition	Vitamins (A, C, E)
		Coenzyme Q10
		Hyaluronic acid
		Lactic acid
		Natural extracts (plants, algae)
Additives	Provide products physical and microbiological stability and improved shelf life	Perfumes
		Antioxidants
		Ethylenediaminetetraacetate (EDTA)
		Citric acid
		Thickening agents (e.g., xanthan)
		Emulsifiers
		Dyes and pigments
		UV filters

material, extraction method, etc.), physical form (powder, paste, gel, liquid), molecular weight, purity (including characterization of the impurities), solubility in water and/or in any other relevant organic solvent, and other additional relevant physical and chemical specifications (e.g., organoleptic properties, flash point, melting/boiling point, etc.).

3.3 Main Cosmetic Vehicles

The term vehicle is used both in cosmetics and pharmaceuticals upon the optimization of products formulation. It consists on the matrix into which the active principles are embedded. The role of the vehicle is to serve as carrier and to deliver the active principles on the application site or on the target organ where the desired effect is to be achieved. Even though cosmetic formulations are not allowed to contain pharmacologically active substances that treat diseases, they present a key role on exerting important effects on skin, such as care, cleansing, hydration, protection, and decoration. Furthermore, many cosmetically used compounds are bifunctional, by acting first as vehicle and then showing a positive effect on the skin

properties when applied. There is a wide range of cosmetic vehicles forms, with diverse physical and chemical properties depending on the application types and forms. The major vehicles systems, in which polysaccharides play an important role, are emulsions, hydrogels, encapsulating structures, and suspensions (Buchmann 2001).

3.3.1 Emulsions

Within the cosmetic care products, emulsions are probably the most commonly used vehicles. They are preferred to waterless oils and lipids due to their more appealing skin feeling and ease of application. If emulsions are flowing fluids, they are generally called lotions. By the contrary, if they occur in semisolid form, they are known as creams (Buchmann 2001).

Emulsions are systems constituted by two immiscible liquid phases composed of lipids (lipophilic components) and water (and/or hydrophilic components), in which one of them is dispersed within the other in the form of small droplets with a size in the range from 0.1 to 100 μm (Dickinson and Stansby 1988). According to the distribution of the two liquids, emulsions may be classified either as oil-in-water (o/w), in which the lipid is dispersed in the aqueous phase, or as water-in-oil (w/o), where droplets of the aqueous phase are dispersed within the lipid phase. In addition, two types of multiple emulsions are encountered in skin care: water-in-oil-in-water (w/o/w), where the internal and external water phases are separated by oil, and oil-in-water-in-oil (o/w/o), where the water phase separates two oil phases (Epstein and Simion 2001).

As the phases are immiscible, these biphasic systems are thermodynamically unstable. However, they are allowed to remain in a metastable mixed state by the addition of amphiphilic components (emulsifiers), which adsorb at the oil-water interface reducing the interfacial tension. There is the formation of a film that stabilizes the emulsion by slowing down the coalescence of the dispersed droplets, preventing phase separation (Epstein and Simion 2001).

In addition to emulsifiers, other classes of components are present on cosmetic emulsions, such as: (i) emollients, to improve sensory properties (e.g., silicon oils, isopropyl myristate); (ii) moisturizers and humectants (e.g., glycerol, urea); (iii) active substances (e.g., UV sunscreens, vitamins); (iv) antimicrobial agents; (v) perfumes and coloring agents; and (vi) viscosity-increasing agents (Buchmann 2001).

Polysaccharides, namely, those produced by bacteria, are especially important in (o/a) cosmetic emulsions. They form extended hydrogel networks, and their solvation properties serve to increase the interfacial viscosity and the viscosity of the continuous phase, which slows down the droplet motion. This enables a secondary stabilization, decreasing the occurrence of phenomena such as creaming (upward movement of oil droplets due to their lower specific gravity) and coalescence. In addition to that, polysaccharides act as rheology modifiers and control the consistency of emulsions. They are extremely important on the optimization of texture properties, which are known to be of great importance, as the consumers' preference is closely connected to it (Gilbert et al. 2013a). As such, texture of cosmetic products must be designed in order to meet sensory expectations of consumers.

Carbopol® polymers (high-molecular-weight homo- and copolymers of acrylic acid cross-linked with a polyalkenyl polyether) and Veegum (aluminum silicate material) are traditionally used as thickeners. In addition, natural biopolymers like alginates and cellulose-based polymers (e.g., carboxymethyl cellulose) are also used (Epstein and Simion 2001).

Xanthan gum is the most referred bacterial polysaccharide applied for these purposes. The physical properties of such cosmetic emulsions have been studied by rheology and texture analysis, in order to perceive the behavior of xanthan gum as viscosity controller and psychosensorial agent, in comparison with other natural and synthetic polymers (Gilbert et al. 2013a, b, c; Isaac et al. 2013). Beyond xanthan gum, Fucogel, hyaluronic acid, and gellan gum have also been applied (Prajapati et al. 2013; Vianna-Filho et al. 2013).

3.3.2 Hydrogels

Gels may be defined as dilute mixtures of at least two components, in which the components form separate continuous phases throughout the system. In most gels the minor component is a polymeric phase which forms a three-dimensional network structure surrounding a liquid phase. The network structure prevents the fluid from flowing, whereas the fluid prevents the structure from collapsing. The coexistence of a polymeric network structure together with a liquid phase differentiates gels from pure solids or fluid materials and gives gels their unique viscoelastic properties (Jung and Shinkai 2004).

Particularly, hydrogels are formed by a three-dimensional network structure made of hydrophilic polymers (e.g., polysaccharides) surrounding water molecules. The mechanical properties of these structures may vary from firm and brittle to soft and elastic, depending on the type of the polymer and on the nature of intermolecular interactions that hold the network structure together (e.g., hydrogen or covalent bonds, ionic interactions).

The bacterial polysaccharide hyaluronic acid has been widely used to develop hydrogels. Berkó et al. (2013) have compared hydrogels based on linear and cross-linked HA as potential semisolid drug delivery forms from the aspects of deep HA penetration through the skin.

Gellan gum has been applied successfully in toothpaste formulations, due to its binding properties and true-gel structure. It presents very good flavor release properties, enabling the use of significantly lower amounts of flavors and sweeteners in such formulations. At typical usage levels, gellan gum imparts a low viscosity during production, allowing the design of fluid formulations that form a gel after packaging. This low viscosity makes manufacturing and packaging easier and allows the incorporation of fragile ingredients, such as encapsulated flavors, that would not normally be possible with other binder systems. In addition, by using blends of low and high acyl gellan, it is possible to design a range of toothpastes with different binding, stand-up, and preparation viscosity (Prajapati et al. 2013).

Emulsion-filled gels are particular type of structures in between true emulsions and gels, widely used in cosmetic products. They are composed of a continuous semisolid phase (gel) with dispersed oil droplets, with an overall oil concentration

ranging between 2 % and 20 % (Buchmann 2001). Due to the elastic characteristics of the gel, oil droplets can be kept in suspension avoiding creaming. Lorenzo et al. (2013) have focused on the bacterial polysaccharide gellan gum to produce emulsion-filled gels. They studied the influence of oil and hydrocolloid concentrations on the viscoelastic behavior of emulsion-filled gels formulated with high acyl gellan gum.

3.3.3 Encapsulating Structures

Many biologically active compounds are unstable and sensitive to pH, temperature, light, and oxidation. As such, they require to be protected against unwanted degradation during formulation, storage, and application of the final product. In addition, topical- and transdermal-controlled delivery of active cosmetic ingredients is advantageous in many cases, which requires safe and nontoxic means of reaching the target sites without causing any irritation (Ammala 2013). This may be achieved with the encapsulation of biologically active compounds within a material forming mico-/nanostructures (e.g., micro-/nanospheres and capsules, micro-/nanoemulsions, liposomes, microsponges) (Patavale and Mandawgade 2008; Ammala 2013). Beyond the protection of the active compounds, reducing their reactivity with external factors, encapsulation also enables an easier handling, to mask the compounds aroma and to promote their dilution in the final product when they are toxic in large quantities.

There is a large variety of active cosmetic substances that have been encapsulated using a wide range of materials. Examples include nylon microparticles loaded with vitamins, sun filters, moisturizers, fragrances, and many other actives such as retinyl palmitate, D-panthenol, ascorbic acid, tocopheryl acetate, dihydroxyacetone, vitamin E, and dimethicone (Patavale and Mandawgade 2008). The attention has been driven to the use of biodegradable polymers as encapsulating agents for cosmetic applications. The main advantages of using biodegradable polymers over those nonbiodegradable are that they are generally nonreactive when in contact with the human body and can be metabolized and removed from the body via normal metabolic pathways. They include not only polysaccharides but also proteins, poly α-esters, polyalkylcyanoacrylates, and polyamidoamine dendrimers (Ammala 2013). As examples, agar is used commercially to produce microspheres containing emollient oils and vitamins for the enhancement of the tactile and visual appearance of cosmetic and personal care products (http://www.lipochemicals.com). Gomaa et al. (2010) used chitosan microparticles to incorporate phenylbenzimidazole sulfonic acid, a hydrophilic sunscreen agent. Egg album microspheres containing vitamin A were applied to prepare O/W creams, resulting in a prolonged release and higher bioavailability of that vitamin (Patavale and Mandawgade 2008). The use of polylactic acid has been recently reported to encapsulate retinyl retinoate as an antiwrinkle treatment (Kim et al. 2012). Furthermore, the encapsulation and stabilization of ascorbic acid using poly(lactic-co-glycolic acid) (PLGA) nanoparticles, as well as PLGA microparticles to stabilize urea (used as moisturizing agent) and provide effective means of controlled release, has been described (Haddadi et al. 2008).

HA is the most tested bacterial polysaccharide in delivery systems. Particularly, HA-based nanoemulsions and their application as transdermal carriers were recently reported. Encapsulation experiments with vitamin E demonstrated that these nanoemulsions were capable of carrying lipophilic additives (Kong et al. 2011a, b). In addition, HA was used successfully to stabilize gold nanoparticles (AuNPS). The biocompatibility of HA, along with the unique attributes of AuNPS, enabled the production of colloidal dispersions that may be potentially applied in cosmetics (Hien et al. 2012).

3.3.4 Suspensions

Suspensions are type of formulations in which particles, generally functional excipients, are dispersed in a liquid or semisolid media that functions as vehicle. As examples, it may be indicated for sun-protection products or nail pearlescent lacquers containing pigments. The main potentially negative phenomenon that may occur in suspensions is the sedimentation of particles with higher density than the liquid. Sedimentation should be reduced, or even hindered, during storage, which may be accomplished by increasing the viscosity of the liquid media. Among the polysaccharides, xanthan gum has been widely used as suspending agent due to its good thickening ability with shear-thinning behavior. In addition, gellan gum can provide effective stabilization of shampoo and conditioner formulas due to its thickening capacity and fluid gels formation (Prajapati et al. 2013).

4 Toxicity Requirements

4.1 Regulatory Requirements

The safety of a cosmetic product is based on the safety of its ingredients, the latter being evaluated by toxicological testing. The determination of the toxic potential of a cosmetic ingredient is based on a series of toxicity studies (Table 3). The minimal base set requirements include the following tests: acute toxicity (if available), irritation and corrosivity, skin sensitization, dermal/percutaneous absorption, repeated dose toxicity, and mutagenicity/genotoxicity (European Directive 93/35/EEC). Further tests (carcinogenicity, reproductive toxicity, toxicokinetics) may additionally be required, while photo-induced toxicity data is only necessary when the cosmetic product is expected or intended for use on sunlight-exposed skin. Although the use of human volunteers is subjected to great ethical concerns, it is extremely useful to have human data since animal tests and alternative methods are of limited predictive value with respect to the human response. Such tests are only performed when the toxicological profiles of the ingredients indicate that they are safe. When such studies are considered not needed or technically possible, a scientific justification is given. For complex ingredients derived from biotechnology, specific data must be available, such as

Table 3 Toxicity tests used for assessment of the safety of cosmetic ingredients, according to the European Directive 93/35/EEC

Toxicity test	Objective	Type of test
Acute toxicity	Health effects resulting from a single exposure to a substance by oral, dermal, or inhalation administration	*In vivo* tests:
		The fixed dose method
		The acute toxic class method (determination of exposure dosages, by performing a complex stepwise dosage with a reduced number of animals)
		The up-and-down procedure (estimation of the LD50-value)
Irritation	Reversible damage of the skin or mucous membranes (namely, the eye) following the application of a test substance	*In vivo* test (with rabbits); for reduction of the number of animals, several prior steps are performed (e.g., evaluation of existing human and animal data, analysis of structure activity relationships, taking *in vitro* and *ex vivo* tests)
Corrosivity	Irreversible damage of the skin (i.e., visible necrosis through the epidermis and into the dermis) or to the eye (i.e., tissue damage or serious deterioration of vision), following the application of a test substance	*In vitro* tests, using culture models for eye and skin cells or reconstructed membranes
Skin sensitization	Development of allergic contact dermatitis upon exposure to a test substance	*In vivo* tests (with mice or pigs)
Dermal/ percutaneous absorption	Passage of compounds across the skin, involving the penetration and permeation of a topically applied substance through the skin/mucous layers, and its resorption into the vascular system (lymph and/or blood vessel)	*In vivo* or *in vitro*
Repeated dose toxicity	Adverse general toxicological effects occurring as a result of repeated daily dosing with, or exposure to, a substance for a specific part of the expected lifespan of the test species	*In vivo* tests:
		Repeated dose (28 days) toxicity (oral, dermal and inhalation)
		Sub-chronic oral toxicity test (90 days) in rodents and in non-rodents
		Sub-chronic dermal toxicity test (90 days) in rodents
		Sub-chronic inhalation toxicity test (90-days) in rodents (rarely used)
		Chronic toxicity test

(*continued*)

Table 3 (continued)

Toxicity test	Objective	Type of test
Mutagenicity/ genotoxicity	Evaluation of mutagenicity of the substance at a gene level, chromosome breakage and/or rearrangements (clastogenicity), and numerical chromosome aberrations (aneugenicity)	*In vitro* (stage 1):
		Tests for gene mutation (bacterial reverse mutation test; in vitro mammalian cell gene mutation test)
		Tests for clastogenicity and aneugenicity (in vitro micronucleus test)
		In vivo (stage 2): depends on the results observed in the in vitro assays
Carcinogenicity	Induction of tumors (benign or malignant) or increase of their incidence, malignancy, or shortening of the time of tumor occurrence when a substance is inhaled, ingested, dermally applied, or injected	*In vitro* and *in vivo* (depending on the type of substance)
Reproductive toxicity	Adverse effects induced by a substance on any aspect of mammalian reproductive cycle (e.g., impairment of male or female reproductive function or capacity, induction of nonheritable adverse effects in the progeny such as death, growth retardation, structural and functional effects)	*In vivo*:
		Two-generation reproduction toxicity
		Teratogenicity test in rodents and non-rodents
		The whole embryo culture test (WEC)
		The MicroMass test (MM)
		The Embryotoxic Stem cell Test (EST)
Toxicokinetic studies	Time-dependent fate of a substance within the body (absorption, distribution, biotransformation, and/or excretion) leading to adverse effects	*In vivo* or *in vitro*
Photo-induced toxicity	Cytotoxicity of a substance when tested in the presence and in the absence of exposure to a noncytotoxic dose of UV/visible light	*In vitro* and *in vivo*
Human data	Confirm that there are no harmful effects when applying a cosmetic product for the first time to human skin or mucous membranes	Human volunteers tests for finished products

the description of the organisms involved, their pathogenicity, toxicity of any metabolites produced by the organisms, fate of viable organisms in the environment, etc. (Chew and Maibach 2001; Robinson et al. 2001; Van Essche 2001).

The last phase in the safety evaluation of a cosmetic ingredient is the determination of the margin of safety (MoS) that is calculated by dividing its lowest NO(A)

EL (no observed (adverse) effect level) value by its possible SED (systemic exposure dosage). NO(A)EL is the highest dosage for which no observed (adverse) effects can be observed, expressed as mg/kg body weight/day. SED is the amount expected to enter the blood stream per kg body weight and per day. The MoS value is used to extrapolate from a group of test animals to an average human being and subsequently from average humans to sensitive subpopulations. For a substance to be considered safe for use as cosmetic ingredient, its MoS must be at least 100 (European Directive 93/35/EEC).

The safety evaluation of cosmetic products is based upon the toxicological profile of the ingredients, their chemical structure, and their exposure level. Nevertheless, in some cases, additional information on the finished product is needed. The exposure level will depend on several factors, including the class of cosmetic product (e.g., hair care, bathing, skin care, etc.), the method of application (rubbed-on, sprayed, applied and washed off, etc.), the concentration of the ingredient in the finished cosmetic product, the quantity of product used at each application, the frequency of application, the total area of skin contact, the site of contact (e.g., mucous membrane, sunburnt skin), consumer target group (e.g., children, people with "sensitive skin"), etc. If the final product is not expected to cause any adverse effect under foreseeable conditions of use, compatibility testing is undertaken on a number of human volunteers prior to product marketing (Bashir and Maibach 2001).

4.2 Toxicity Assessment of Bacterial Polysaccharides

The Cosmetic Ingredient Review (CIR) Expert Panels have concluded that HA and its salts, xanthan gum, gellan, and levan are safe for use in cosmetic formulations (Becker et al. 2009; Bergfeld et al. 2012). BC was also considered nontoxic and biocompatible in several studies (Jeong et al. 2010; Moreira et al. 2009).

No adverse reactions were reported to HA applied topically, and the adverse reactions reported to injected HA for the treatment of osteoarthritis and tissue augmentation do not raise safety concerns regarding its use in cosmetics (Becker et al. 2009). Nevertheless, it can enhance the penetration of other ingredients through the skin. Hence, caution should be taken for its use in mixture with substances for which dermal absorption is a concern.

The toxicity of BC nanofibers was evaluated in vitro in human umbilical vein endothelial cells using viability and flow cytometric assays and in vivo using C57/Bl6 mice (Jeong et al. 2010). No toxicity was observed, indicating that BC a biomaterial suitable for biomedical and cosmetic applications. Moreira et al. (2009) evaluated that BC nanofibers were not genotoxic.

Levan is accepted as a nontoxic biopolymer, with a lethal dosage of 7.5 g/kg body weight and a NOEL value of 1.5 g/kg/day (Kang et al. 2009). The cytotoxicity of levan has been evaluated using human fibroblast and keranocyte cell lines. No cytotoxicity was shown in the human fibroblast cell line up to 100 μg/mL, and a considerable cell proliferation effect was observed in the keranocyte cell line at concentrations above 1 mg/mL (Kim et al. 2005).

The acute toxicity of xanthan gum was assessed orally in mice, rats, and dogs. There was no notable toxicity observed in those studies for xanthan gum concentrations up to 20 g/kg body weight (Booth et al. 1963; Eastwood et al. 1987; McNeely and Kovacs 1975; Robbins et al. 1964; Woodard et al. 1973). The reproductive and developmental toxicity of xanthan was evaluated in a three-generation study with albino rats fed with xanthan (0–0.5 g/kg body weight). There were no significant differences in the developmental parameters between test and controls, and no malformations were observed (Woodard et al. 1973). The dermal irritation and sensitization potentials of xanthan gum were evaluated in animal studies. Xanthan gum was neither irritating to rabbit and rat skin (in concentrations up to 1 %) nor a sensitizer in guinea pigs (at a concentration up to 0.1 %) (Bergfeld et al. 2012; Booth et al. 1963; Guillot et al. 1982). Xanthan gum (1 %) was also considered as nonirritant to the eyes of rabbits (Booth et al. 1963; Guillot et al. 1982).

The oral and acute toxicity of gellan gum, assessed in rats, dogs, and monkeys, demonstrated that it was not toxic (Anderson et al. 1988; Bergfeld et al. 2012). Gellan gum was considered noncarcinogenic based on studies with mice and rats fed with the polysaccharide (0–5 g/kg body weight), wherein no neoplastic or nonneoplastic lesions were observed (Bergfeld et al. 2012). Gellan gum (0.2–0.8 %) was also considered as nonirritant to the eyes of rabbits (Liu et al. 2010; Ramaiah et al. 2007).

Finally, in human studies, the daily ingestion of xanthan gum (150 mg/kg body weight) and gellan (175–200 mg/kg body weight) was shown not to have any significant adverse dietary, physiological, or allergenic effects (Eastwood et al. 1987). Gellan gum was also shown to be nonirritating to human eyes (Liu et al. 2010).

5 Conclusions

Although many bacterial polysaccharides possess physical-chemical properties and/or biological activity suitable for their use in cosmetic applications, only a limited number of such biopolymers are currently used as cosmetic ingredients. The most relevant is xanthan gum that is widely used in cosmetic products due to its high viscosity-enhancing ability at low concentrations. Other bacterial polysaccharides used in cosmetics are hyaluronic acid, bacterial cellulose, and levan, which are used as bioactive ingredients, and gellan gum that is used to produce hydrogels and as rheology modifier. Beyond its biological activity, hyaluronic acid has also been applied in cosmetic vehicles, such as hydrogels and nanoemulsions. Since there are no extensive regulatory requirements for marketing new cosmetic products, it is likely that other bacterial polysaccharides, with new or improved properties, may be developed as cosmetics ingredients. Nevertheless, their safety must be established by toxicological testing.

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Effects of Polysaccharide-Based Formulations on Human Skin

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Contents

Abstract

The polysaccharides, mainly from natural sources, have been widely used in cosmetic area due to their multifunctional properties, playing a large role in the research and development of cosmetics products.

The knowledge of the skin biology allows the identification of which active ingredients are important for the maintenance of its integrality and barrier function, essential to skin health.

So due to the polysaccharides' protective effects on the skin, the development of cosmetic formulations with these ingredients like hyaluronic acid, algae, and others obtained by biotechnology process could improve the efficacy of the formulations, maintaining the skin in good conditions. In addition, some polysaccharides can also improve formulation stability and sensorial properties.

The data from clinical studies showed that the polysaccharide-based formulations promoted the transepidermal water loss reduction, protecting the skin

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barrier function. This way, they are interesting active ingredients to develop effective cosmetic formulations for skin protection and maintenance of its structure integrality.

Keywords
Polysaccharides • Cosmetic formulations • Clinical efficacy • Skin hydration • Skin protection • Rheology • Biophysical techniques

1 Introduction

Many new active ingredients are being used in topical formulations due to the development of technology in the cosmetic industry and a better comprehension of the skin biology. However, the search for different active ingredients to be used in cosmetic is very important to develop stable, safe, and effective cosmetic formulations. The choice of adequate raw materials that will be part of its composition is fundamental, as they need to be compatible among themselves, and the selected ones also need to follow the product-use methodology, and that leads to the need of studies to assure this stability, safety, and efficacy in real conditions of use.

The skin is a complex and heterogeneous organ that covers the human body with a surface area of approximately 2 m^2, which is 10–15 % of the corporal mass, being, that way, the largest human organ. The skin is essentially composed of three layers: the first one, the epidermis, an intermediate, the dermis, and a deeper one, the hypodermis (Leonardi 2004).

The epidermis is the outermost layer and acts as a protective sheath against environmental influences. It consists of several layers starting with the stratum corneum to the basal cell layer and is continually being regenerated. The dermis lies beneath the epidermis and contains collagen and glycosaminoglycans. This layer is where collagen and elastin are synthesized and contains the blood vessels, nerves, sweat glands, hair follicles, and sebaceous glands. The hypodermis or subcutaneous layer contains the adipose tissue (subcutaneous fat) and provides a thermal barrier (Ammala 2013).

Skin aging is a result of the influence of a few factors that can be divided as intrinsic (or chronologic) and extrinsic (the early aging). The intrinsic aging happens as a result of the "genetically programmed" decline of vital functions that guarantee the organism's well functioning and can result in the epidermis thickening and its fragility, the thickness of the dermis, the decrease of the tissue vascularization, the reduction of fibroblasts and their metabolic capacity, and also a smaller response to growth factors. The extrinsic aging on the other hand is caused by external influence, especially UV radiation, atmospheric pollution, trauma, smoking habits, and ingestion or inhalation of metabolic substances (Sayre et al. 1994).

The relation between the intrinsic aging and the intensification of solar damages is called photoaging, which appears as an intensification of the chronological aging and also to the appearance of different characteristics from the usual aging beyond the pigmentation irregularity, wrinkles, skin texture modifications, and some malignant lesions (Beitner 2003; Yaar et al. 2002; Enjelke et al. 1997).

So the maintenance of the structural integrality of the skin as well as its barrier function is essential to skin health (Buraczewska et al. 2007), and cosmetic products are very important to preserve the good condition of the skin.

This way, new products containing different active substances appear every year, and the ones with natural origin are being widely used in association with vitamins in cosmetic formulations.

Natural-originated products have been broadly used in the cosmetics industry and have a good acceptance by the consumers that seek benefits related to these natural substances' properties. The antioxidant characteristic of botanical extracts and their composition, which is very rich in moisturizers, emollients, softeners, and others, could minimize the effects of aging from free radicals and skin dryness and also decrease damages caused by irritation.

Among the products that have a natural origin, the polysaccharides, which can be translated into "many sugars," can have either a natural origin or polymeric origin and are a highlight in cosmetic formulations for their properties that can provide different benefits to the skin (Bravo 1998). This way, they are a new trend in cosmetic formulations, bringing benefits as emulsion stabilizer, film former, binder, viscosity-increasing agent, rheology modifiers, and suspending agents. They can also have moisturizing effect on the skin (Gruber 1999).

Historically, their use is due to their availability from natural sources and multifunctionality, which is a growing research cosmetic area and very rare to find in a natural ingredient, playing then a large role in the cosmetic formulation technology.

Focusing on dermatological activities of carbohydrates, it has to be kept in mind that polysaccharides are often used as vehicles of dermatological formulations for the development of gels, ointments, or lotions (Deters et al. 2005).

Another benefit that justifies the use of polysaccharides in the development of cosmetic formulations is their interaction with other ingredients in a formulation as active substances, surfactants, and salts and their facile chemical modification which has allowed their preeminent use in cosmetics as well (Gruber 1999). In addition, polysaccharides, of natural and polymerical origin, are renewable and have a safety profile not accorded with synthetic-based polymers (Guillot et al. 1982).

Some studies have related the importance of polysaccharides to the stability of cosmetic formulations once the rheological properties are positively influenced when these polymers are used (Camargo Junior 2014).

According to Camargo and Maia Campos (2012), polysaccharide-based formulations have shown clinical effects in the transepidermal water loss reduction, protecting the skin barrier function and promoting a good sensorial.

In this context, the polysaccharides from different sources have a great potential to be applied in cosmetics as they can improve skin protection and hydration as well as for the development of stable formulations with pleasant sensorial properties.

2 Sources and Types Used in Topical Formulations

The polysaccharides that can be utilized in cosmetic formulations and have a natural origin can be found in many different sources.

Although genetic engineering has been offering new and interesting polysaccharides, the ones with cosmetic functions are still mostly from natural and seminatural origins.

The behavior of polysaccharides can be influenced by the nature of the substituent groups bound to the individual monosaccharides. These substituents can be of natural origin or may be synthesized, being called seminatural. Whether they are natural or seminatural, the polysaccharides can be organized into five categories, based primarily on its charge. These include: anionic, cationic, nonionic, amphoteric, and hydrophobic (Gruber 1999).

There are two cosmetically interesting polysaccharides available today that are made by human intervention, the cellulose gum (carboxymethylcellulose) and carboxymethylchitin (Gruber 1999), and among the polysaccharides used in cosmetic products, alginates, pectins, gums, and chitosan can be mentioned for their large application as rheological additives (Stelzer and Klug 1980). In addition, they have also been used for their protective effects on the skin, as different types of seaweed, as polysaccharides from *Klebsiella pneumoniae* and *Myrtus communis* extracts, and others.

Alginates are a class of polysaccharides that originated from the marine brown algae and from bacterial fermentation. They consist of two types of monosaccharides, the β-D-(1,4)-mannuronic acid and α -L-(1,4)-guluronic acid, and have the function to provide cellular support to the bacteria and algae cells, preventing them to collapse (Gacesa 1988).

Pectins are also polysaccharides that provide cellular support and can be industrially obtained by extraction from citrus fruit peels. Their structure is similar to the alginic acid except that pectins are composed of a repeating α-D-(1,4)-galacturonic acid unit which is interrupted at times by an α-L-(1,2)-rhamnose. Pectins are indicated for formulations with low pH, considering their citrus source (Gruber 1999).

Carrageenans are cellular structural polysaccharides with anionic characteristic and they are isolated from the marine red algae. They are indicated for topical formulations as they are very abundant in polysaccharides and with that, being a gelling, stabilizing, and thickening agent as they are biocompatible, with biodegradability and high capacity of water retention and mechanical strength of gels (Prajapati et al. 2014).

There are at least seven known varieties of carrageenans and their differentiation is made by a Greek letter. Due to its anionic substituents, the carrageenans are very functional and its structure is also quite diverse. The carrageenans that are more

utilized commercially are the kappa, iota, and lambda carrageenans and consist primarily of repeating monosaccharides as the β-D-(1,3)-galactose and α-D-(1,4)-galactose. The kappa carrageenan has an interesting and unique rheological feature, the synergistic viscosifying effect observed in blends with either a galactomannan, as the konjac mannan, or a glucomannan, as the locust bean gum. Some formulators have observed that although the addition of a glucomannan or a galactomannan to a solution with kappa carrageenan can increase the viscosity, the mixture of two synergistic polysaccharides and the salts of the formulation must be controlled with attention to achieve the maximum performance desired (Gruber 1999; Prajapati et al. 2014).

The xanthan gum is another polysaccharide that has bacterial fermentation origin and is available commercially. Its structure is made by a complex polysaccharide composition of a primary chain of β-D-(1,4)- glucose, which has, on alternating glucose halves, a branching trisaccharide side chain of β-D-(1,2)-mannose, attached to β-D-(1,4)-glucuronic acid, ending in a β-D-mannose. It was first discovered in the late 1950s in the United States Department of Agriculture (USDA) and is widely used today for many different areas for its unique rheological properties (Song et al. 2006).

As the xanthan has an anionic character, it is not compatible with most cationic surfactants. When a nonionic surfactant is present, the xanthan solution's viscosity is also relatively unaffected regardless of surfactant concentration. This fact helps the suspending capability of xanthan gum. The xanthan gum is also popularly combined with colloidal magnesium aluminum silicate (Veegum), an inorganic clay frequently employed to thicken cosmetic formulations, having then the xanthan's thickening efficiency synergistically enhanced (Laba 1993; Gruber 1999).

Wide varieties of the applications of β-glucan have been reported, including as thickening and stabilizing agents in chemical industries and immunostimulating and antitumor agents in clinical uses. Apart from these applications, β-glucan has been used as a substance that enhances the skin's natural ability to heal and protect itself against infection (Bae et al. 2005, Du et al. 2014).

The β-glucan is also used as a component of various cosmetics and is mainly produced from *Saccharomyces cerevisiae* as a water-soluble particulate or its chemically modified soluble forms such as carboxymethyl or phosphorylated glucan (Baschong et al. 2009). Another β-glucan, schizophyllan, which is produced from *Schizophyllum commune*, has been used as an immunotherapeutic agent for cancer treatment in Japan since 1986 (Lee et al. 2003).

The hyaluronic acid and chondroitin sulfate are polysaccharides with anionic characteristics isolated from animal tissue. The hyaluronic acid, specially, is much utilized in cosmetic formulations and can be obtained by bacterial fermentation. Its composition involves a linear polysaccharide formed from disaccharide units containing *N*-acetyl-D-glucosamine and glucuronic acid.

The hyaluronic acid has also interesting viscoelastic properties that are influenced by its polymeric and polyelectrolyte characteristics. Present in almost all biological fluids and tissues, it is a natural compound in the human skin and has

the capacity to immobilize water in tissue and, this way, change dermal volume and compressibility. It can also influence cell proliferation and differentiation and tissue repair (Kogan et al. 2007).

Naturally, the hyaluronic acid and the chondroitin sulfate make covalent bonds with several proteins in the animal tissue, and this union is called "proteoglycans," which have an important and diverse psychological role in the animal tissue. The hyaluronic acid, which is known to appear in the dermis of the skin, vitreous fluids, and in the body's synovial fluids, has many functions, with cellular turgidity and lubrication being the most important ones. Because of that, the hyaluronic acid and also the chondroitin sulfate, which have a similar function, are important ingredients to improve the appearance of the skin. This polysaccharide is also completely biocompatible with human tissue, but as it has an anionic charge, the glycosaminoglycans are not completely compatible with the anionic surfaces of the skin and hair (Sarpotdar 1993; Robbins 1994; Idson 1988). But it has further been shown that when combined with cationically charged polymers, the hyaluronic acid can enhance the binding to keratinized surfaces like the skin (Band et al. 1991; Pavlichko 1990).

The development of cosmetic formulations containing hyaluronic acid must be done very carefully as it establishes polyelectrolyte complexes with the interaction of anionic and cationic polymers, which is known to frequently form water-insoluble complexes (Gruber 1999).

The arabic, karaya, and tragacanth gums are very traditionally used gums and perhaps the most well-established anionic polysaccharides commercially available, but their complex molecular identities are still requiring study, as they are only superficially known (Whistler 1993). Their main difference from the previous polysaccharides is that they are a group of polysaccharides, with different molecular weight and structure, making it hard to determine their chemical identity (Gruber 1999).

The arabic gum consists of a chain of galactose sugars. Because of its low molecular weight, the arabic gum is mainly used in formulations where a high level of sugar solids is desired without a significant viscosity buildup (Gruber 1999).

The karaya gum is not a truly water-soluble material (Meer 1980; Clark 1993). It is composed primarily of a highly branched D-galacturonic acid backbone. Many of the acidic monosaccharides are acetylated, which causes the water insolubility.

Tragacanth gum is, perhaps, the best characterized of these commercial gums (Stauffer 1993; Clark 1993). It is composed of a highly branched complex of polysaccharides, the arabinogalactan, a water-soluble, neutral polysaccharide having a D-(1,6)-galactose backbone, and tragacanthic acid, an anionic polysaccharide polyglycan based on α-D-(1,6)-galacturonic acid (Gruber 1999). It can be used in cosmetic formulations as a stabilizer and as a protective coating, providing a smooth handfeel. When combined with glycerin, it has the function of a binding agent and when combined with water, forms viscous solutions, even at low concentrations (Verbeken et al. 2003).

The functional characteristics of many natural polysaccharides can be dramatically improved by chemical modification of the native polysaccharide. Cellulose gum, more precisely termed sodium carboxymethylcellulose (CMC), is a chemically altered cellulose (Feddersen and Thorp 1993; Idson 1988). Cellulose is a naturally occurring, structural polysaccharide found in all plants (BeMiller 1992, Majewicz and Podlas 1993). It is isolated commercially from cotton linters and also from wood after the removal of various other components such as lignin and hemicelluloses. Cellulose is composed of one repeating monosaccharide, β-D-(1,4)-glucose (Draget et al. 1997; Gruber 1999).

Chitosan is distinct among the cationic polysaccharides: it is the only naturally occurring, commercially available polyglycan that can carry a cationic charge. Chitosan, naturally available in noncommercial quantities from insect exoskeletons and various bacterial parasites, is more practically isolated by alkaline hydrolysis of the acetamide of crustacean chitin (Djabourov 1991).

Chitosan, employed as its salt, has strong substantivity to anionic surfaces like the skin and hair (Brode et al. 1991; Watcher et al. 1997; Julia et al. 1996; Onsoyen and Dybdahl 1991; Lochead 1988). It is also a film-forming polysaccharide that has been used in fixative formulations and 2-in-1 shampoos. Because chitosan is one of the few naturally occurring cationic polysaccharides, and because of its biocompatibility with the human body, it has been employed extensively in the development of anionic/cationic polysaccharide matrices for drug delivery (Onishi et al. 1997).

The hydroxyethylcellulose (HEC) is successfully used as a conditioning polysaccharide. Cellulose is composed of β-D-(1,4)-glucose monosaccharides and has a water-insoluble nature. Hydroxyethylcellulose is very utilized in industrial and personal care applications as an aqueous viscosifier (Laba 1993).

Guar gum is a polysaccharide that is commercially isolated from the seeds of several leguminous plants (Maier et al. 1993; Seaman 1980; BeMiller 1992). It is composed of linear chains of β-D-(1,4)-mannose (Yalpani 1988), with alternating mannose sugars having branching α-D-(1,6)-galactose units (BeMiller 1992). Guar is often referred to as a galactomannan because of its constituent monosaccharides (Gruber 1999). It is highly utilized in the cosmetic industry, especially in emulsions and lotions, as a protective colloid, increasing emulsion stability, water loss, and phase separation. An example is some shaving creams; beyond its use as a stabilizer, it can also improve facial skin feel after the shaving process (Chudzikowski 1971).

Cyclodextrins, on the other hand, are used extensively in cosmetic and drug delivery applications (Amann 1993; Szejtli 1984; Citernesi and Sciacchitano 1995; Romberger 1998; Motwani and Zatz 1997). Cyclodextrins, as the name implies, are cyclic amyloses composed of 6 (α-), 7 (β-), and 8 (γ-), α-D-(1,4)-glucose monosaccharides. They are also important components in many cosmetic formulations as they function as solubilizing agents for water-sensitive or water-insoluble materials (Gruber 1999).

Sclerotium gum or scleroglycan can be obtained from an exocellular fermentation product (Brigand 1993). Its composition is based on a main chain of

β-D-(1,3)-glucose residues to which, on approximately every third glucose, a branching β-D-(1,6)-glucose is attached. The combination of (1,3)-linkages and the branching glucose moieties reduces its crystallinity and increases its solubility compared to cellulose (Gruber 1999).

3 Cosmetic Formulation Development: Rheology

Systems with polysaccharides are interesting to scientists and technologists from different areas for their structural characteristics, rheological properties, and applicability.

Polysaccharides, as established before, have many cosmetic functions, such as the capacity of being a formulation rheology modifier. The flow limit values from emulsions are increased with the addition of the polysaccharides (Vianna Filho 2009).

This way, the polysaccharides are important ingredients to obtain cosmetic formulations with desired rheological properties. For example, emulsions with sodium hyaluronate and *Klebsiella pneumoniae* extract had low thixotropy when compared to the rest, and xanthan gum solutions are insensitive to a wide range of salt concentration, pH, and temperature. The most basic property of xanthan gum is its ability to form highly viscous solutions at zero or low shear rates, even at low polymer concentrations.

Figure 1 shows the rheological behavior of a gel cream formulation with and without (placebo) polysaccharides from a biotechnological source. The presence of polysaccharides in formulations increases the viscosity and improves its stability. Both formulations had pseudoplastic behavior, with a flow index below 1.

The rheological answers were influenced by the different types of polysaccharides and the other blends utilized. This way, the polysaccharides could increase the time-related stability of the emulsions under study and also increase the elastic modulus values (Vianna Filho 2009).

In the cosmetic industry specifically, the demand for natural-origin raw materials that add desired characteristics in terms of benefits in the physical, sensorial, and visual characteristics and functional compatibility with the skin and other components of the formulation is continually increasing.

The study of the effects caused by these biopolymers in the rheological properties in a specific formulation makes it possible to establish a connection between their physical, chemical, and sensorial characteristics and their rational and functional use in the industry. Also, the structural diversity and physical-chemical properties of the polysaccharides provide an alternative selection to the proper ingredients that leads to the development and improvement of new products.

The mannans and glucomannans are polysaccharides utilized to the emulsification of O/W cosmetic formulations and can have a greater emulsification capacity when compared to commercial emulsifiers like the Glyceryl Stearate and PEG-100 Stearate (Kuncheva et al. 2007). The polysaccharides, proteins, and lipopolysaccharides produced by bacteria such as *Klebsiella* sp., *Acinetobacter calcoaceticus*

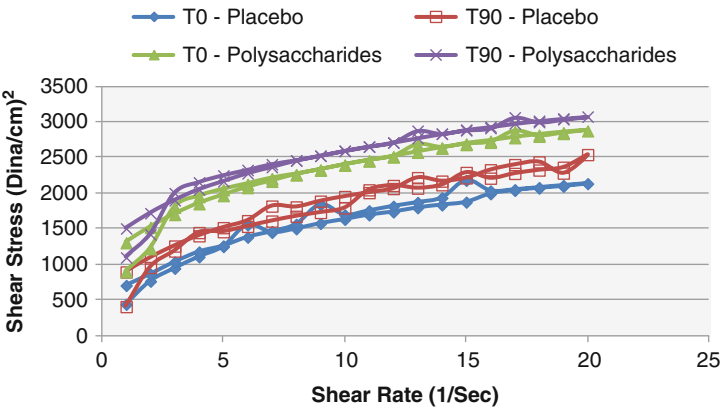

Fig. 1 Rheograms of placebo formulations and formulations with polysaccharides obtained by biotechnological process, stored at 45 °C, 90 days after preparation

RAG-1, *Candida utilis*, and *Methanobacterium thermoautotrophium* can stabilize and emulsify O/W emulsions used in the cosmetic and food industry (Rosemberg and Ron 1997). The whey protein can be used alone or combined with polysaccharides to form emulsified systems that are thermodynamically stable avoiding phase separation (Vianna Filho 2009).

The polysaccharides present numerous advantages over synthetic polymers besides being abundant and easily obtained from renewable sources as algae, plants, and microbial cultures selected and produced by recombinant DNA technique strains. These biopolymers have a wide variety of properties and compositions that can be easily obtained in laboratories (Vianna Filho 2009).

The sclerotium gum, xanthan gum, and polysaccharides from *Klebsiella pneumoniae* extract have shown a positive influence in the rheology of gel cream formulations as well as sensorial properties (Camargo Junior et al. 2011; Camargo Junior 2014).

Polysaccharides extracted from algae such as D-glucose, D- mannose, D-galactose, and D-glucuronic acid have been used as excipients in cosmetic formulations because of its high gelling, bonding, and viscosity-increasing properties (Kim et al. 2008).

Properties as high viscosity, gelling, compatibility with a variety of salts and a wide range of pH and temperature, great water solubility, and synergy with other polysaccharides are some of the features that differentiate the commercially available microbial polysaccharides from other gums.

Polysaccharides were used primarily as thickening, suspending, and stabilizing agents in aqueous systems. The industrial viscosity-increasing polysaccharides have been derived from algae and plant sources traditionally (Paul et al. 1986).

The fact that most polysaccharides can be easily obtained contributes to a lower price when compared to synthetic polymers (Nishinari and Takahashi 2003; Coviello et al. 2007), and they are also biodegradable and can be converted into several derivates (Renaud et al. 2005).

4 Clinical Studies

4.1 Clinical Evaluation by Biophysical and Skin Image Techniques

The clinical studies are essential to demonstrate the efficacy of polysaccharide-based formulations on the skin.

Noninvasive skin biophysical techniques are often used in the clinical studies since they allow evaluation of cosmetic products under actual conditions of use. The skin biophysical methods typically measure selected properties that depend on the measuring principle applied. Hydration, skin viscoelasticity and microrelief, barrier function, and skin thickness are examples of parameters that can be quantified noninvasively using these techniques (Maia Campos et al. 2008).

Some clinical efficacy studies of the cosmetic formulations by biophysical techniques and skin image have been used according to the following parameters: stratum corneum water content, transepidermal water loss (TEWL), viscoelastic characteristics (Ua/Uf, gross elasticity; Ur/Ue, neto-elasticity of the skin; Ur/Uf, biological elasticity; and Uv/Ue, the ratio of viscoelastic to elastic distension), and skin microrelief (roughness – RT, number and width of the wrinkles – SEW, skin smoothness – SESM) techniques (Maia Campos et al. 2008; Wissing and Muller 2003).

The stratum corneum moisture content is determined with a noninvasive, skin capacitance meter (Corneometer® CM 825, Courage-Khazaka, Cologne, Germany), which measures capacitance and is entirely dependent on the water content of the skin (Dal'Belo et al. 2006). Different capacitance changes are converted into a digital-measured value (arbitrary units), which is proportional to the skin's humidity.

Skin barrier function can be evaluated by measuring the TEWL (g/cm2h) using the Tewameter TM 210 (Courage-Khazaka, Cologne, Germany). TEWL is considered an important measure of epidermal barrier function. Evaporimetry consists of applying a probe with two sensors directly to the skin, with one sensor pair measuring humidity and the other temperature. The acquired data are used by integrated microcomputer to compute the water vapor partial pressures at the two parallel levels of each sensor pair and, via the partial pressure gradient, the rate of evaporation. To minimize outside interference, the measurements were carried out in an open-top chamber with closed sides (Huang and Chang 2008).

Skin microrelief parameters are evaluated using Visioscan® VC98, which is a special UVA light video camera with high resolution developed specially to study the skin surface directly, and the SELS (surface evaluation of the living skin) method. The images show the structure of the skin and the level of dryness and the gray level distribution of the image is used to evaluate the following skin roughness parameters: skin roughness (RT), skin smoothness (SESM – proportional to the width and form of the wrinkles), and number and width of the wrinkles (SEW) (Maia Campos et al. 2012).

The viscoelastic properties of the skin are investigated with a Cutometer SEM 575 (Courage-Khazaka, Cologne, Germany) and its measuring principle is by suction/elongation. An optical system detects the decrease of infrared light intensity

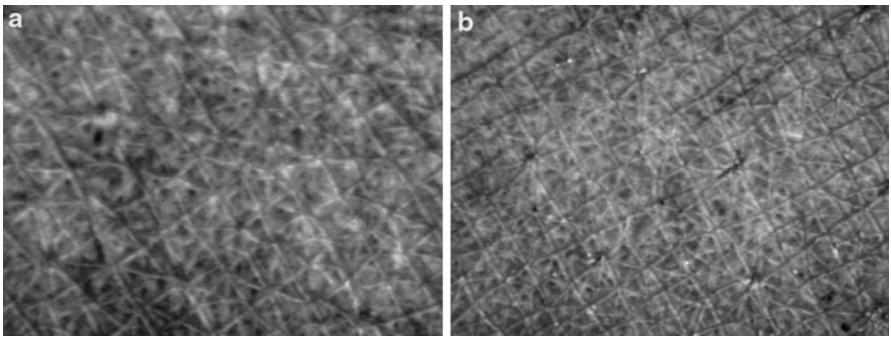

Fig. 2 Skin surface evaluation (skin microrelief). Surface of the skin before (**a**) and after 30 days (**b**) of treatment with a formulation containing polysaccharides. Skin surface was evaluated by Visioscan® VC98 software

depending on the distance the skin is being sucked into the probe. A probe with a 2-mm opening is usually used, and a pressure of 450 mbar is applied in order to suck the skin into the probe. Each measurement consists of five suction cycles (3 s of suction followed by 3 s of relaxation). The parameters Ua/Uf (gross elasticity), Ur/Ue (elasticity of the skin), Ur/Uf (biological elasticity), and Uv/Ue (the ratio of viscoelastic to elastic distension) are evaluated (Dobrev 2000).

Dermal echogenicity and morphological and structural epidermal features can be determined with a DermaScan C (20 MHz ultrasound) and a VivaScope 1500 reflectance confocal microscopy, respectively (Gonzalez and Gilaberte-Calzada 2014; Gianeti and Maia Campos 2014).

The 20 MHz ultrasound is a safe, noninvasive technique for the evaluation of the skin alterations in the dermis as thickness and echogenicity (Unholzer and Korting 2012).

The development of laser scanning confocal microscopy enabled the utilization of this technique in the noninvasive evaluation of the skin. Nowadays, in vivo laser confocal microscopy has a wide range of applications, because it obtains microscopic images in real time, with a resolution close to conventional histology (Gianeti and Maia Campos 2014).

The images below represent the skin microrelief (Fig. 2) and high-resolution photograph of the face (Fig. 3) before and after treatment with a formulation containing polysaccharides from algae. The polysaccharide-based formulation improved the skin microrelief and appearance.

5 Clinical Efficacy of Polysaccharide-Based Formulations on the Skin

In the cosmetic industry, there is a demand for natural-origin raw materials that can add desired characteristics in terms of benefits in the sensorial and visual characteristics and functional compatibility with the skin and other components. This way,

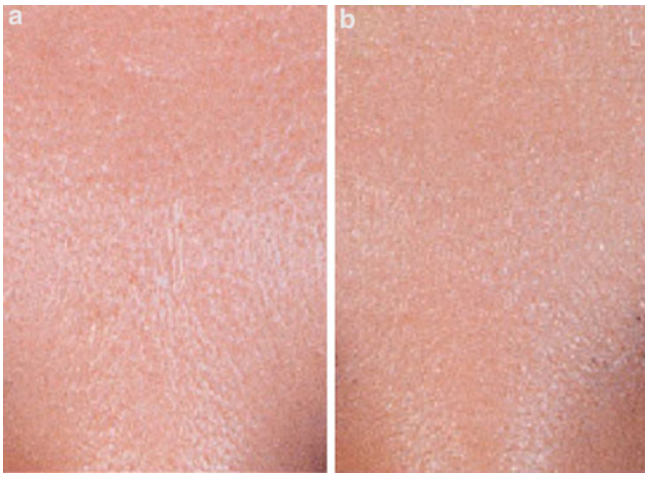

Fig. 3 High-resolution photographic images obtained from an inter-sourciliar area of the face before (**a**) and after (**b**) the application of a gel formulation with polysaccharides from a natural source

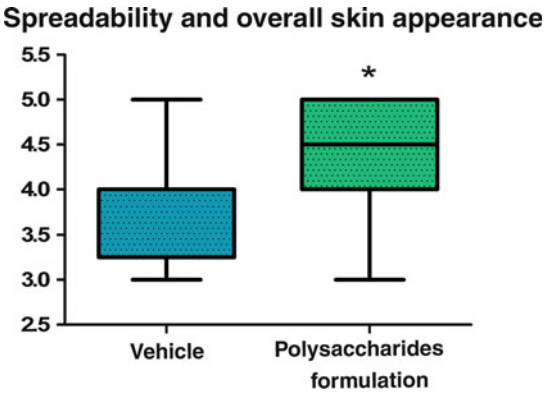

Fig. 4 Spreadability and overall skin appearance after the application of the formulations with and without (vehicle) polysaccharides obtained by biotechnological process. *Significantly different for vehicle formulation ($p < 0.05$)

the formulations containing polysaccharides lead to a higher degree of acceptance by the volunteers and, consequently, better sensorial attributes (Fig. 4)

Skin application of hyaluronic acid-containing cosmetics has been reported to achieve antiwrinkle effects via its ability to effectively moisturize and enhance the elasticity of the skin (Basavaraj et al. 2010; Pavicic et al. 2011). Hyaluronic acid is a very effective topical humectant, attracting water from the air to plump the skin. It is also commonly used as an injectable dermal filler.

In addition to its moisturizing properties, the biodegradability of hyaluronic acid also makes it very attractive for wound healing applications (Price et al. 2007; Teh et al. 2012; Abbruzzese et al. 2009). It is used as a delivery system to transfer keratinocyte cells from tissue culture to skin wounds, in particular burns, with high rates of healing (Price et al. 2007).

The stability of hyaluronic acid-based nanoemulsions as transdermal carriers was also recently reported (Kong and Park 2011). Electrostatic, steric, and hydrophobic effects were found to play a key role in the stability of the emulsions. Encapsulation experiments with vitamin E also demonstrated that the emulsions were capable of successfully carrying lipophilic additives.

Although the exact mechanism of action of topically applied hyaluronic acid has not been fully explored, Brown and Jones (2005) have described some key factors in a recent review article. It is known that the degree of hydration of the stratum corneum influences skin permeability and, with the excellent hydrating properties of hyaluronic acid, can lead to enhanced topical delivery of actives across the skin (Ammala 2013).

Chitosan has been widely reported for use in topical and transdermal delivery systems largely due to its nontoxicity and susceptibility to degradation. It has been reported that chitosan has the ability to enhance permeation across the skin by altering the structure of keratin. It also increases the water content of the stratum corneum and cell membrane fluidity. Further, due to its positive charge under slightly acidic conditions, it can depolarize the negatively charged cell membrane, and in doing so, it decreases the membrane potential and drives the active component or drug through the skin (Pawar and Babu 2010).

In addition to the structural changes to keratin described previously, the use of chitosan and its derivatives has also been demonstrated to increase the water content of the stratum corneum. The derivative *N*-trimethyl chitosan had the greatest effect in increasing water content. A study also demonstrated that the increased amount of water was able to be retained in the stratum corneum for significant lengths of time (He et al. 2009).

The encapsulation of active ingredients using chitosan is also possible. Controlled release has been demonstrated by increasing the viscosity of the polymer matrix (Cattaneo 2005). A skin permeability study confirmed that the encapsulation of retinoic acid with chitosan delivered the retinoic acid at much slower and controlled rates as compared to free retinoic acid (Cattaneo 2005). This ability to control the delivery of retinoic acid is important to reduce skin irritation (Ammala 2013).

Cyclodextrins and chemically modified cyclodextrins have been utilized for a wide range of applications in cosmetics and personal care markets including controlled release of fragrances, stabilization of drugs, encapsulation of vitamins, reduction of the dermal penetration of preservatives, and masking of odors (Matsuda and Arima 1999; Gommet et al. 2010).

Cyclodextrins have also been reported to enhance the effectiveness of sunscreen formulations (Scalia et al. 1998). The study reported an in vitro model, using ethanol-propylene glycol solution, as well as a more realistic model using an oil-in-water emulsion. It was demonstrated that the photostability of sunscreen agents could be improved by encapsulation (Taramici 2011).

Polysaccharides can also come from other sources, as algae, being the red and brown algae the ones most utilized in the industry (Cunha et al. 2009).

Chondrus crispus is an example of red algae rich in polysaccharides and minerals, including manganese, zinc, calcium, and magnesium which attributed to hydrating, soothing, healing, moisturizing, and conditioning effects (Kim et al. 2008).

Fucoidan, which is an active ingredient found mainly in various brown algae such as *Laminaria japonica*, *Fucus vesiculosus*, *Undaria pinnatifida*, *Cladosiphon okamuranus*, and *Hizikia fusiforme*, had its antioxidant and antiaging properties studied in epidemiological and experimental studies (Kim et al. 2008). It also inhibits ultraviolet B (UVB)-induced matrix metalloproteinase-1 (MMP-1) enzyme activity in human skin fibroblasts (Moon et al. 2008; Oresajo et al. 2008). Matrix metalloproteinases (MMPs) are responsible for the degradation and the inhibition of synthesis of collagenous extracellular matrix in connective tissues (Moon et al. 2008) and have demonstrated that fucoidan can mainly inhibit UVB-induced MMP-1 expression by inhibiting the extracellular signal-regulated kinases (ERK) pathways. That way, fucoidan is emerging as a popular natural cosmetic ingredient to be used as a potential agent in skin cosmetics for the prevention of skin photoaging (Wijesekara et al. 2011).

New clinical efficacy studies with *Spirulina*, which has a rich composition in provitamin A, B vitamins, proteins, and polysaccharides such as glucose, galactose, mannose, and ribose, have shown that an emulsion containing this type of seaweed has a potential to increase the stratum corneum water content and reduce the transepidermal water loss. Also shown was a significant reduction of the scaliness and the roughness of the skin (Maia Campos et al. 2013).

The polysaccharide from *Klebsiella pneumoniae* is also commercially available which is supposed to enhance cell renewal and improve skin hydration and microrelief. In addition, extracts of *Myrtus communis* leaves, which contain different types of sugars, may also provide the same benefits. Galacturonans, the main polysaccharides of hydrolyzed *Myrtus communis* extract, are polymers formed by units of galacturonic acid that can form gels in the presence of water. The enhancement of skin firmness (tensile strength) is related to galacturonan film formation (Gillon et al. 1999).

A clinical study showed that both polysaccharides added in gel formulation provoked an enhancement to the skin hydration and isolated or combined the active substances can improve the skin barrier function. So these results suggest that the daily use of formulations containing these substances is important for the protection of the skin barrier function. In the same study, a sensorial analysis also showed that formulations with the polysaccharides previously mentioned had a better acceptance regarding spreadability, skin appearance, and sense of skin hydration when compared to placebo formulation. The tests are useful to study the sensorial properties of the product by volunteers, predicting its future acceptance in the market (Camargo Junior et al. 2012).

Other studies from our research group (data not published) that evaluated the transepidermal water loss (TEWL) showed that formulations supplemented with polysaccharides from natural sources reduced this parameter after 15 and 30 days of daily application, resulting in an improvement of the skin barrier function (Fig. 5). In addition, those formulations presented better sensorial attributes regarding skin appearance, skin feeling, and skin hydration after the application.

Pillai et al. (2005) studied the penetration of oat β-glucan in human skin models to evaluate clinically its efficacy for reducing fine lines and wrinkles. After 8 weeks

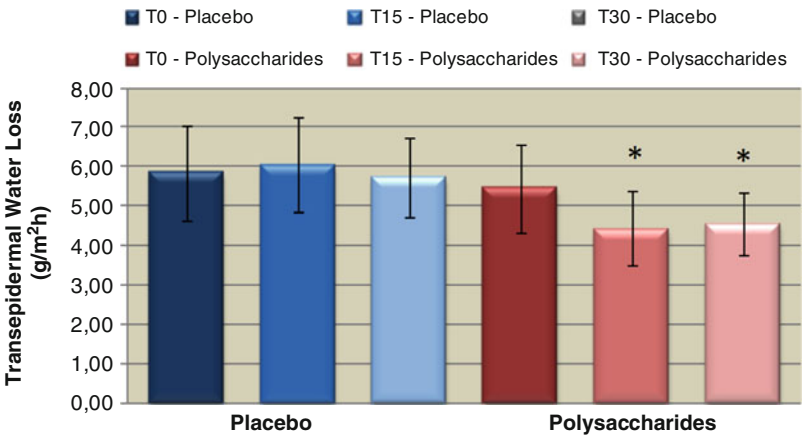

Fig. 5 Transepidermal water loss on the volunteers' forearm skin before (baseline values – T0) and 15 (T15) and 30 (T30) days after the application of the formulations: placebo formulation and a formulation containing polysaccharides from a natural source. * Significantly different for baseline values ($p < 0.05$)

of treatment, the results indicated a significant reduction of wrinkle depth and height and overall roughness (Humbert et al. 2012a). Nevertheless, the cause and occurrence mechanism of the coarser lines on an aging individual's skin are not fully understood yet (Humbert et al. 2012b).

The water content of the stratum corneum and skin surface lipids are important factors in the appearance and function of the skin (Cheng et al. 2007). Moisturizers decelerate the loss of skin humidity and minimize fine wrinkles (Baumann 2007). They also increase hydration of the stratum corneum and improve physical and chemical properties of the skin surface, making it moist, smooth, and soft (Kim et al. 2007). Traditional moisturizing ingredients, such as pantothenic acid (Kobayashi et al. 2011) and hyaluronic acid (Pavicic et al. 2011), are known to possess an effective moisturizing activity in cosmetic formulations. Additionally, some natural extracts from the root of *Lithospermum erythrorhizon* (Chang et al. 2008), okra polysaccharide (Kanlayavattanakul et al. 2012), and β-glucan (Kim et al. 2008) play important roles in the cosmetic and pharmaceutical industry.

Zulli et al. (1998) suggested that glucan from the baker's yeast cell wall could be used as an active ingredient for cosmetic and pharmaceutical purposes. This experiment showed that carboxymethyl glucan (CM-glucan) protected skin cells against the depletion of antioxidant molecules upon UVA irradiation.

6 Conclusion

The effects of polysaccharide-based formulations on the human skin have been demonstrated in some clinical studies. Sometimes these effects are associated with the improvement of the skin barrier function and hydration. However, the polysaccharides can also act on the skin viscoelasticity and microrelief.

In addition, the use of polysaccharides in cosmetic formulations is important to formulation technology, once it can act on the rheological properties and increase the formulation stability. The cosmetic sensorial properties can also be enhanced by the use of these ingredients in the formulation.

As previously mentioned, the polysaccharides obtained from different natural sources like hyaluronic acid and algae, obtained by biotechnological process and others, are interesting active ingredients to develop effective cosmetic formulations for skin protection and maintenance of its structure integrality and physiology.

So according to the benefits on the human skin described, polysaccharides have been extensively used in cosmetic formulations for their diverse range of benefits.

Finally, the use of polysaccharide-based formulations on the human skin is very important to maintain physiological skin conditions and to prevent skin disorders related to barrier function alterations.

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Major Advances in the Development of Synthetic Oligosaccharide-Based Vaccines

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Damiano Cancogni, Matteo Fusari, Niccolò Poggi, and Luigi Lay

Contents

Abstract

Because of their involvement in a variety of different biological processes and their occurrence onto pathogens and malignant cell surface, carbohydrates have been identified as ideal candidates for vaccine formulation. However, as free oligosaccharides are poorly immunogenic and do not induce immunological memory in the most at risk population (infants and young children, elderly and immunocompromised patients), glycoconjugate vaccines containing the same carbohydrate antigen covalently linked to an immunogenic carrier protein have gained a prominent role. Accordingly, a number of glycoconjugate vaccines mostly directed against infections caused by bacterial pathogens have been licensed and are currently available on the market. However, also glycoconjugate vaccines suffer from significant drawbacks. The challenging procedures required for the isolation and purification of the carbohydrate antigen from its natural source often lead to poor homogeneity and presence of biological contaminants, resulting in batch-to-batch variability. Moreover, in some cases, the overwhelming immunogenicity of the carrier protein may induce the

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carbohydrate epitope suppression, causing hyporesponsiveness. The development of synthetic oligosaccharide-based vaccine candidates, characterized by the presence of pure and well-defined synthetic oligosaccharide structures, is expected to meet the requirement of homogeneous and highly reproducible preparations.

In the present chapter, we report on the major advances in the development of synthetic carbohydrate-based vaccines. First of all, we describe different strategies developed during the last years to circumvent the inherent difficulties of classical oligosaccharide synthesis, such as the one-pot glycosylation and the solid-phase synthesis, and their application to the preparation of carbohydrate antigens apt to conjugation with protein carriers. Next, we discuss the most representative methodologies employed for the chemical ligation of oligosaccharide structures to proteins. Finally, in the last section, we report significant examples of fully synthetic vaccines exploiting the multivalency effect. These constructs are based on the concept that the conjugation of multiple copies of synthetic oligosaccharide antigens to multivalent scaffolds, such as dendrimers, (cyclo)peptides, gold nanoparticles, and calixarenes, raises cooperative interactions between carbohydrates and immune receptors, leading to strong enhancement of the saccharide antigen immunogenicity.

Keywords

Vaccines • Immunology • Oligosaccharide • One-pot oligosaccharide synthesis • Solid-phase oligosaccharide synthesis • Glycoconjugates • Protein conjugation • Multivalency

Abbreviations

Ac_2O	Acetic anhydride
AcOH	Acetic acid
CDI	Carbonyldiimidazole
CPS	Capsular polysaccharide
CRM_{197}	Nontoxic cross-reactive material of diphtheria toxoid
CuAAC	Cu(I)-catalyzed azide-alkyne cycloaddition
DMAP	*N*,*N*-dimethylaminopyridine
DMTST	Dimethyl(thiomethyl)sulfonium trifluoromethanesulfonate
DT	Diphtheria toxoid
ECA	*Erythrina cristagalli* agglutinin
EDC or EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Fmoc	Fluorenylmethyloxycarbonyl
GNP	Gold nanoparticle
GPI	Glycosylphosphatidylinositol
GSL	Glycosphingolipid
HbPG	Hyperbranched polyglycerol

HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
KLH	Keyhole limpet hemocyanin
LAM	Lipoarabinomannan
Lev	Levulinoyl
LM	Lipomannan
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization with time of flight detector
mRNA	Messenger ribonucleic Acid
NCL	Native chemical ligation
NIS	*N*-iodosuccinimide
NMR	Nuclear magnetic resonance
OMP	Outer membrane protein from *N. meningitidis* serogroup B
OST	Oligosaccharyltransferase
OVA	Ovalbumin
PADRE	Pan HL ADR binding epitope
PAMP	Pathogen-associated molecular pattern
PEG	Polyethylene glycol
PGCT	Protein glycan coupling technology
PV	Polio virus
RAFT	Regioselective Addressable Functionalized Template or Reversible Addition Fragmentation chain Transfer polymerization Technology
rEPA	Recombinant exoprotein from *Pseudomonas aeruginosa*
RRV	Relative reactivity value
SAM	Self-assembled monolayer
SAR	Structure-activity relationship
SPOS	Solid-phase oligosaccharide synthesis
SPPS	Solid-phase peptide synthesis
STol	ρ-methylphenylthio-
TACA	Tumor-associated carbohydrate antigen
TEC	Thiol-ene coupling
TfOH	Trifluoromethanesulfonic acid
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
tRNA	Transfer ribonucleic acid
TT	Tetanus toxoid
TYC	Thiol-yne coupling
UAAs	Nonnatural Amino Acids
VNTR	Variable number of tandem repeats
WHO	World Health Organization
ZPS	Zwitterionic polysaccharide

Glossary

Adaptive immune response	The (antigen-specific) **immune response** is the response of antigen-specific lymphocytes to antigen, including the development of the immunological memory. The adaptive response is initiated and promoted by the innate immune system cells. The activated APCs travel into the draining lymph nodes where they present the peptide antigen to recirculating naïve T cells (immunological synapse), eliciting a T cell-dependent immune response. Depending on the antigen exposed on APC surface, the immunological synapse raises a complex cascade of events culminating in the activation and differentiation of T cells into **cytotoxic T lymphocytes** (CTL or CD8+ cells) and/or **T helper cells** (T_H or CD4+ cells). CTL are effector T cells responsible for the **cellular immunity** that destroy target cells infected by intracellular viruses and bacteria. T_H cells initiate the maturation process of resting B cells driving their differentiation into **plasma cells** (antibody-forming cells) and **memory B cells** (**humoral immunity**). While plasma cells secrete low-affinity IgM-type antibodies, memory B cells survive for a long time in the body and respond rapidly to subsequent exposures of antigen by eliciting high-affinity IgG antibodies, which represents the overall objective of the vaccination practice.
Adjuvant	**Adjuvant** is any substance that enhances the immune response to an antigen. Adjuvants can be categorized in delivery systems and immune potentiators. The delivery systems play the main function to localize vaccine components and to target them to APCs, whereas immune potentiators directly activate APCs acting as agonists of PRRs (mostly TLRs) facilitating the antigen uptake.
Antibody	An **antibody** is a protein that binds specifically to a particular substance, called **antigen**. While each antibody has a unique structure that enables it to bind specifically to its corresponding antigen, all antibodies have the same overall structure and are known collectively as **immunoglobulins** (Ig).

Antigenic determinant	An **antigenic determinant**, or **epitope**, is the portion of an antigenic molecule that is bound by a given antibody.
Antigen-presenting cells	**Antigen-presenting cells** (APCs): highly specialized cells that can process antigens and display their peptide on the cell surface. Antigen-presenting cells include **macrophages** and **dendritic cells** (DC), but also B cells can act as APCs.
B cell epitope	**B cell epitope** is the minimal portion of an antigenic molecule needed to induce the production of specific antibodies in the humoral immune response.
Hapten	**Hapten** is any small molecule usually unable to activate an immune response unless conjugated to an immunogenic carrier.
Innate immunity	The **innate immunity** is based on a variety of innate resistance mechanisms that recognize and respond to the presence of a pathogen. The innate response is mediated by APCs and it acts during the early stages of the infection. APCs detect and respond to pathogen-associated molecular patterns (PAMPs), structurally and chemically diverse compounds that are unique to foreign microorganisms, via a multiple set of pathogen recognition receptors (PRRs), including peptidoglycan recognition proteins, scavenger receptors, **C-type lectin** receptors typically including a carbohydr-binding domain, and **Toll-like receptors** (**TLRs**). Binding to PRRs induces the pathogen engulfment by APC and its intracellular processing, i.e., enzymatic degradation into short peptides that eventually bind the **major histocompatibility complex** (**MHC**) molecule and are exposed on cell surface to be presented to T cells.
T and B cells	**T and B cells** are the two subsets of lymphocytes defined by their development in the thymus and bone marrow, respectively.
T cell epitope	**T cell epitope** is the minimal portion of an antigenic molecule (typically a peptide) needed to elicit a T cell-dependent immune response.

1 Introduction

Together with proteins and nucleic acids, carbohydrates are a major class of biopolymers that mediate a wide range of biological processes in living organisms. It is now well established that, for example, carbohydrates play crucial roles in viral and bacterial invasion, angiogenesis and tumor cells metastasis, embryogenesis, inflammatory reactions, and, more generally speaking, in a large number of fundamental molecular recognition phenomena (Varki et al. 2009; Dwek 1996; Varki 1993). In particular, there is a growing interest towards what is known as glycoimmunology: this term derives from the merger of glycobiology with immunology, and it deals with the peculiar interactions of carbohydrates with the immune system, emphasizing the importance of using carbohydrates to induce protective immunity against microbial infections but also cancer metastasis. This relatively new field is based on the concept that diverse pathogens and malignant cells expose on their surface a dense array of often unique glycan structures that exert a protective function against the host's immune defense and are essential for their pathogenicity. Typical examples are lipopolysaccharides (LPS) of Gram-negative bacteria, the polysaccharide coat (capsular polysaccharides, CPS) of encapsulated bacteria, and diverse glycoproteins/glycolipids specifically expressed on viral and cancer cell surface. On the other hand, all these glycoforms are capable of interacting with the immune system, acting as cell antigenic determinants, and raising carbohydrate-specific antibodies production, and therefore they represent attractive targets for vaccine development.

Vaccination has certainly been a key breakthrough in the history of medicine and produced a tremendous impact on health-care systems. It is therefore not surprising that, despite the advent of more and more sophisticated antibiotics, vaccination is still considered by the World Health Organization as the most cost-effective approach for preventing infectious diseases and protecting public health, especially in developing countries. There is however a general concern about carbohydrate-based vaccines. Vaccines based on free and purified polysaccharides are poorly immunogenic in infants, in young children (under 2 years of age), in the elderly, and in immunocompromised patients, since they induce only short-lasting antibody responses in adults and fail to generate conventional B cell-mediated immunological memory (Gonzalez-Fernandez et al. 2008; Segal and Pollard 2004). Polysaccharide-based vaccines thereby possess limited clinical efficacy that has been largely ascribed to the T cell-independent immune response, hallmarked by the exclusive production of low-affinity IgM antibodies and no class switch to high-affinity protective IgG antibodies. This kind of immune response is typically triggered by antigens with polymeric structure composed of multiple repeating units, such as polysaccharides (Mond et al. 1995). The discovery that the polysaccharide immunogenicity can be strongly enhanced by their conjugation to an immunogenic carrier protein led to the development of glycoconjugate vaccines (Lesinski and Westerink 2001; Weintraub 2003). This strategy opened a new era in the field of vaccinology. Glycoconjugate antigens are able to induce T cell recruitment and immune memory B cell proliferation, with the production of long-lasting IgG antibodies specifically directed towards the carbohydrate strictly associated to

the pathogen (Ada and Isaacs 2003). The isolation and purification of carbohydrate antigens from their natural sources, and their subsequent manipulation for protein conjugation, are however a challenging task. In addition, the material available is often heterogeneous and not sufficient for the inclusion in a vaccine, and these represent major limitations to further expansion in this field. Hence, the development of cost-effective, fully synthetic carbohydrate vaccines would be of great importance, as witnessed by the spectacular success of the Cuban vaccine against *Haemophilus influenzae* type b (Verez-Bencomo et al. 2004). Synthetic carbohydrate antigens have, indeed, defined composition, and they can be produced as homogeneous compounds in a controlled manner with little or no batch-to-batch variability, affording highly reproducible biological properties. In addition, some microorganisms express carbohydrate structures that have close similarity to mammalian tissue-specific structures, and this molecular mimicry may induce tolerance by the host's immune system. One approach to evade this immune tolerance is to use a chemically modified version of the carbohydrate, an option available only with the recourse to the chemical synthesis. The unnatural structure will be perceived as a foreign antigen by the host, but at the same time, it should elicit antibodies able to cross-react with the natural glycan expressed on the pathogen cell surface.

On the basis of these considerations, the present chapter deals with the major advances in the formulation of synthetic carbohydrate-based vaccines, with a particular focus on the strategies and relevant synthetic methodologies that emerged during the last years.

First and foremost, for the sake of clarity and with the purpose to facilitate the comprehension of the contents by nonspecialized readers, we report a glossary defining the most significant terms referred to the complex machinery of the immune response and used throughout this chapter.

The following section highlights the most significant synthetic methodologies applied to the preparation of carbohydrate-based vaccine candidates. Finally, the new strategies (or new applications of the existing ones) carried out for the design of new, more efficient, and safer vaccines based on the conjugation of synthetic oligosaccharides with protein carriers and polyfunctional scaffolds (in order to exploit the multivalency effect) are described and critically discussed. It should be mentioned that a comprehensive review focused on the preparation of vaccines or vaccine candidates based on fully synthetic carbohydrate antigens but classified according to the target disease (infections from bacteria, viruses, parasites, fungi, and cancer) has been published by our group in 2011 (Morelli et al. 2011).

2 New Methodologies for the Synthesis of Oligosaccharides as Vaccine Candidates

Due to the enormous importance of carbohydrates in different biological processes (Varki et al. 2009; Dwek 1996; Varki 1993), the study of their functions and structure-activity relationships (SARs) needs homogeneous and well-defined

oligosaccharides. As stated above, the achievement of carbohydrate structures in high purity and quantity from natural sources is a tough and complex process. To overcome these problems, significant efforts have been carried out for the development of chemical and enzymatic synthesis of well-defined oligosaccharides and conjugates (Zhu and Schmidt 2009; Boltje et al. 2009; Fraser-Reid et al. 2008; Kamerling 2007; Wong 2003; Ernst et al. 2000; Nicolaou and Mitchell 2001; Koeller and Wong 2001; Seeberger and Werz 2007; Bertozzi and Kiessling 2001).

The synthesis of carbohydrates is more difficult than the synthesis of the other two major classes of biopolymers (peptides/proteins and nucleotides/DNA and RNA). In the construction of oligosaccharides, two major challenges have to be taken into consideration: the regioselective protection/deprotection of polyhydroxy groups and the stereoselective formation of glycosidic linkages.

The chemical synthesis of oligosaccharides requires monosaccharide building blocks with appropriate protecting groups and anomeric leaving group. The mammalian glycome includes a limited number of monosaccharides and linkages, so using only a limited number of building blocks, a large variety of oligosaccharides could be prepared by a proven synthetic strategy (Werz et al. 2007a). This will also be true for viruses, which use the host glycan machinery for the synthesis of their oligosaccharides. On the contrary, the bacteria glycome comprises much more complex structures (Adibekian et al. 2011), attainable only in assembling a large number of building blocks.

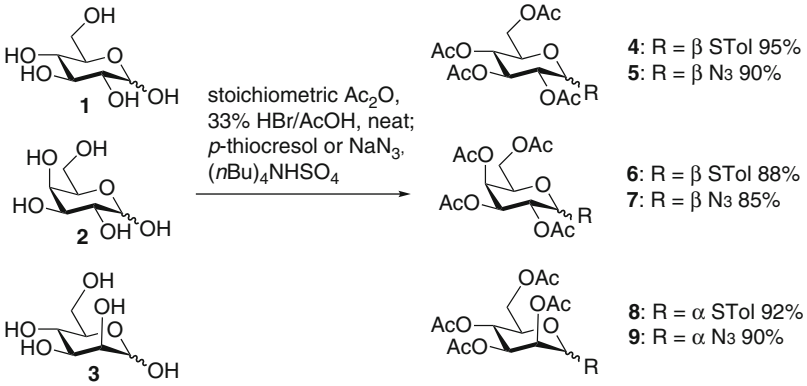
The chemical synthesis of oligosaccharides, as well as the development of new methodologies for the orthogonal protection of building blocks used in saccharide chain assembly, made enormous progress during the last two decades. Some significant examples of these techniques and their application to the preparation of carbohydrate antigen are described in the following section.

2.1 Regioselective Protection of Monosaccharides

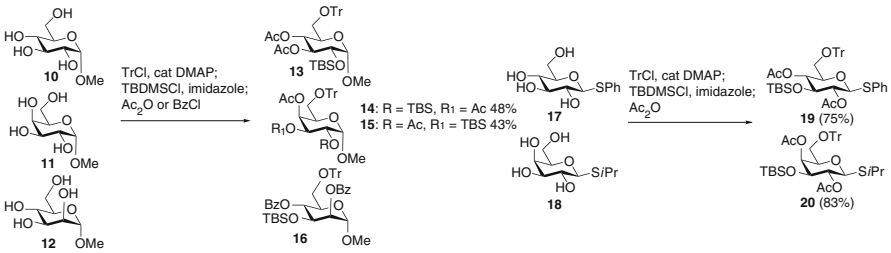
The correct choice of the protecting groups is the basis to design an ideal synthesis of complex oligosaccharides. One of the most common problems is the regioselective installation of orthogonal protecting groups. The protection of the different hydroxy groups of a monosaccharide influences the reactivity of a glycosyl donor/acceptor, the stereoselectivity of the newly formed glycosidic bond, and the ease of the final deprotection.

Besides the classical stepwise protection strategy, during the last years, different methodologies for the one-pot protection of monosaccharides have been developed.

An example of one-pot protection is the per-*O*-acetylation of sugars: with this reaction it is possible to obtain important precursors of monosaccharide synthons. In 2003 Hung and coworkers developed a procedure to obtain fully acetylated hexoses: the reaction proceeds under solvent-free conditions using a stoichiometric amount of acetic anhydride and a catalytic amount of $Cu(OTf)_2$ (Tai et al. 2003). Subsequently, another one-pot procedure to prepare thioglycosides and glycosyl azides from unprotected sugars was developed (Kumar et al. 2006). The reaction



Scheme 1 One-pot full acetylation and subsequent synthesis of thioglycosides or glycosyl azides



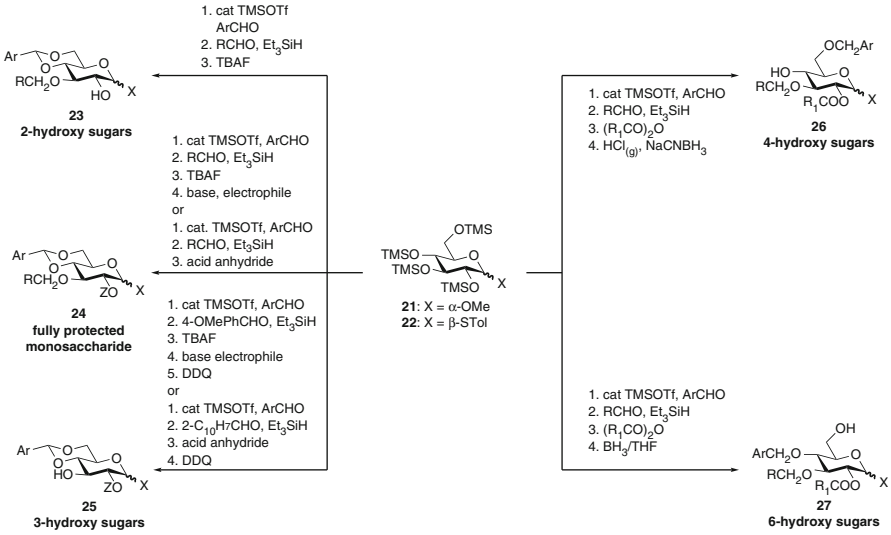
Scheme 2 One-pot tritylation/silylation/acetylation

proceeds under phase-transfer conditions using a stoichiometric amount of Ac_2O and 33 % HBr in AcOH: thioglycosides **4**, **6**, and **8** were obtained by further addition of *p*-thiocresol and $(nBu)_4NHSO_4$, while glycosyl azides **5**, **7**, and **9** were prepared by adding NaN_3 instead of *p*-thiocresol (Scheme 1).

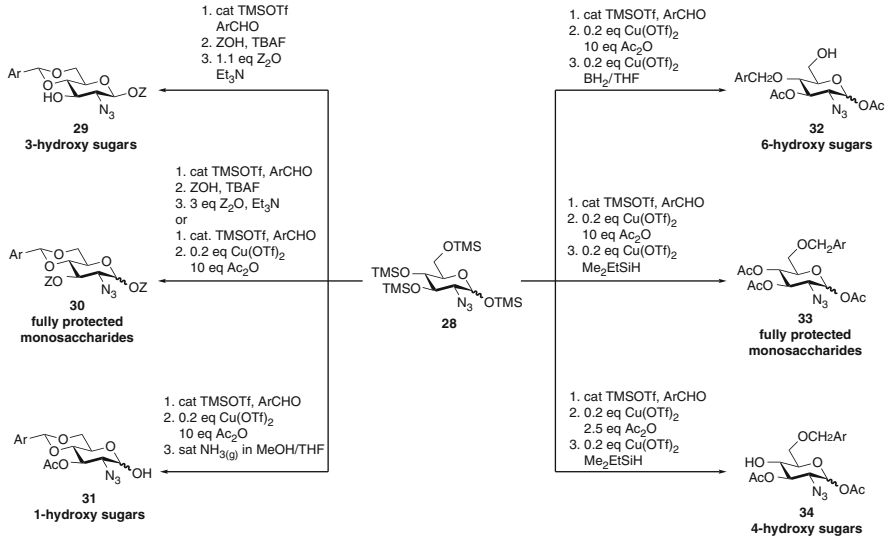
In order to prepare monosaccharides containing different and orthogonal protecting group, a one-pot tritylation/silylation/acylation sequence was employed (Scheme 2) by treatment with a catalytic amount of DMAP. Different results were obtained using methyl glycosides (**10**, **11**, **12**) or thioglycosides (**17**, **18**), but in all cases the fully protected monosaccharide was obtained in good yield (71–86 %) (Du et al. 2000).

Another possibility to obtain partially or fully protected glycosides is the combinatorial and regioselective one-pot protection. In this case the reaction is performed using 2,3,4,6-tetra-*O*-trimethylsilylated glucosides (α-Me-glucopyranoside **21** and β-*ρ*-tolylthioglucopyranoside **22**) (Wang et al. 2007, 2008). Starting from the fully silylated sugar, it is possible to follow five different routes to obtain 2-hydroxy sugars **23**, fully protected monosaccharides **24**, 3-hydroxy sugars **25**, 4-hydroxy sugars **26**, and 6-hydroxy sugars **27** (Scheme 3).

The same procedure can be also applied to the synthesis of fully protected monosaccharides (**30** and **33**) and 1-, 3-, 4-, 6-OH sugars (**31**, **29**, **34**,



Scheme 3 Synthesis of different orthogonally protected monosaccharide building blocks using combinatorial and regioselective one-pot protection



Scheme 4 Synthesis of different orthogonally protected glycosyl azide building blocks using combinatorial and regioselective one-pot protection

32, respectively) starting from 2-azido glucopyranoside **28** (Scheme 4) (Chang et al. 2010). The authors selected the azido group at C-2 because it can be converted into an *N*-acetyl or free amino group (found in different biologically important compounds) and it can control the stereoselective formation of the glycosidic bond.

2.2 Programmable One-Pot Oligosaccharide Synthesis

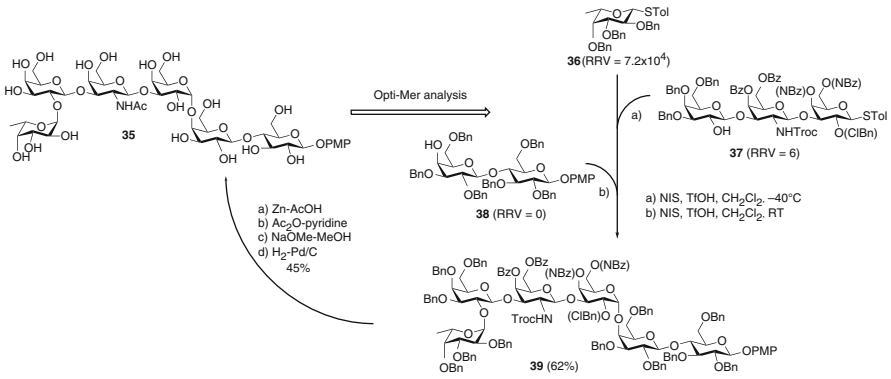
The one-pot glycosylation strategies enable the preparation of oligosaccharides without the isolation and purification of reaction intermediates that are time-consuming procedures typically affecting classical synthesis. For this purpose three different one-pot methodologies have been developed:

1. The chemoselective strategy: the less reactive donor is glycosylated with the more reactive activated donor providing a new glycoside that can act as a new donor itself (Raghavan and Kahne 1993; Ley and Priepke 1994; Yamada et al. 1994a).
2. The preactivation strategy: a donor is activated alone; then it is reacted with a second donor/acceptor with the same aglycon at the reducing end (Crich and Sun 1998; Codee et al. 2003; Huang et al. 2004).
3. The orthogonal strategy: a donor is selectively activated over another donor with different leaving group (Kanie et al. 1994; Yamada et al. 1994b).

Here we focus our attention on the first methodology, which in principle can become an automatic process.

The reactivity of the glycosyl donor and acceptor can be tuned by the manipulation of the protecting groups. The basic concept of the chemoselective glycosylation is that the product obtained using this strategy can be directly used in a subsequent glycosylation without modification of the aglycon at the reducing end. In 1990 the Fraser-Reid group developed the concept of "armed/disarmed" donor. Monosaccharides containing 2-*O*-ether protecting groups (armed) react preferentially than those with 2-*O*-ester group (disarmed) (Fraserreid et al. 1990). In 1998 the Ley research group developed a new concept, the "semi-disarmed" sugar. In this case the reactivity of the thioglycosyl donor is tuned by using a diacetal protecting group (Douglas et al. 1998).

The one-pot glycosylation proceeds starting from the nonreducing end to the reducing one. The first donor used is the most reactive one, while the last one is the less reactive. To design an ideal one-pot glycosylation, it is important to know the "relative reactivity" of a variety of glycosides. In 1999 Wong and coworkers performed a competitive HPLC experiment in order to obtain informations on the reactivity of different glycosyl donors (Zhang et al. 1999). The study was carried out using STol glycosides (easy to prepare) for their intrinsic characteristics, such as stability during protecting groups manipulations and the possibility to study the reactivity via HPLC due to the presence of an aromatic ring. The relative reactivity values (RRVs) for each thioglycosyl donor were obtained by comparison of peracetylated tolylthiomannoside (RRV = 1.0). In particular, high values of RRV represent donors with high reactivity. The authors revealed interesting trends in the reactivity of thioglycoside donors, in particular: (1) pyranosides show reactivities that differ as a function of the sugar (fucose > galactose > mannose > glucose > sialic acid), (2) the reactivity of amino sugars can be tuned by the *N*-protecting groups (NHCbz > NHTroc > NHPhth > N_3 > NHAc), (3) the protecting groups



Scheme 5 Programmable one-pot synthesis of Globo H antigen via [1 + 3 + 2] protocol using the OptiMer analysis

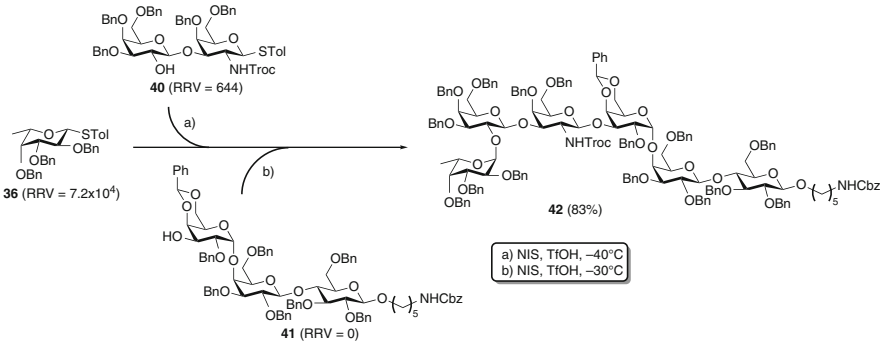
greatly influence the reactivity properties (in particular the substitution at C-2 plays a significant role), (4) the position that mostly affects pyranosides reactivity is not always the same for all sugars, (5) the magnitude of any protecting group reactivity effect is influenced by its position on the pyranoside, and (6) influence of leaving group like steric effects (the reactivity of the glycosyl donor can be tuned by changing the size of its leaving group) and electronic effects. In addition, it was found that, for each sugar, conformational effects (torsional effects) and solvent effects may be crucial to determine the RRVs.

The results of this study led to a computer database (OptiMer) in which RRVs of many donors and donors/acceptors are reported. Starting from the structure of the target oligosaccharide, the computer generates the best combination of building blocks for its preparation. Using this program/database, it is possible to prepare oligosaccharides containing three to six monosaccharides in minutes or hours, without isolation and purification of the intermediates.

An example is the synthesis of the Globo H hexasaccharide **35**, a carbohydrate antigen occurring in many epithelial tumors. The analysis of the oligosaccharide by the OptiMer program showed that the synthesis can be performed using three building blocks (**36**, **37**, **38**), tuning the different reactivity by electron-donating and electron-withdrawing groups (Scheme 5) (Burkhart et al. 2001).

The trisaccharide donor/acceptor **37** can be prepared by one-pot glycosylation. The glycosylation between the fucosyl donor **36** and the trisaccharide **37** and the subsequent glycosylation with the disaccharide acceptor **38** promoted by NIS/TfOH system provided the fully protected hexasaccharide **39** in 62 % yield that after deprotection led to the desired Globo H antigen **35**. The same authors developed another approach for the one-pot synthesis of Globo H hexasaccharide (Huang et al. 2006). In this case the synthetic strategy is based on [1 + 2 + 3] one-pot synthesis (Scheme 6). The challenging Galα(1 → 4)Gal bond was formed in a previous preparation.

Using this approach the fully protected hexasaccharide **42**, precursor of Globo H antigen, was obtained in 82 % yield. The trisaccharide building block **41**, bearing a



Scheme 6 Programmable one-pot synthesis of Globo H hexasaccharide via [1 + 2 + 3] protocol. Conditions: (**a**) NIS, TfOH, −40 °C and (**b**) NIS, TfOH, −30 °C

proper linker, is a valuable building block for the synthesis of a set of truncated Globo H sequences, used for the fluorescent-based binding analysis of the two monoclonal antibodies VK9 and Mbr1 and the identification of the optimal structure for the development of vaccines.

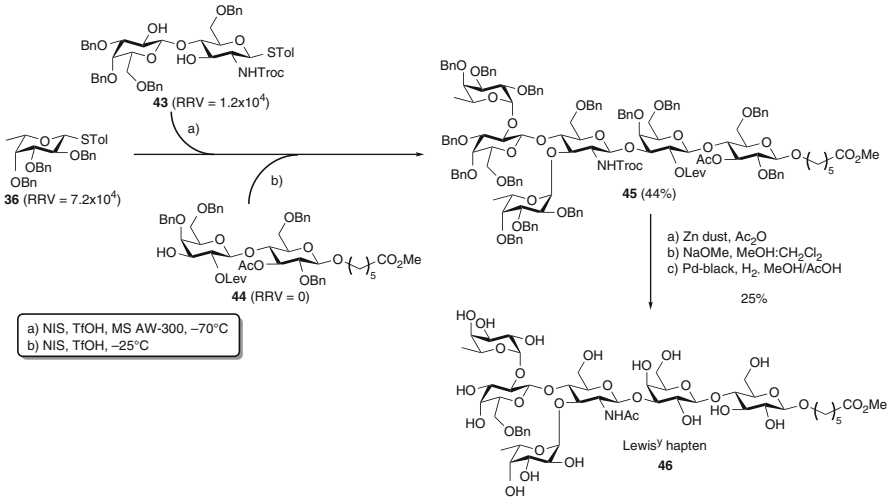
Based on the high reactivity of the fucosyl donor **36** used in the synthesis of Globo H fragment, Wong and coworkers developed another one-pot synthesis of fucose-containing oligosaccharides. In particular they synthesized the Lewis^y hapten **46**, a carbohydrate antigen expressed on the surface of many carcinoma cells. With the OptiMer analysis, three building blocks have been identified for the hexasaccharide synthesis (Scheme 7) (Mong and Wong 2002).

Also in this case the one-pot synthesis was performed using the NIS/TfOH system as a promoter. The first glycosylation was carried out at −70 °C in order to obtain the α glycosidic bond, while the second reaction was performed at −25 °C to facilitate the coupling between the two large and less reactive sugars. The hexasaccharide **45** was obtained in 44 % yield (81 % per glycosylation).

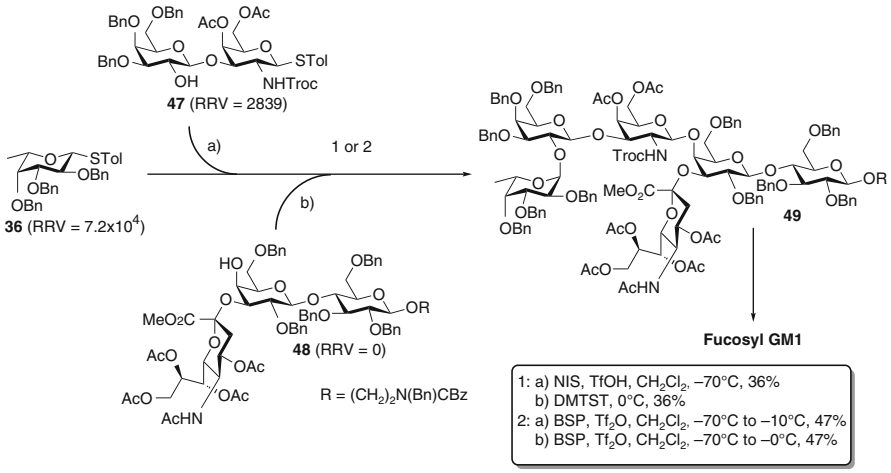
Although the NIS/TfOH system is used to promote the programmable one-pot glycosylation of thioglycosyl donors, in some cases, the succinimide generated by a stoichiometric amount of NIS can compete in the subsequent glycosylation. To overcome this problem, Crich and Smith used the reagent system 1-(benzensulfinyl) piperidine/trifluoromethanesulfonic anhydride (BSP/Tf_2O). The applicability of this reagent mixture was confirmed in the one-pot synthesis of fucosyl-GM1 **49** (Scheme 8), a tumor marker for small-cell lung cancer (Mong et al. 2003; Lee et al. 2006a).

Using the BSP/Tf_2O system, the overall yield increased from 13 % to 22 % and the reaction time decreased from 1 day to 5 h. This promoter system was also applied to the one-pot synthesis of the tumor-associated antigen N3 octasaccharide **52** (Scheme 9) (Lee et al. 2006b).

The one-pot oligosaccharide synthesis was then used for the preparation of oligomannoses, precursors of antigens recognized by 2G12, a human monoclonal antibody (mAb) specific for the oligomannose residues present on gp120



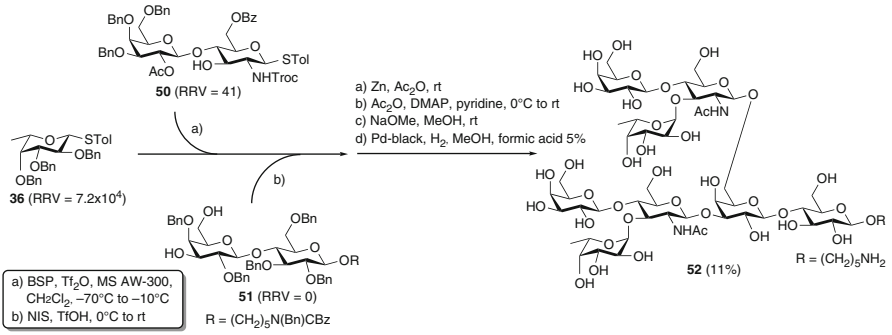
Scheme 7 Programmable one-pot synthesis of Lewis^y hapten



Scheme 8 Programmable one-pot synthesis of fucosyl-GM1 and comparison of NIS/TfOH and DMTST route and BSP/Tf_2O strategy

(the surface glycoprotein of HIV-1 virus) (Lee et al. 2004). On the basis of binding study between 2G12 and gp120, Wong and coworkers synthesized five different oligomannose structures **53**–**57** (Fig. 1) using the one-pot synthesis and a minimal number of building blocks.

The dimannose **59** and trimannose **60** structures, precursors of compounds **53**–**57**, were synthesized using a novel one-pot methodology based on the self-condensation of a mannosyl donor/acceptor (Scheme 10). The authors obtained



Scheme 9 Programmable one-pot synthesis of N3 antigen

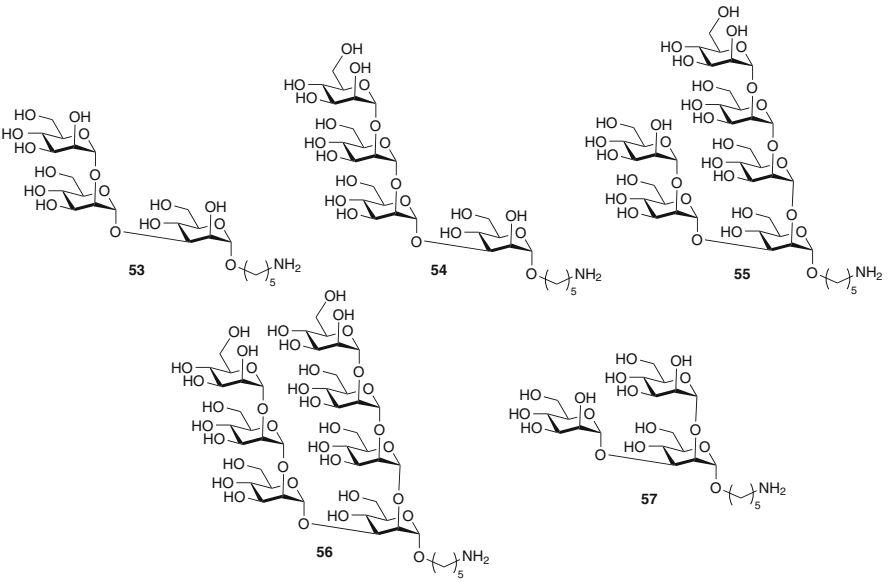
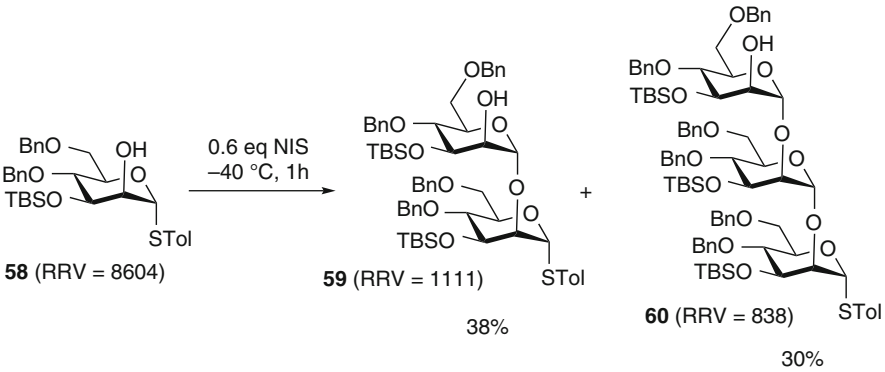


Fig. 1 Structures of oligomannose targets A–E

disaccharide **59** in 38 % yield and trisaccharide **60** in 30 % yield. As expected by RRV values, the most reactive monosaccharide provided the less reactive disaccharide by self-condensation, which could react with another monosaccharide providing the trimer. With the three donors in hand (mono-, di-, and trisaccharide), compounds **53**–**57** were prepared using a mannosyl acceptor bearing a suitable linker. Next the inhibition in the interaction between 2G12 and gp120 was studied using **53**–**57**. Compounds **53** and **57** were the less active, while **54** and **55** showed the highest inhibition values (79 % inhibition at 2.0 nM). Surprisingly, the heptamannose **56** showed a low value of inhibition, probably due to a nonoptimal conformation for 2G12 recognition.

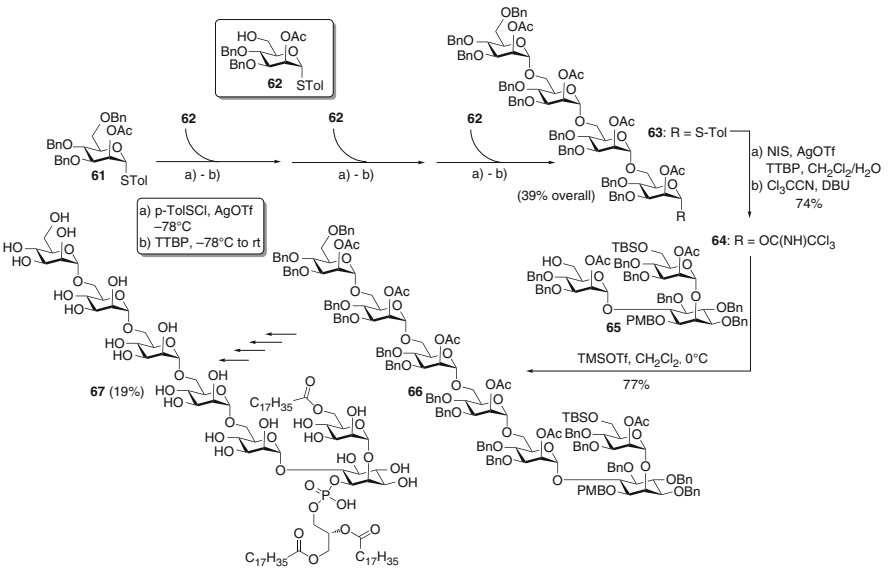


Scheme 10 One-pot self-condensation of mannosyl donor/acceptor

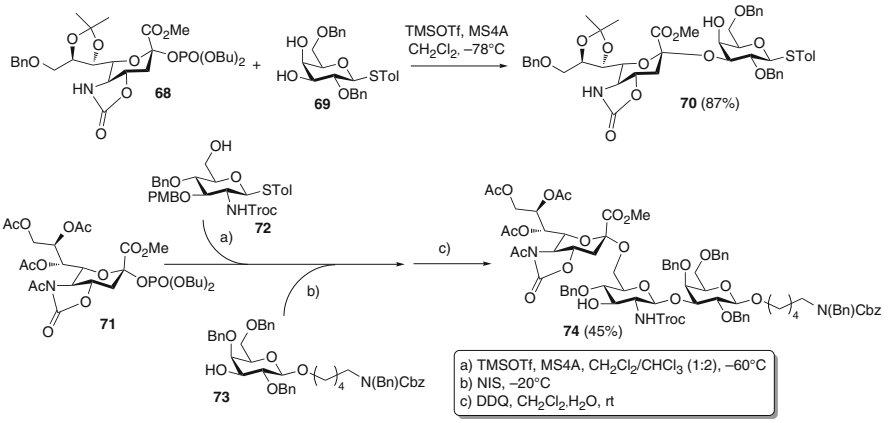
More recently, the synthesis of a lipomannan (LM), suitable for vaccine formulation, was developed using a convergent and efficient methodology (Gao and Guo 2013). Lipomannans (LMs), together with lipoarabinomannans (LAMs), are the major virulent factors of *Mycobacterium tuberculosis* (*Mtb*), the pathogen causing tuberculosis (TB), one of the most life-threatening diseases worldwide (more than two million deaths per year). The LM synthesized in this work is composed by a tetramannose residue and a trisaccharide containing a *myo*inositol derivative **67** (Scheme 11). The one-pot synthesis of the tetramannose building block **63** was carried out using thioglycoside monosaccharides. Each donor was preactivated with *p*-TolSOTf (generated from *p*-TolSCl and AgOTf) at −78 °C; then the glycosylation was performed by addition of 2,4,6-tri-*tert*-butylpyridine (TTBP) as a scavenger for TMSOTf generated during the reaction. Tetramannose **63** was obtained in 39 % overall yield (73 % for each glycosylation step) in a total time of 6 h. Due to the low reactivity of the tetramannose thioglycoside **63**, this compound was first converted into trichloroacetimidate **64** and then coupled with the trisaccharide acceptor **65** providing the fully protected LM precursor **66** in 77 % yield. The desired LM compound **67** was obtained after deprotection and derivatization, and its structure was confirmed by NMR and MALDI-TOF analysis. Starting from the fully protected intermediate **66**, it is possible to regioselectively introduce a proper linker in order to prepare LM-based vaccine by conjugation with carrier protein or other scaffolds.

In 2013 Wong and coworkers reported an elegant synthesis of the RM2 antigen, a glycosphingolipid (GSL) occurring in renal cell carcinoma (cell line TOS-1), and prostate cancer cell lines LNCap and PC-3 (Chuang et al. 2013). The authors developed a [1 + 2 + 3] one-pot synthesis of the hexasaccharide precursor. Initially, the disaccharide donor/acceptor **70** was synthesized using a regioselective glycosylation and the trisaccharide acceptor **74** was prepared itself by a one-pot strategy (Scheme 12).

Next an orthogonal [1 + 2 + 3] glycosylation allowed to obtain the desired hexasaccharide **76** in 32 % overall yield in a total time of 23 h (Scheme 13).

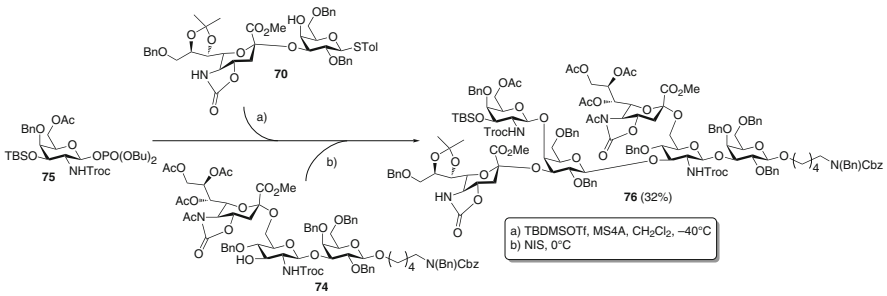


Scheme 11 One-pot synthesis of tetramannose and subsequent synthesis of *Mtb* lipomannan



Scheme 12 Regioselective synthesis of the disaccharide donor/acceptor and one-pot synthesis of the trisaccharide acceptor

Together with the hexasaccharide **76**, the authors also synthesized a panel of fragments of RM2 antigen. The deprotection of these compounds followed by the conjugation to a carrier protein (nontoxic cross-reactive material of diphtheria toxin, CRM_{197}) allowed to study the activity as vaccine. The biological tests were performed by immunization of mice with 2 μg of glycoconjugate together with 2 μg



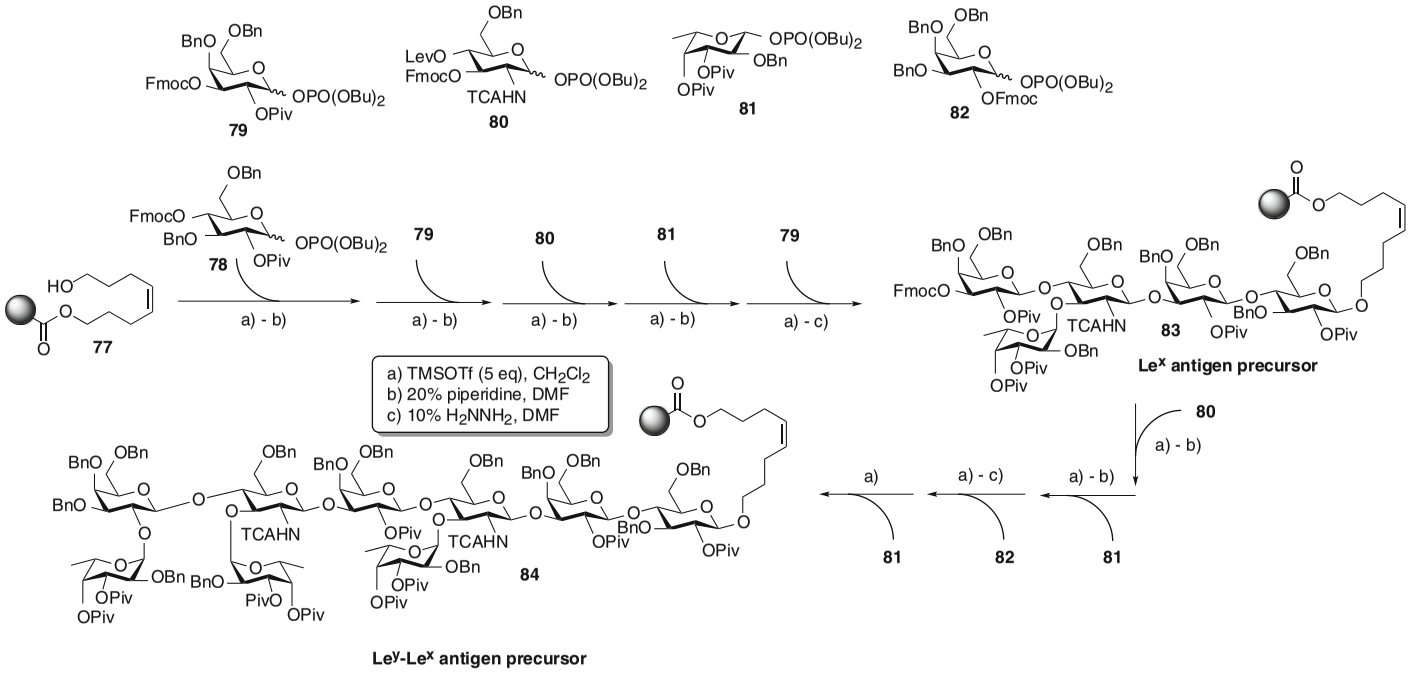
Scheme 13 One-pot [1 + 2 + 3] synthesis of hexasaccharide precursor of RM2 antigen

of adjuvant: the vaccination was carried out by giving three doses at 2-week intervals. After 2 weeks from the last injection, the sera were collected and analyzed with glycan microarray. The authors observed that the anti-RM2 IgG titers increased as vaccination proceeded: in particular the best results were obtained when glycolipid C34 (an α-galactosylceramide derivative) was used as adjuvant. In addition, the strongest anti-RM2 antigen titers were obtained with an antigen/protein ratio of 4.7 (CRM_{197}-$RM_{4.7}$), using the hexasaccharide RM2 as antigen (Chuang et al. 2013).

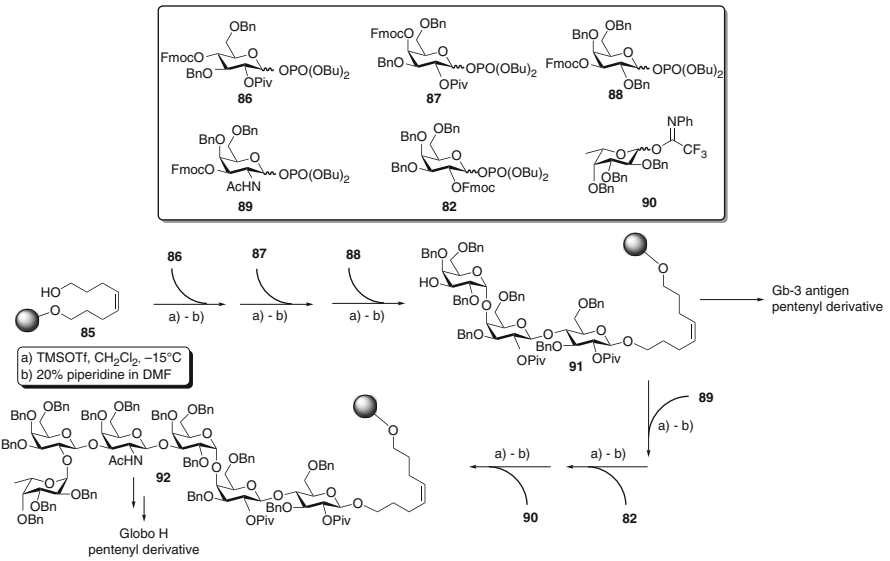
2.3 Solid-Phase Oligosaccharide Synthesis

Together with the programmable one-pot synthesis and other methodologies for the preparation of oligosaccharides (like fluorous-tag assisted solution-phase synthesis (Jaipuri and Pohl 2008)), the solid-phase oligosaccharide synthesis (SPOS) is another approach developed during the last years. SPOS offers two main advantages: (1) only a single purification by chromatography is needed in most cases at the end of the synthesis and (2) unwanted reagents and side products can be easily removed by washing and filtering. Due to the significant improvements in carbohydrate chemistry, during the past decade, there has been a great development of solid-phase oligosaccharide synthesis, confirmed by the numerous contributions reported in the literature (Seeberger and Haase 2000; Seeberger 2001; Danishefsky et al. 1993; Randolph et al. 1995; Rademann and Schmidt 1996; Roussel et al. 2000; Jonke et al. 2006; Nicolaou et al. 1997, 1998). In particular, an automated oligosaccharide synthesizer was developed by Seeberger and coworkers by modification of a peptide synthesizer (Plante et al. 2001; Seeberger 2008) and employed for the synthesis of the nonasaccharide of Le^y-Le^x (KH-1) antigen derivative **84** (Scheme 14) (Love and Seeberger 2004).

With a careful choice of the leaving group (glycosyl phosphates) and orthogonal protecting groups (permanent and temporary like Fmoc and Lev), the authors synthesized the nonasaccharide precursor **84**. The general strategy can be rationalized as follows:



Scheme 14 Automated solid-phase synthesis of Le^x and Le^y-Le^x precursors



Scheme 15 Automated solid-phase synthesis of Gb-3 and Globo H antigens

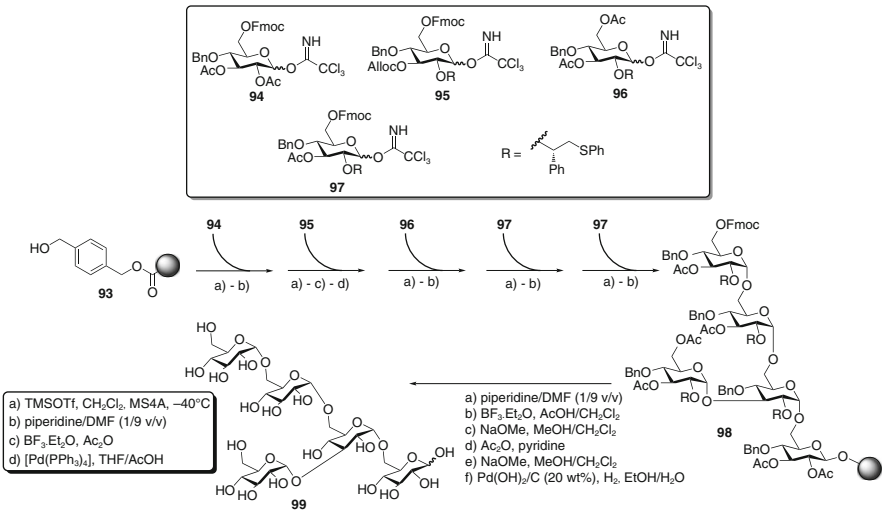
1. Coupling using TMSOTf (5 eq) as a promoter: the glycosyl phosphate was added twice to drive the reaction to completion.
2. Deprotection of the temporary group: Fmoc was removed by treatment with 20 % piperidine in DMF, while Lev was selectively removed using 10 % hydrazine in DMF.
3. Release of the oligosaccharide from the resin using sodium methoxide.

After purification by HPLC, the nonasaccharide was obtained in 6.5 % overall yield in a total time of 23 h. Using this strategy it is also possible to synthesize the Le^x antigen **83**.

Another application of the automated solid-phase oligosaccharide synthesis is the preparation of the tumor-associated carbohydrate antigens Gb-3 and Globo H by Seeberger group (Scheme 15) (Werz et al. 2007b).

In a total time of 12 h, the fully protected Gb-3 antigen was obtained in 46 % overall yield (after cleavage from the solid support and purification by chromatography), while the synthesis of the fully protected Globo H antigen required 23 h (30 % overall yield).

A significant challenge in SPOS is the formation of 1,2-*cis*-glycosides. Different progresses have been achieved in the synthesis of α-galactosidic (Gb-3 and Globo H SPOS) and β-mannosidic linkages (Codee et al. 2008). In 2010 Boons and coworkers developed a 1,2-*cis*-glycosylation strategy driven by the protection at C-2 with a chiral auxiliary (Scheme 16) (Boltje et al. 2010). Using *(S)*-(phenylthiomethyl)benzyl chiral auxiliary as a protecting group at position 2 of the glycosyl donor, the glycosylation proceeds via a trans decalin ring



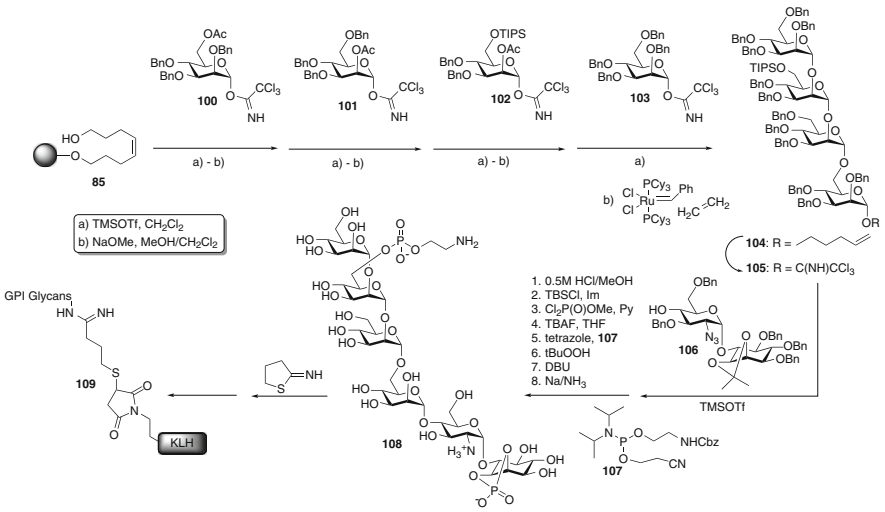
Scheme 16 Stereoselective solid-phase synthesis of tetraglucose residue

intermediate (equatorial sulfonium ion) allowing the stereoselective formation of 1,2-*cis*-glycosidic bond. In particular, with this procedure, the authors were able to synthesize a branched pentasaccharide **99** with different 1,2-*cis*-glycosidic linkages in 13 % overall yield (corresponding to 86 % yield per step). Another advantage of the chiral protecting group is that it can be converted into an acyl group on the solid support under conditions compatible with the removal of the temporary groups.

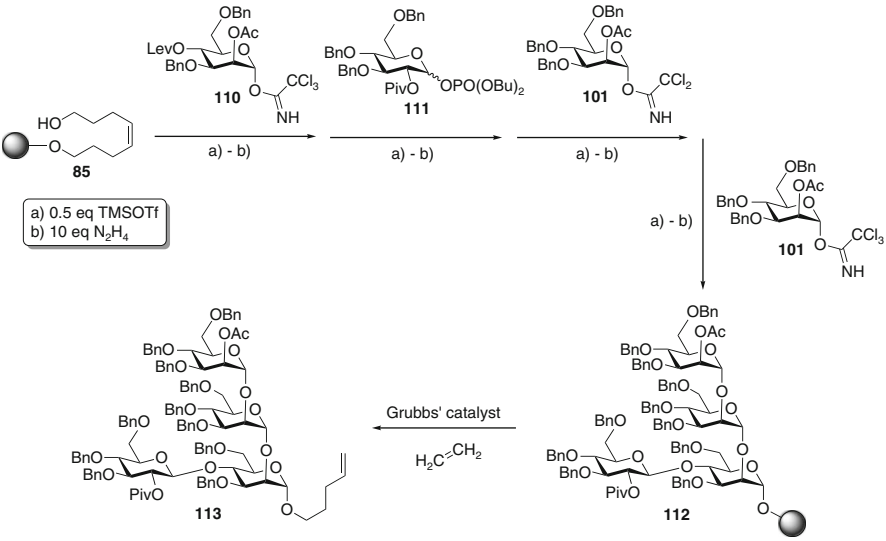
The solid-phase oligosaccharide synthesis was also employed for the synthesis of the *Plasmodium falciparum* glycosylphosphatidylinositol (GPI). This protozoan parasite is responsible for 95 % of the malaria deaths worldwide. Seeberger and coworkers synthesized the tetramannose **104** precursor of the hexasaccharide **108** (Scheme 17), which was then converted into the conjugate **109** by the treatment with maleimide-activated keyhole limpet hemocyanin (KLH) (Schofield et al. 2002).

The conjugate **109** was administrated to mice. The authors observed significant protection against the parasite-induced pathology (malarial acidosis, pulmonary edema, cerebral syndrome).

In 2001 Seeberger and coworkers reported the automated solid-phase synthesis of the branched *Leishmania* cap tetrasaccharide (Scheme 18) (Hewitt and Seeberger 2001). *Leishmania* parasites cause the leishmaniasis, a tropical disease affecting over 12 million people worldwide. The lipophosphoglycan (LPG) antigen of the parasite is composed of a GPI anchor, a repeating phosphorylated disaccharide, and an oligosaccharide cap. Previous promising immunological studies indicated the cap tetrasaccharide as an attractive vaccine target. The automated SPOS was performed using both glycosyl trichloroacetimidates (**110** and **101**) and the glycosyl phosphate **111** as donors with different esters as temporary protecting groups. The desired tetrasaccharide **112** was synthesized using three monosaccharide building



Scheme 17 Automated solid-phase synthesis of tetramannose residue and subsequent solution-phase synthesis of *P. falciparum* GPI-base malaria conjugate vaccine



Scheme 18 Automated solid-phase synthesis of *Leishmania* Cap tetrasaccharide pentenyl precursor

blocks (**101**, **110**, **111**) in a modified ABI 433A peptide synthesizer. After cleavage with the Grubbs catalyst and ethylene, the cap oligosaccharide **113** was obtained in 50 % yield (HPLC) in a total time of 9 h in the synthesizer and less than 4 days starting from the monosaccharide precursors.

3 Carbohydrate-Protein Conjugate Vaccines

As mentioned before, carbohydrate antigens could induce a strong, long-lasting, and protective immune response only after their transformation into T cell-dependent immunogens by conjugation of multiple copies of the saccharide antigen to the surface of a carrier protein (Lesinski and Westerink 2001; Weintraub 2003).

Carrier proteins must be nontoxic and non-reactogenic and should not share T cell epitopes with relevant capsular pathogens. Diphtheria toxoid (DT), CRM_{197}, keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), recombinant exoprotein from *Pseudomonas aeruginosa* (rEPA), outer membrane protein from *N. meningitides* serogroup B (OMP), and more recently protein D derived from non-typable *H. influenzae* are among the most used proteins.

Commonly employed conjugation methods make use of chemoselective reactions between a suitable functional group available on the protein and another one on the carbohydrate moiety. Anchoring sites on the protein can be lateral amines (lysine residues), carboxylic acids (aspartic or glutamic acid), or sulfhydryls (cysteine). On the contrary, attachment sites on the carbohydrate portion can be the intrinsic carbonyl group at the reducing end or aldehyde groups inserted by random periodate oxidation of oligo- or polysaccharides. These random strategies however do not allow an accurate control of the conjugation reaction. Moreover, the precise structure of the carbohydrate portion eliciting the immune response cannot be established a priori, leading to low batch-to-batch reproducibility and difficult characterization of the products. The use of synthetic carbohydrates provided with a suitable linker ending with amino, carboxylic, sulfhydrylic, or olefinic groups ensures to preserve the structural integrity of the saccharide moiety. Examples of chemoselective conjugation of these functional groups are shown in Fig. 2 (Adamo et al. 2013). However, care must be taken in the choice of the linker. In particular, it has been shown that these moieties could be highly antigenic and therefore suppress antibody responses to weakly immunogenic saccharide antigens such as self-antigens (Buskas et al. 2004; Costantino et al. 2011).

Many well-known glycoconjugates employed for antibacterial vaccine formulation were synthesized, exploiting these approaches. For instance, vaccines against *H. influenzae* type b (PRP-TT, Sanofi-Pasteur) and *N. meningitidis* serogroup A (Menafrivac™) (Frasch et al. 2012) are approved and available on the market, whereas a 15-valent-CRM_{197} conjugate against *S. pneumoniae* from Merck and a 3-valent-CRM_{197} conjugate targeting Group B *Streptococcus* from Novartis are in advanced clinical trials. Other recent examples of glycoconjugates currently under study as vaccine candidates include the CRM_{197} conjugate of synthetic fragments of *Clostridium difficile* polysaccharide (Danieli et al. 2011; Adamo et al. 2012) and carba-analogues of *N. meningitidis* A capsular polysaccharide (Gao et al. 2012, 2013).

However, the conjugation chemistry still remains difficult to control, with random regio- and stereochemistry and unpredictable stoichiometry. Different methods have been recently developed in order to obtain well-defined

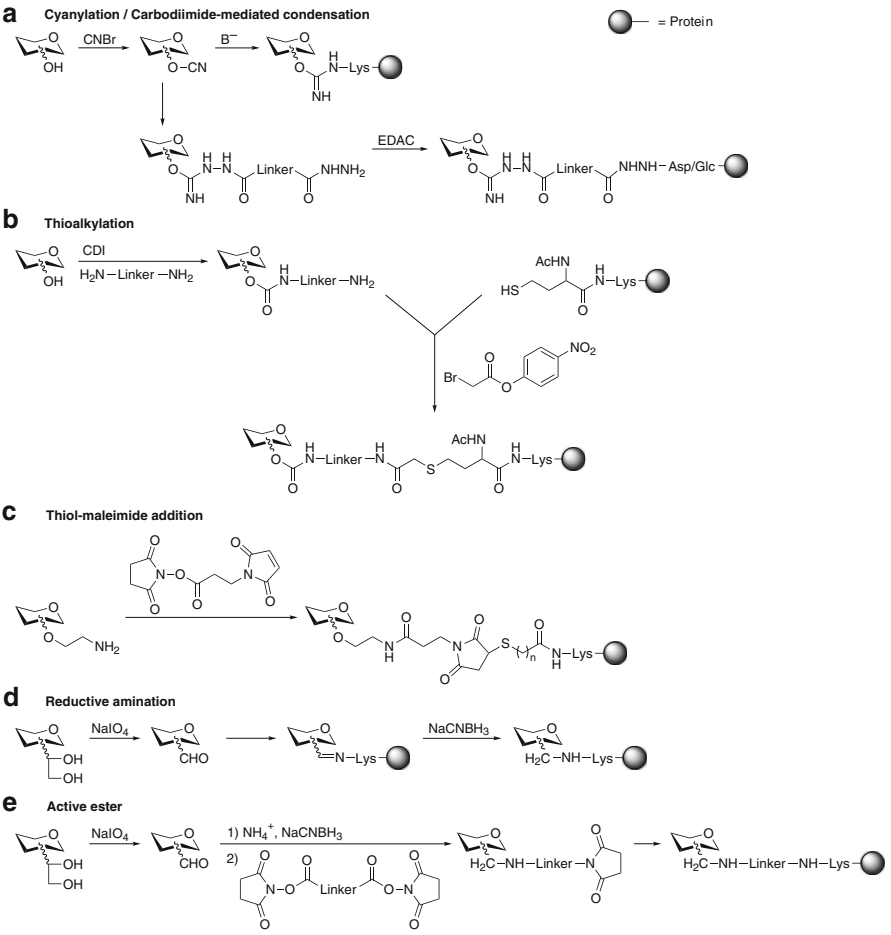
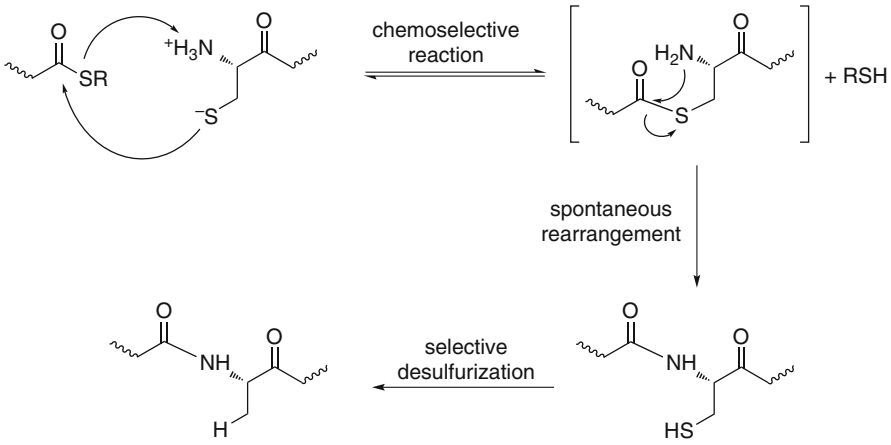


Fig. 2 Commonly employed strategies for the conjugation of carbohydrate antigens to carrier proteins

protein-carbohydrate conjugates. Among them, native chemical ligation, site-selective protein modification, and chemoenzymatic approaches are worthy of note (Adamo et al. 2013).

3.1 Native Chemical Ligation

Native chemical ligation (NCL) is a methodology that permits the construction of small proteins through the convergent chemoselective linkage of two unprotected peptide fragments: the first one containing a thioester as *C*-terminal residue and the



Scheme 19 Mechanism of the native chemical ligation

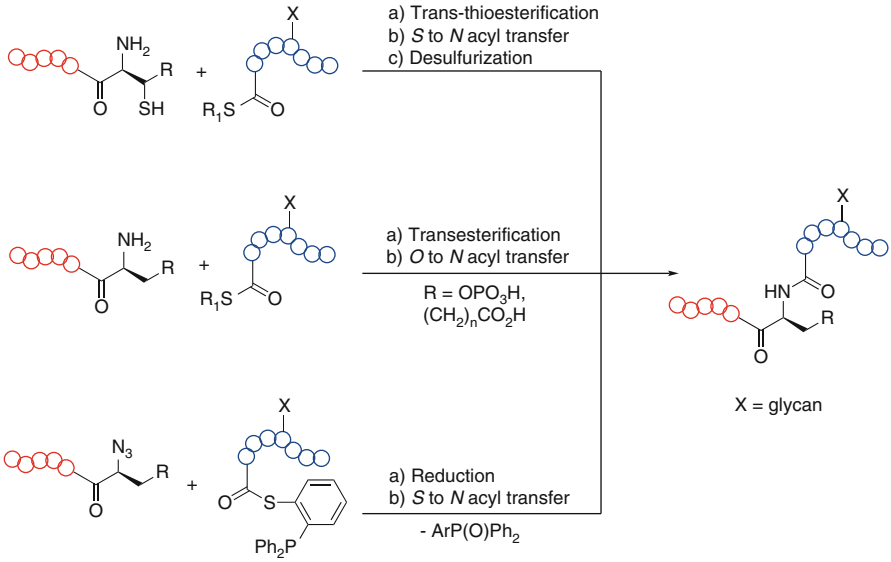
other one bearing a free cysteine at the *N*-terminus (Dawson et al. 1994; Schnolzer and Kent 1992). The reaction mechanism is shown in Scheme 19.

Unlike conventional strategies based on automated solid-phase peptide synthesis (SPPS), which usually rely on long linear reaction sequences and protecting group manipulations, the benefits arising from a convergent total synthesis approach that uses unprotected (glyco-)peptide fragments make NCL a leading methodology for the preparation of glycoconjugates.

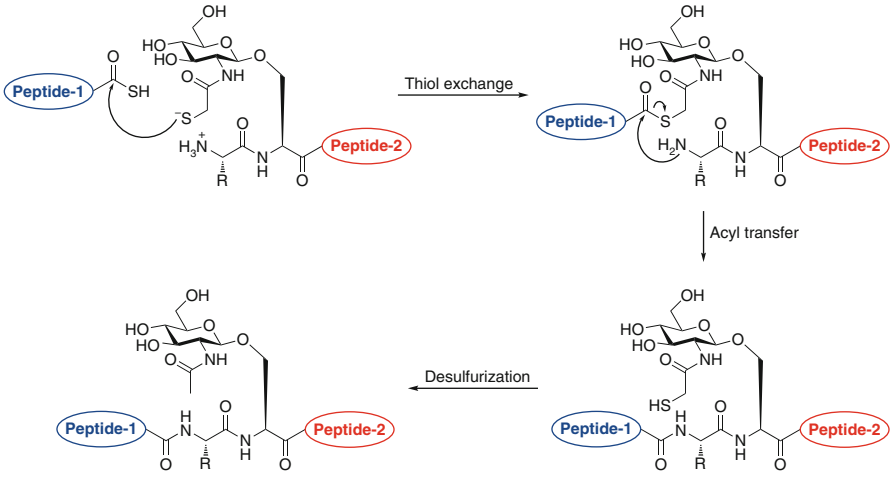
The first examples of NCL-mediated glycoprotein synthesis were reported in 2008 (Yamamoto et al. 2008; Becker et al. 2008). Firstly, a glycan was linked to an amino acid, followed by incorporation into a polypeptide by SPPS. Finally, the resulting glycopeptide chain was coupled via NCL with another peptide sequence, allowing for a fast and chemoselective chain elongation.

This protocol has been studied in detail, and many other types of chemical ligations have been reported in the literature (Payne and Wong 2010). Relevant examples are ligation involving thiol-free moieties such as phosphates (of phosphoserine or phosphothreonine), aspartate or glutamate residues (Thomas et al. 2011), and azides (Scheme 20) (Nilsson et al. 2003).

In addition, a protocol developed by Wong and coworkers, the so-called sugar-assisted chemical ligation, allows for a traceless ligation using thiol-containing glycosyl auxiliaries, which is particularly relevant for the preparation of glycopeptide fragments. It is based on the ligation promoted by β-2-(thioacetamide)-glucosamine-*O*-serine, β-2-(thioacetamide)-glucosamine-*O*-threonine, and β-2-(thioacetamide)-glucosamine-*N*-asparagine residues, which represent favored handles for subsequent (chemo)enzymatic attachment of more complex glycan epitopes for vaccine preparation (Scheme 21) (Brik et al. 2006).



Scheme 20 Different chemical ligation strategies



Scheme 21 Mechanism of the sugar-assisted chemical ligation

3.2 Site-Selective Protein Modification

A complementary protocol to the stepwise insertion of glycosylated amino acid building blocks in the early stages of NCL-like approaches is the site-selective protein modification.

The key aspect is the presence of a bioorthogonal functionality, commonly named "tag," at a specific point in the protein, that allows for a late-stage homogenous glycosylation (Chalker et al. 2011a; Gamblin et al. 2009). The tagging can be obtained either by site-selective protein modification or by selective protein tagging. The former approach involves site-specific chemical modification under mild conditions of natural residues, such as cysteine, its elimination product, and dehydroalanine, which are well suited for thiol-ene reaction and Michael addition (Bernardes et al. 2008; Lin et al. 2008; Chalker et al. 2011b). Even more interesting is the selective protein-tagging approach. This methodology relies on the insertion of nonnatural amino acids (UAAs) into proteins by genetically reprogramming the translation cascade in *E. coli*. Translation has a great intrinsic capacity to be adapted to accommodate new building blocks, since the relationship between the template (mRNA) and the product (polypeptide) is defined by the genetic code, which utilizes aminoacyl-tRNA adapters to establish the map between mRNA and the protein sequence.

The use of modified aminoacyl-tRNA thus led to the incorporation of UAAs in the expressed protein with excellent consistency (Liu and Schultz 2010). Some of the most representative examples include homoallylglycine for thiol-ene reactions (Floyd et al. 2009); *S*-allyl- or *Se*-allylselenocysteine for cross-metathesis reactions (Lin et al. 2008); azidohomoalanine, suitable for the well-known Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Boutureira et al. 2010); Staudinger and traceless Staudinger ligations (Loka et al. 2010; Bernardes et al. 2011); and also recent strained alkyne-based Cu-free cycloaddition protocols (Agard et al. 2004). Other important transformations include the use of carbonyl handles, typically employed in oxime bond formation (Hudak et al. 2011); homopropargylglycine and other alkyne handles for CuAAC, thiol-yne reactions (Li et al. 2013); and Sonogashira as well as Suzuki cross-couplings with p-halophenylalanine tags (Fig. 3) (Spicer and Davis 2013).

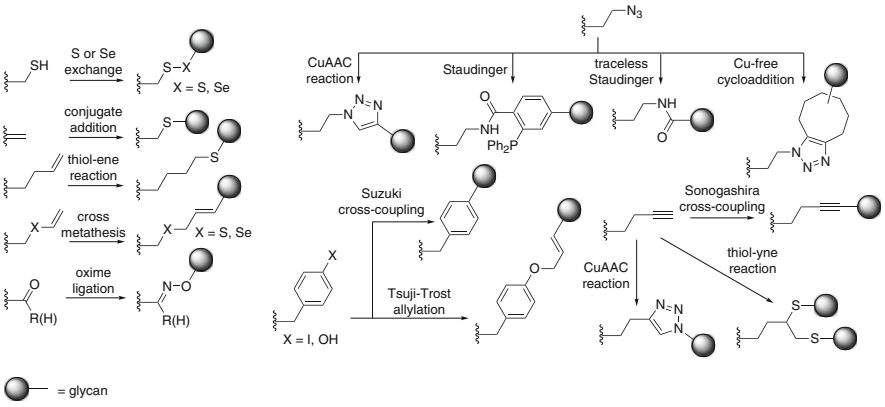


Fig. 3 Significant examples of selective protein-tagging techniques

Excellent orthogonality of different tags permits differentiation of the conjugation sites for multiple presentations of different antigens (van Kasteren et al. 2007).

If needed, the glycan pattern can be extended by sequential addition of further monosaccharide units using a chemoenzymatic approach based on endoglycosidases (Fernandez-Gonzalez et al. 2010).

3.3 Protein Glycan Coupling Technology

The discovery that some bacteria, such as *Campylobacter jejuni*, possess a *N*- or *O*-glycosylation machinery that can modify several proteins opened a new way towards selective glycoconjugation (Nothaft and Szymanski 2010). The key enzyme of this pathway was found to be an oligosaccharyltransferase (OST) named PglB, which demonstrated relaxed glycan specificity and the ability to transfer a number of polysaccharides to acceptor proteins containing the extended glycosylation sequence recognition pattern D/EYNXS/T (where X and Y are any amino acids except proline). When *E. coli* was genetically modified to express the general glycosylation locus, it was established, for the first time, that recombinant glycoproteins could be obtained (Wacker et al. 2002).

These findings were recently translated into a novel approach, named protein glycan coupling technology (PGCT), where selected glycans are coupled to target proteins using an *E. coli* recombinant system to produce an unlimited and readily purified supply of vast combinations of glycoconjugate vaccine candidates. Moreover, in a more advanced vision, both the glycan and protein syntheses could be triggered inside the *E. coli* host, avoiding the long multistep synthesis of the glycan and the need of manipulating pathogenic bacteria for protein isolation (Terra et al. 2012). To this end, the loci encoding the glycan and the target carrier protein (containing the appropriate consensus sequon; otherwise it can be directly engineered (Ihssen et al. 2010)) have to be cloned and expressed in *E. coli*, usually on a suitable plasmid. Finally, the coupling enzyme has to recognize the reducing end of the target glycan in order to transfer it to the protein (Fig. 4).

Although still in its infancy, PGCT is however a very promising technique. Striking examples concern glycoconjugate vaccine candidates obtained by conjugation of *Shigella dysenteriae* O antigen 1 to two different carrier proteins (Ihssen et al. 2010).

3.4 Peptides as T Cell Epitopes

In addition to the abovementioned problems, another important aspect should also be taken into account when designing glycoconjugate vaccines. Due to the presence of diverse T cell epitopes, carrier proteins are highly immunogenic and therefore elicit strong T cell responses, which can overcome that of the saccharide moiety (especially in the case of very short oligosaccharide fragments) causing hapten suppression (Herzenberg and Tokuhisa 1980). The use of a single peptide as a T cell

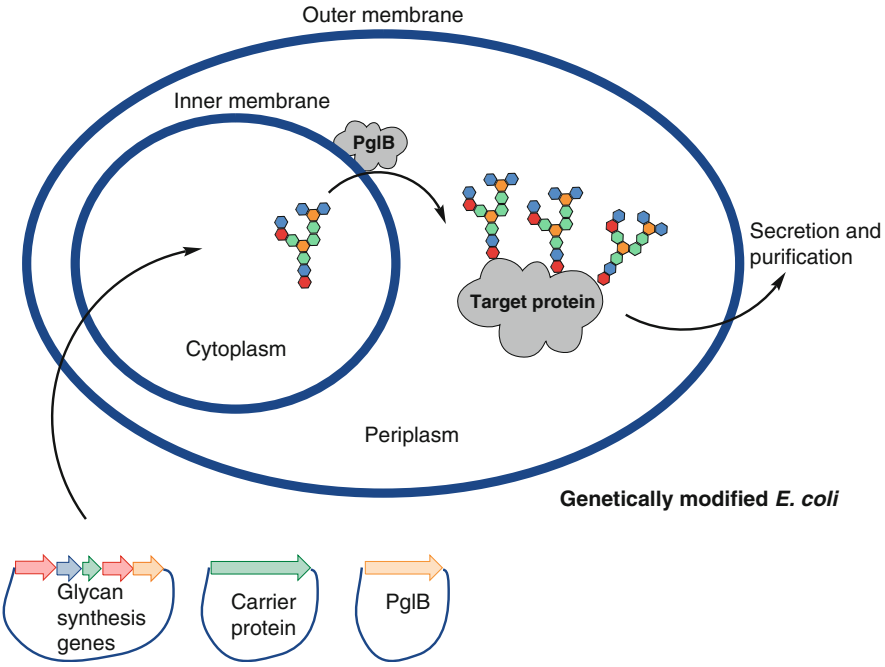
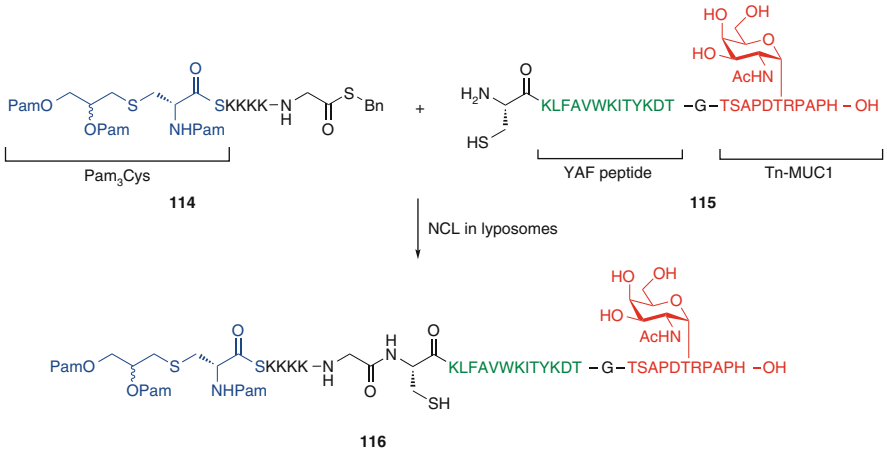


Fig. 4 Protein glycan coupling technology mechanism

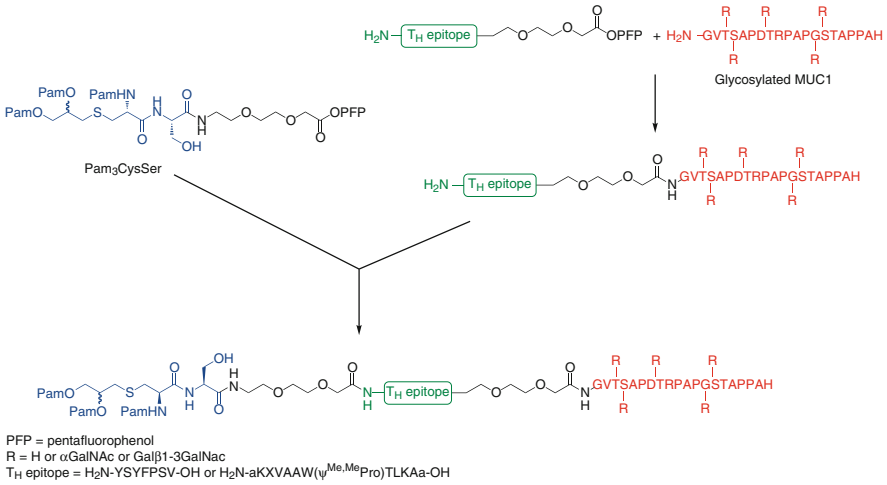
epitope should give rise to a more robust immune response, more specifically directed towards the carbohydrate antigen. To address this issue, over the last decade, a number of computer algorithms that map the locations of MHC-restricted T cell epitopes within proteins of various origins have been developed. As a result, it has been found that some sequence regions of protein antigens might sensitize CD4+ cells (Weber et al. 2009). This finding led to the identification of small and simple T_H peptide epitopes that are chemically defined, easily synthesized by SPPS, and able to generate effective helper T cell responses in humans. Among them, the Pan HLA DR-binding Epitope (PADRE), $OVA_{323–339}$ and YAF peptides, TT heptapeptide, and tridecapeptide from poliovirus are widely used (Panina-Bordignon et al. 1989; Diethelm-Okita et al. 1997, 2000).

Boons recently developed a tricomponent synthetic anticancer vaccine **116** incorporating the Tn antigen (αGalNAc) linked to YAF peptide and also containing the immunoadjuvant Pam_3Cys (Scheme 22) (Ingale et al. 2009).

Other two examples were reported by the Payne group (Wilkinson et al. 2011, 2012). Both are anticancer vaccines containing the Tn and TF (Galβ1-3GalNAc) antigens as carbohydrate epitopes, commonly overexpressed in cancer cells. In both vaccines the B cell epitope is linked to the T cell epitope, tetanus toxoid peptide (YSYFPSV) or PADRE, via an ethylene glycol spacer. The peptide *N*-terminus is connected via the same spacer to the Pam_3Cys moiety terminated with a serine residue. The three building blocks were assembled by means of a pentafluorophenyl



Scheme 22 Synthesis of tricomponent synthetic anticancer vaccine by NCL

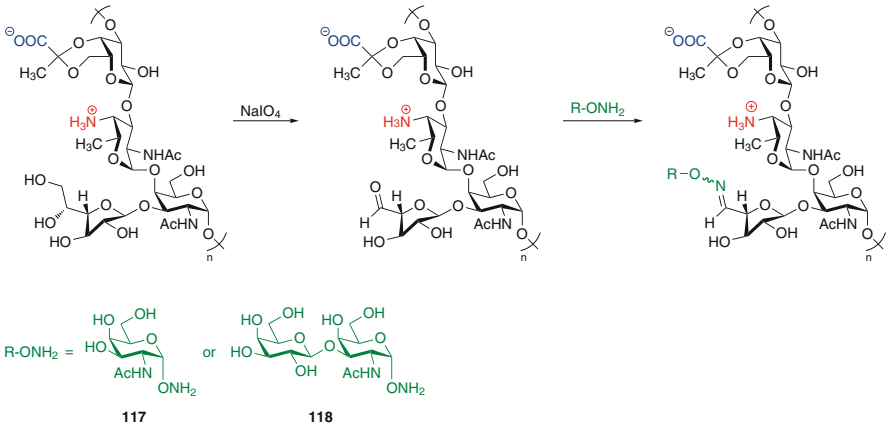


Scheme 23 Synthesis of the three-component anticancer vaccine candidates

ester-mediated condensation (Scheme 23). Mice immunization confirmed the strong adjuvant role of the $Pam_3CysSer$ and the superior immunogenicity of tricomponent vaccines compared to others lacking the T cell epitope.

3.5 Zwitterionic Polysaccharides

Zwitterionic polysaccharides (ZPS) are a class of structurally distinct bacterial polysaccharides that possess a zwitterionic charge motif distributed along the



Scheme 24 Synthesis of the TACA-PSA1 conjugates

chain, within the repeating unit. Typical examples are the Sp1 capsular polysaccharide from *S. pneumoniae* type 1 and capsular polysaccharides A1, A2, and B from *Bacteroides fragilis*. A number of recent reports described the ability of such compounds to behave like traditional T cell-dependent antigens, activating CD4+ T cells both in vitro and in vivo through the traditional MHC-dependent mechanism, without protein conjugation (Cobb and Kasper 2008; Surana and Kasper 2012; Avci et al. 2013; Bloem et al. 2013; Berti and Adamo 2013).

Due to their unique behavior, ZPSs are gaining attention as potential scaffolds for vaccine assembly, acting both as carrier and adjuvant. Andreana and coworkers recently reported the first two examples of entirely carbohydrate vaccine constructs containing the α-aminooxy derivatives of Tn and TF tumor-associated cancer antigens attached to PSA1 polysaccharide (**117** and **118**, Scheme 24) (De Silva et al. 2009, 2012; Bourgault et al. 2014). Immunization studies conducted on the Tn-PSA1 conjugate **117** showed the production of high titers of specific IgG antibodies, even in the absence of any external adjuvants.

4 Fully Synthetic Vaccines

The use of sugars as ligands for protein targeting (such as antibodies) is a challenging task because carbohydrate-protein interactions are typically weak, with dissociation constants in the mM range. In living cells, this issue is circumvented by multivalent receptor-ligand interactions, where multiple ligands interact simultaneously with multimeric receptors inducing an increase of the binding affinities of several orders of magnitude (multivalency effect). When this phenomenon is referred to carbohydrate-receptor interactions, it is commonly named "cluster glycoside effect" (Lundquist and Toone 2002).

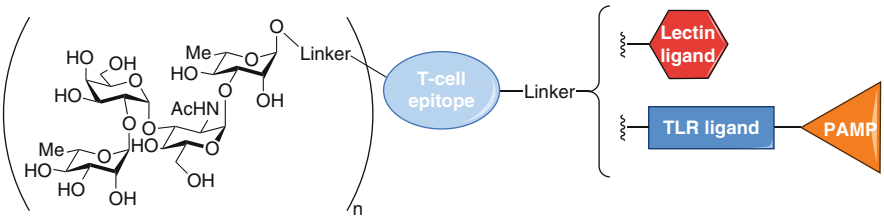


Fig. 5 Composition of fully synthetic vaccines

A major goal pursued by the vaccinology research is to reproduce the multivalency effect into fully synthetic systems using polyfunctional scaffolds capable of displaying multiple copies of carbohydrate antigens and that can be univocally characterized from a chemical point of view (mass and NMR spectroscopy). Under this point of view, the traditional glycoconjugate vaccines can be considered as an example of replication of the cluster glycoside effect, since they contain a large number of carbohydrate antigens exposed on the protein surface that are collectively much more immunogenic than the corresponding monovalent forms.

Fully synthetic vaccines are based on a modular architecture and incorporate into the same molecule different functional units: a synthetic glycan or a multivalent glycocluster, a synthetic T cell epitope, and an adjuvant, such as a ligand of TLR or C-type lectin receptor onto dendritic cell surface. Each of them is synthesized independently and then chemically conjugated in a convergent way, providing total control on the chemical structure (Fig. 5).

During the last years, a wide range of synthetic clustered glycosides has been designed to interfere in an array of biological processes. Among them, glycopeptides (Lee and Lee 1994; Krauss et al. 2007; Ohta et al. 2003; Pujol et al. 2011), glycodendrimers (Chabre and Roy 2010; Andre et al. 2001; Heidecke and Lindhorst 2007; Mintzer et al. 2012; Touaibia and Roy 2007; Turnbull and Stoddart 2002), glycopolymers (Kiessling et al. 2006; Bes et al. 2003; Otsuka et al. 2010; Ponader et al. 2012; Rieger et al. 2007), glyconanoparticles (Marradi et al. 2010; Wang et al. 2011), glycofullerenes (Cecioni et al. 2011; Nierengarten et al. 2010; Compain et al. 2010), glycocalixarenes (Dondoni and Marra 2010; Sansone et al. 2011; Baldini et al. 2007; Cecioni et al. 2009; Andre et al. 2011), and sugar-functionalized carbon nanotubes (Hong et al. 2010) and quantum dots (Kikkeri et al. 2010; Robinson et al. 2005; Yang et al. 2010) were shown to be efficient biomimetics of natural glycoclusters, often reaching low nM activities. This continuously growing topic is well covered by some recently published reviews (Bernardi et al. 2013; Galan et al. 2013; Marradi et al. 2013; Peri 2013; Shiao and Roy 2012; Blanco et al. 2013; Sansone and Casnati 2013). In the following sections, we will focus on systems with different architectures, specifically designed for vaccine developments.

4.1 Dendrimers

Dendrimers are repetitively branched molecules. The name comes from the Greek word δένδρον (dendron), which translates to "tree" (Astruc et al. 2010). Dendritic molecules are characterized by structural perfection: they are monodisperse and usually highly symmetric around the core, adopting a globular three-dimensional morphology. Dendritic molecules can be categorized into low-molecular weight and high-molecular weight species. The first class includes dendrimers and dendrons, while the latter comprises dendronized polymers, hyperbranched polymers, and polymer brushes. Dendrimers can be considered to have three major portions: a core, an inner shell, and an outer shell. In principle, a dendrimer can bear different functionalities in each of these portions to control properties such as solubility, thermal stability, and attachment of compounds for specific applications. Synthetic processes can also precisely control the size and number of branches on the dendrimer. Such molecules can be assembled following two different approaches. In the divergent method, the dendrimer is assembled from a multifunctional core, which is extended outward by a series of reactions. However, each step must be driven to completion in order to avoid mistakes in the dendrimer, which can cause trailing generations (some branches are shorter than others, Fig. 6a). On the other hand, in the convergent methods, dendrimers are built from

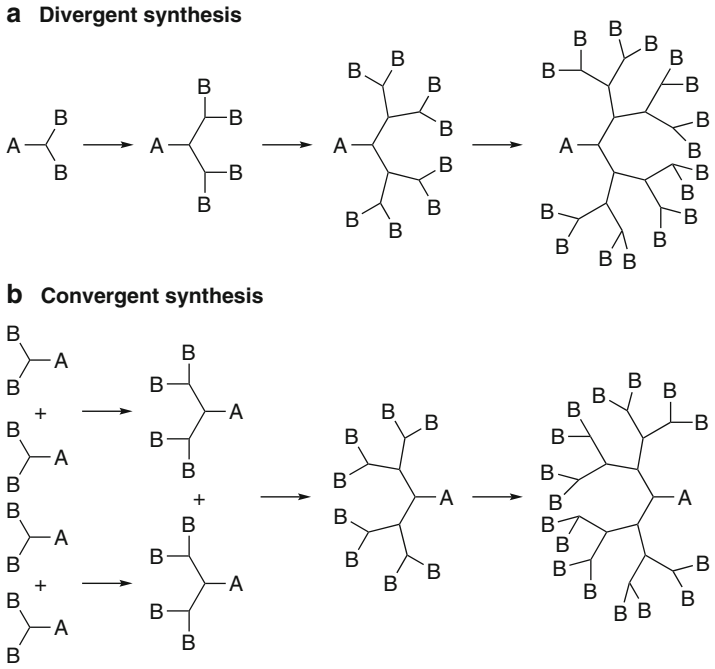
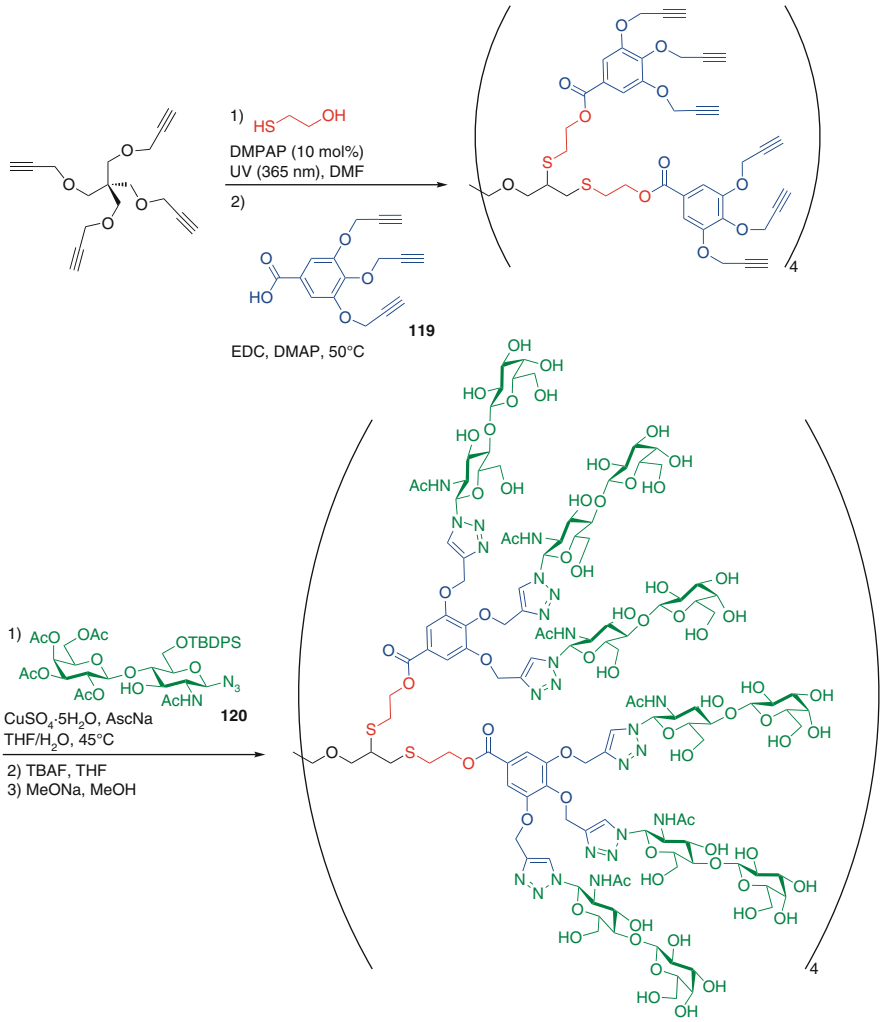


Fig. 6 Outline of convergent and divergent syntheses of dendrimers

small molecules that end up at the surface of the sphere, and reactions proceed inward to the center core (Fig. 6b). This method makes it much easier to remove impurities and shorter branches. However dendrimers made this way cannot be as large as those made by divergent methods because of crowding due to steric effects around the core.

The possibility to incorporate a huge variety of building blocks containing orthogonal functional groups at each layer allows for the construction of original dendritic architectures that differ in terms of constitution, valency, and peripheral functionalities, exemplified by Roy et al. in a recent paper (Sharma et al. 2014). They proposed a new family of model glycoclusters and glycodendrimers, decorated with *N*-acetyllactosamine (LacNAc) termini, that can be assembled through a divergent original "onion peel strategy." High chemo- and regioselective photolytic thiol-ene coupling (TEC) and thiol-yne coupling (TYC) were used to rapidly and sequentially grow the dendritic architectures and to install a high degree of branching (Dondoni and Marra 2012; Gingras et al. 2013). Further branching (up to 36 valences) was added by EDC-mediated esterifications (or amidations) of polypropargylated dendrons (such as **119**), to which the LacNAc azide derivative **120** was attached by "click chemistry" (Scheme 25). As far as the biological relevance is concerned, LacNAc represents a part of the Lewis^x and Lewis^y tumor-associated carbohydrate antigens (Heimburg-Molinaro et al. 2011), and it is known to possess strong binding affinities towards galectins, a cancer-associated family of proteins (Zhou 2003; Liu and Rabinovich 2005; Salatino et al. 2008; Rabinovich and Toscano 2009). Therefore, a protein binding study was carried out with a model leguminous lectin from *Erythrina cristagalli* agglutinin (ECA). Interestingly, the glycodendrimers exhibited high relative potencies, with an up to 216-fold enhancement in global affinity with respect to the reference monomer.

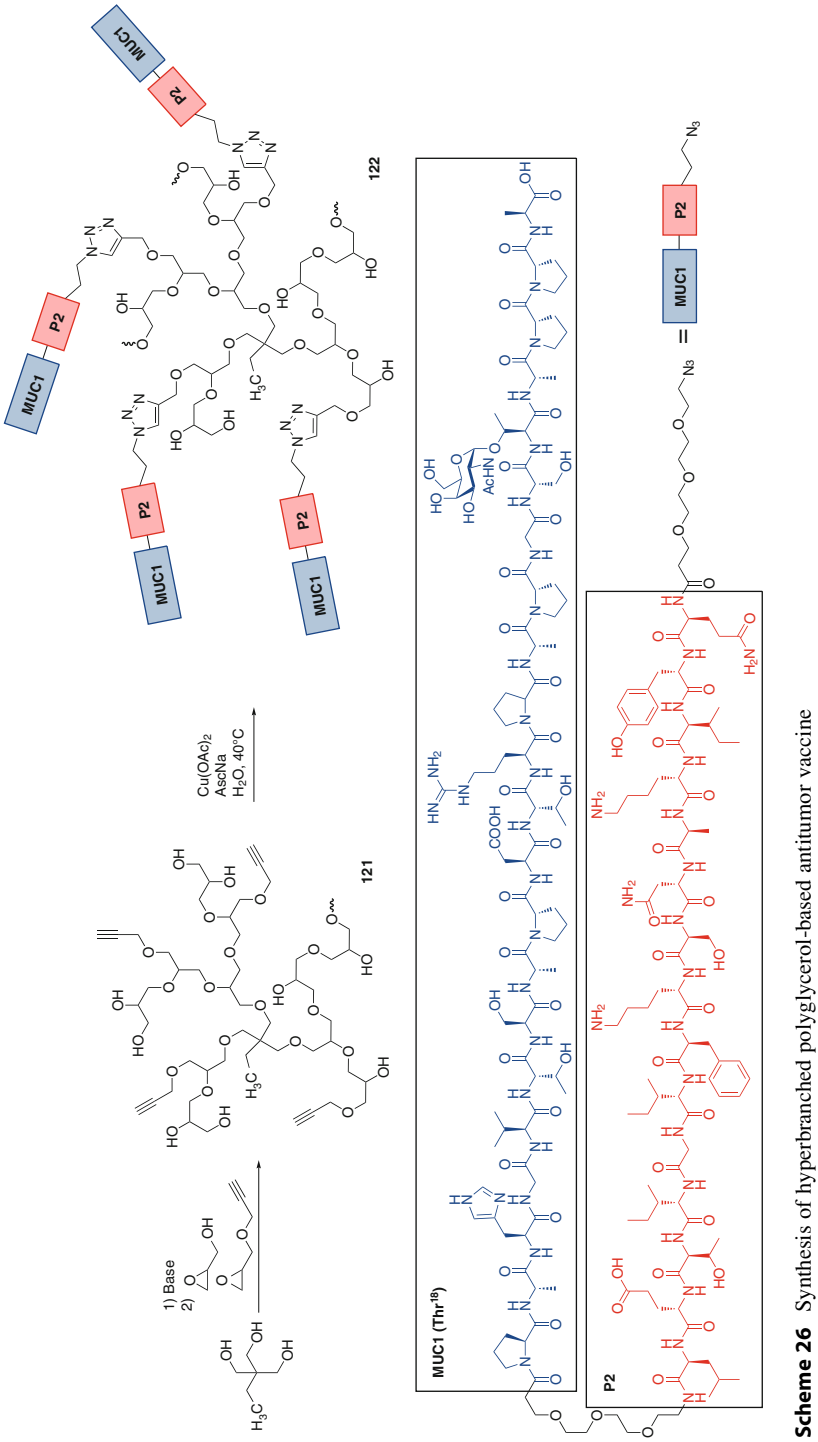
An example of the use of high-molecular weight species was reported by Kunz (Glaffig et al. 2014). The authors employed a water-soluble and readily available polymer, which is the multi-alkyne-functionalized hyperbranched polyglycerol (hbPG) **121**, obtained in a one-step copolymerization of glycidol and its propargyl ether. The scaffold presents a globular shape and a flexible dendrimer-like structure, which allows an optimal antigen presentation to the immune system. Moreover, polyglycerols are suitable as inert carriers for biomedical applications, as they are considered biocompatible and non-immunogenic. A tumor-associated MUC1 glycopeptide combined with the immunostimulating T cell epitope P2 from tetanus toxoid was coupled to the terminal alkyne groups by "click chemistry" (Scheme 26). MUC1 is a tumor-associated glycoprotein member of the mucins family, densely glycosylated high molecular weight proteins implicated in a variety of epithelial cancers. Typical TACAs of mucins include Tn, sTn (NeuAcα2-6GalNAc), and TF, α-*O*-glycosylated to serine/threonine residues in the peptide backbone (Heimburg-Molinaro et al. 2011; Pashov et al. 2011). The resulting construct **122** was shown to induce a strong immune response in mice, raising IgG antibodies able to recognize human breast cancer cells.



Scheme 25 Representative synthesis of LacNAc decorated "onion peel" dendritic structures via an accelerated divergent strategy

4.2 (Cyclo)peptide Scaffolds

Membrane-bound tumor-associated glycoproteins like MUC1 are known to be highly overexpressed on tumor cells. Therefore, they represent an attractive target for anticancer vaccine formulation (Pashov et al. 2011; Beatson et al. 2010). Over the years, considerable efforts have been devoted to the synthesis of glycopeptide assemblies that include either a carrier protein or T cell epitopes in order to induce stronger immunological responses.



Scheme 26 Synthesis of hyperbranched polyglycerol-based antitumor vaccine

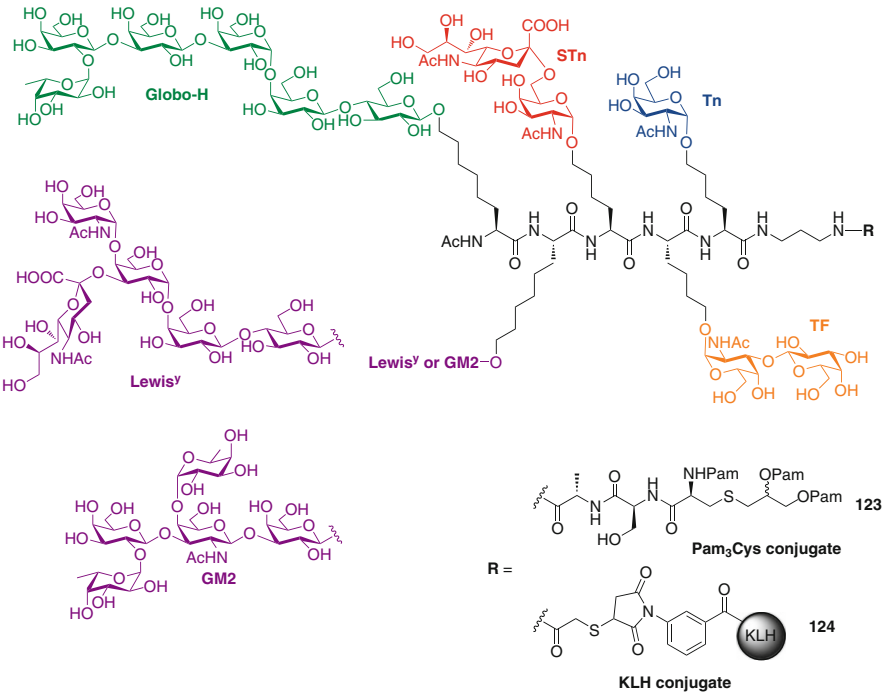


Fig. 7 Structure of the pentavalent vaccine candidates **123** and **124**

The major contribution to this field came from Danishefsky and coworkers. They reported the synthesis and immunological evaluation of several multivalent, fully synthetic, glycopeptide-based antitumor vaccines (Ragupathi et al. 2006). The authors speculated that the combination of several carbohydrate antigens closely associated with a particular cancer type could induce a more robust immune response, decreasing the percentage of tumor cells that can elude an immunological response. The constructs were assembled following a unimolecular multivalent approach, where several distinct TACAs are displayed on a single peptide backbone (Kim and Varki 1997). A sequential assembly of the glycopeptide in order of carbohydrate size, from the smallest (Tn) to the largest and most complex one (Globo H), was performed. The functionalized carbohydrate antigens were prepared either by manipulation of glycal epoxides or sulfonylaziridines, whereas the spacers were built through Horner-Emmons reactions followed by asymmetric hydrogenation or by cross-metathesis (Fig. 7). The results coming from mice immunization point out that the immunological properties of the individual antigens are preserved in the multivalent structures. However, it seems also that the immunogenicity is influenced by the position of the antigen on the peptide backbone. Overall, the antibodies showed significant reactivity with all three cell lines tested (MCF-7, LSC, DU-145). These vaccine candidates are now at the stage of evaluation in clinical trials.

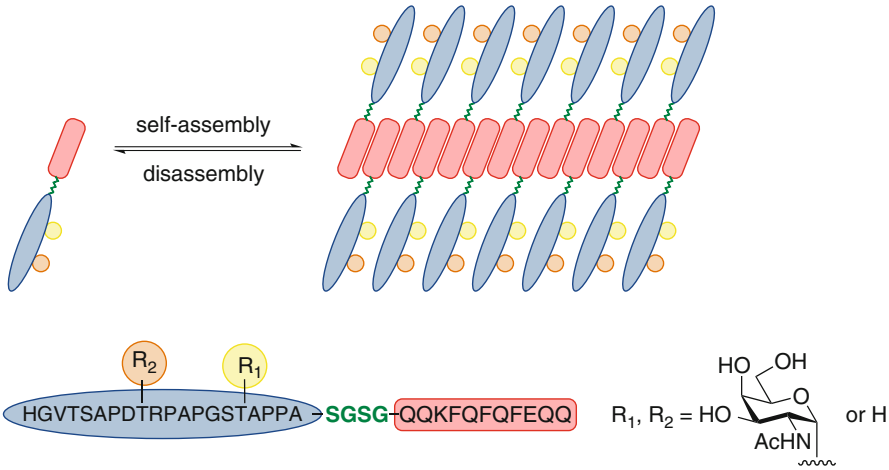
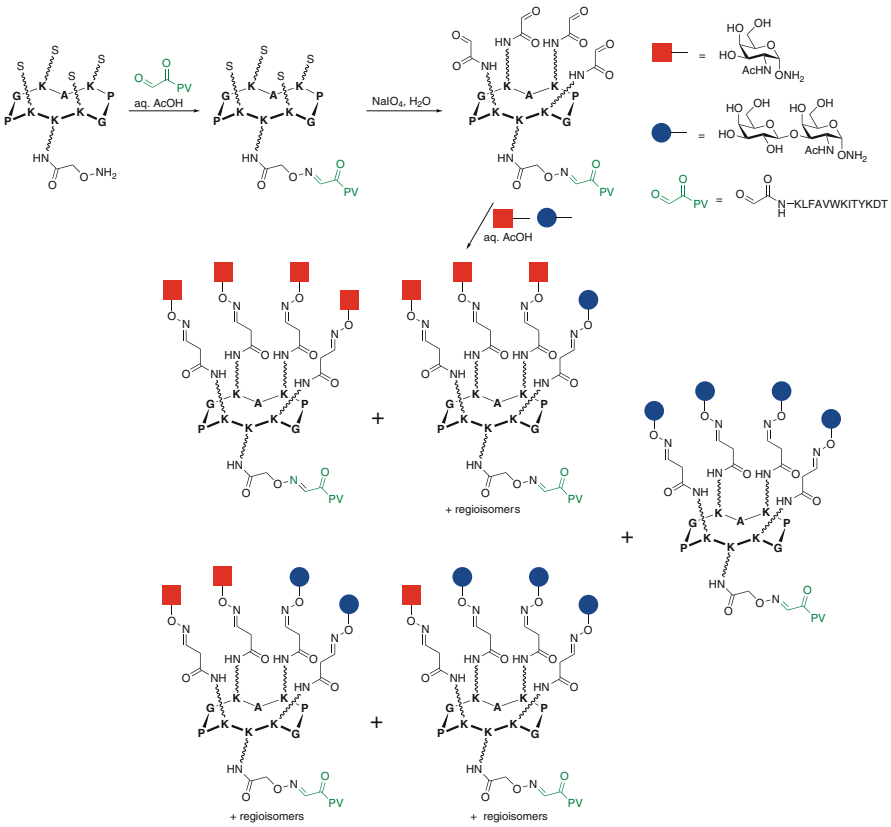


Fig. 8 Structure of self-adjuvanting, self-assembling vaccine candidates with different MUC1 glycosylation pattern

Another exciting approach was reported by the Li team (Huang et al. 2012). They synthesized self-adjuvanting vaccine candidates containing Tn-glycosylated full-length MUC1 VNTR (variable number of tandem repeats) domains (Pashov et al. 2011) conjugated to a self-assembled peptide sequence (Q11 domain, Fig. 8) (Jung et al. 2009). The Q11 domain could aggregate in solution, under mild conditions, into fibers over 200 nm long, thus displaying multivalent B cell epitopes on the fiber surface, and it served both as adjuvant and vaccine carrier (Rudra et al. 2010). Immunological studies revealed the production of high titers of antibodies capable of recognizing human breast tumor cells.

Conversely, the major advantage in the use of small cyclopeptides over linear ones is certainly their locked and rigid conformations that allows for a well-defined spatial orientation of functional groups, thus preventing hindrances between the assembled elements. Up to now, the most widely used architecture is Mutter's Regioselectively Addressable Functionalized Templates (RAFTs), extensively explored by Dumy and Renaudet (Dumy et al. 1995; Renaudet and Dumy 2003; Grigalevicius et al. 2005; Renaudet and Dumy 2008). RAFTs are cyclic decapeptides constrained in an antiparallel β-sheet by two L-proline-glycine β-turns and stabilized by intramolecular hydrogen bonds in solution. They display two functional faces that can be independently functionalized.

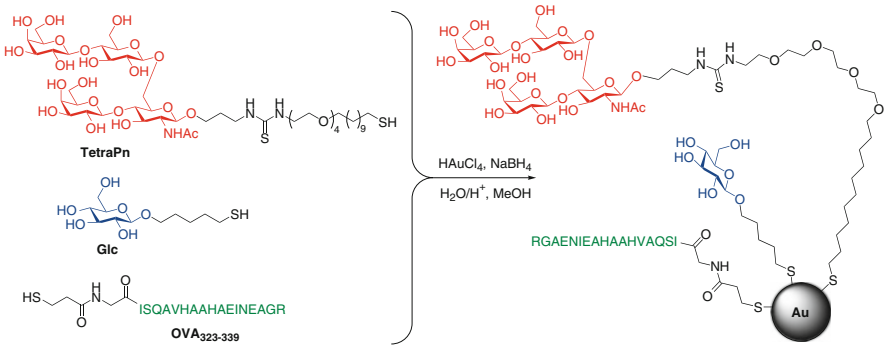
One of the most recent applications of RAFTs was the combination of various TACAs into a modular synthetic vaccine candidate (Fiore et al. 2013). One face of the scaffold was decorated with a mixture of the Tn and the TF carbohydrate antigens, by means of a randomized chemoselective ligation of aminooxy carbohydrates to glyoxoaldehydes anchoring sites under mild conditions. The bottom side was provided with an immunostimulating T cell peptide sequence derived from polio virus type 1 (PV, Scheme 27).



Scheme 27 Chemoselective synthesis of RAFT-based anticancer vaccine candidates

4.3 Gold Nanoparticles

In 2001 the Penadès group reported for the first time the use of gold nanoparticles (GNPs) as scaffolds for the multivalent presentation of biologically relevant oligosaccharides through self-assembled monolayers (SAMs) (de la Fuente et al. 2001). Since then, comprehensive studies have been carried out on the influence of loading, presentation of the ligands (i.e., type and length of the linker), and core size on the interaction with the specific receptor (Marradi et al. 2013; Fallarini et al. 2013; Reynolds et al. 2013; de la Fuente and Penades 2006; Manea et al. 2008). In 2012 the same group reported the first example of a fully synthetic carbohydrate vaccine based on GNPs (Safari et al. 2012). By using the "direct" synthesis, they were able to prepare gold nanoparticles (2 nm average diameter) coated with thiol-ended linkers bearing, respectively, a synthetic tetrasaccharide (the single repeating unit of the *Streptococcus pneumoniae* type 14 capsular polysaccharide), the T cell epitope OVA peptide, and β-D-glucopyranoside in variable



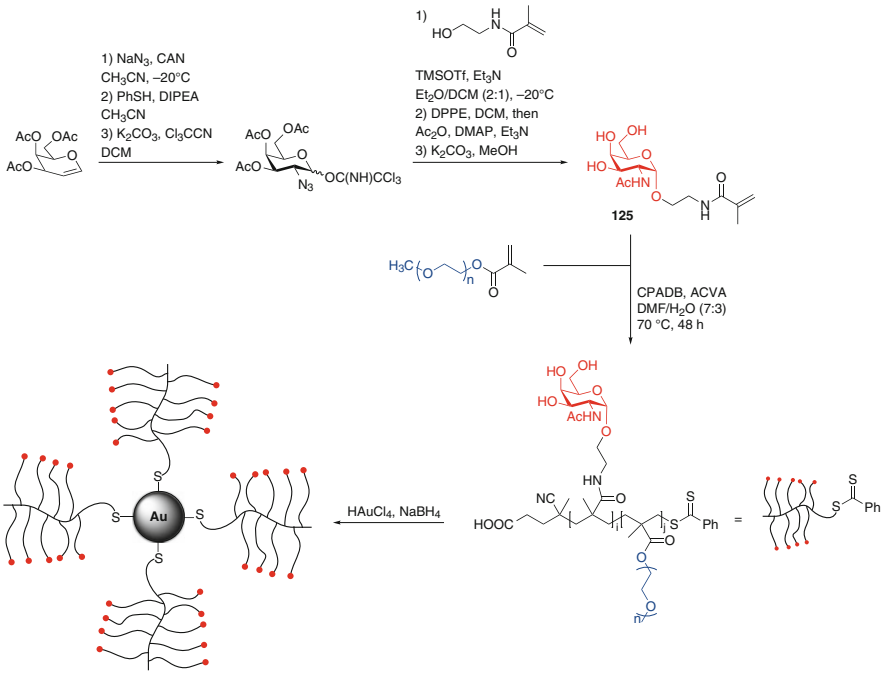
Scheme 28 "Direct" synthesis of sugar-decorated GNPs

ratio (Scheme 28). The authors found out that the molar ratio between the three components and the simultaneous presence of the OVA peptide and the tetrasaccharide antigen were critical for the induction of high titers of specific and functional IgG antibodies.

Another interesting example was reported by Davis group (Parry et al. 2013). The aim of the work was to closely mimic, on gold nanoparticles surface, the overexpression of truncated core 1 mucin-type glycans such as the Tn antigen occurring on the surface of cancer cells. To this end, a glycopolymer displaying multiple copies of the Tn antigen was used to coat and stabilize the surface of GNPs. First of all, a Tn-antigen glycan bearing a polymerizable linker **125** was prepared and then converted by Reversible Addition Fragmentation chain Transfer polymerization technique (RAFT) into various PEG block copolymers with different glycan densities and chain lengths (Scheme 29). Sodium borohydride was finally used to reduce the dithioester end groups of the polymers to thiol and to simultaneously produce GNPs in situ. Immunological studies showed a strong and long-lasting production of antibodies that are selective for the Tn antigen and cross-reactive towards mucin proteins displaying Tn.

4.4 Calixarenes

Calixarenes are a family of cyclic oligomers obtained by the condensation of phenols and formaldehyde (Sansone and Casnati 2013). The even-numbered macrocycles ($n = 4, 6, 8$) are easily obtained using cheap reagents through well-consolidated synthetic procedures and are also commercially available. The odd-numbered homologues ($n = 5, 7, 9$), although known in the literature, are rarely used, as they require more complicated syntheses. The considerable success met by calixarenes, especially calix[4]arenes, as scaffolds for the construction of multivalent ligands can be ascribed to a combination of different factors.



Scheme 29 Preparation of glycopolymer-stabilized GNPs

Among them, the ease to modulate valency, size, structure, and topology of the binding group presentation, together with the possibility to keep them conformationally mobile to exploit an induced-fit binding, or, in contrast, to lock them in a more preorganized specific structure. In fact, while the tetramethoxy and tetraethoxy derivatives are conformationally mobile, the selective introduction of larger groups can lock the macrocycle into one of the four possible conformations. These structures were named by Gutsche as cone, partial cone, 1,3-alternate, and 1,2-alternate (Fig. 9) and are characterized by a remarkably different spatial orientation of the phenolic units.

Functionalization of the calixarene scaffold can be addressed both at the phenolic oxygen atoms (lower rim) and at the para position (with respect to the OH group) on the aromatic nuclei (upper rim), and chains of variable length and containing most of the functional groups used for the glycoconjugation can be introduced.

An interesting example of such an approach is represented by an anticancer vaccine candidate **126** (Geraci et al. 2008). A cone calix[4]arene bearing four Cys-linked tumor-associated Tn antigens at the upper rim was functionalized at the opposite lower rim with the immunoadjuvant Pam_3Cys moiety (Scheme 30). When mice were immunized with the tetravalent calixarene, a fourfold increase in antibody production was observed with respect to a monovalent analogue **127** exposing the same immunoadjuvant but with a single antigen.

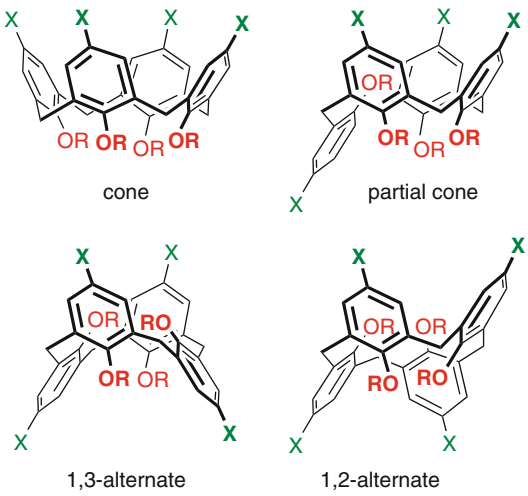
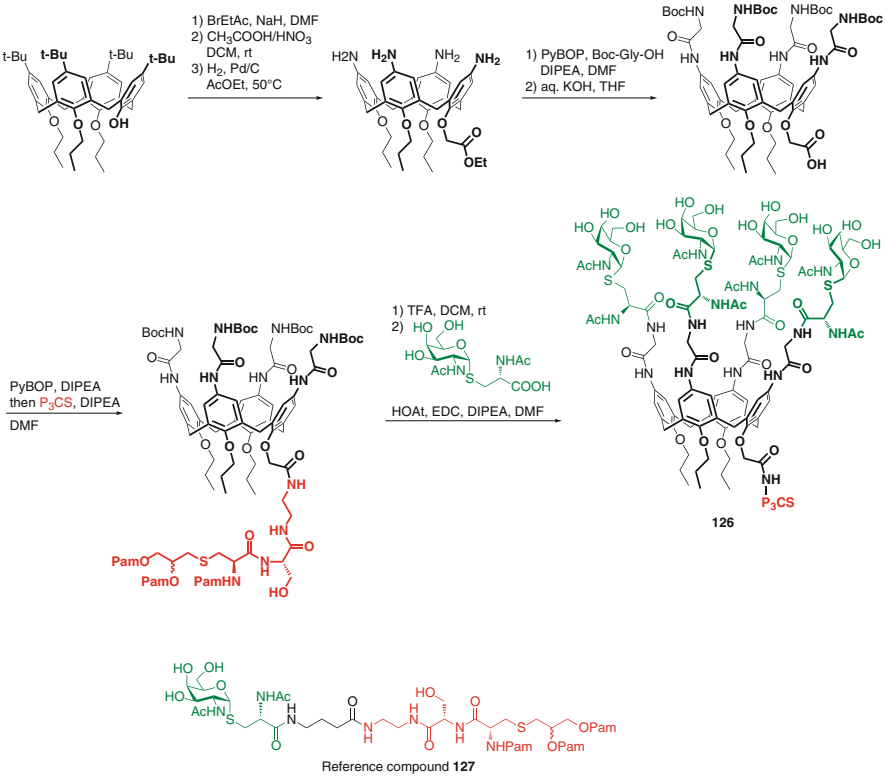


Fig. 9 Stable conformations of calix[4]arenes



Scheme 30 Synthesis of Tetra-*S*-TnAg-Gly-CX-P_3CS

5 Conclusions

Infectious diseases have still strong impact on public health, both in industrialized and developing countries, due to their significant health-related costs for clinical treatment. According to the WHO, the most cost-effective strategy for controlling infections caused by pathogenic microorganisms is the vaccination practice, which should confer long-term protective immunity on the population. Carbohydrate-based vaccines, specifically designed to target pathogen-specific cell surface saccharide structures, showed enormous potential benefits for human health. In particular, it has been ascertained that optimal protection of the population, including newborns and young children, elderly, and immunocompromised patients, may be achieved with the administration of glycoconjugate vaccines that convert the T cell-independent immune response typical of plain polysaccharides into a T cell-dependent response, thereby establishing immunological memory.

However, the manufacture of glycoconjugate vaccines based on carbohydrate antigens isolated from their natural sources is a great challenge that may lead to heterogeneous compositions and batch-to-batch variability, thus raising severe issues of quality assurance and potential difficulties for their approval by the licensing authority.

Over the recent years, the synthesis of complex glycans has made significant progress and is becoming a relevant way to provide oligosaccharide antigens with well-defined chemical structure and devoid of bacterial contaminations which could derive from purification of biological materials. This could be a crucial feature to improve batch-to-batch consistency in vaccine manufacturing and to confer a better safety profile. In addition, synthetic oligosaccharides can be valuable tools for epitope recognition, i.e., to identify within the polysaccharide antigen the minimal structural requirements needed to elicit a protective immune response.

However, glycoconjugate constructs containing synthetic oligosaccharides may encounter a problem of low immunogenicity, as the multi-epitope protein carrier could overlook and weaken the immune response against the saccharide antigens. Some of the most innovative approaches that emerged during the last years in the field of vaccinology are therefore based on the concept that a fully competent immune response targeting poorly immunogenic synthetic carbohydrate antigens may be achieved by including in the vaccine setting only those elements strictly required to elicit a robust carbohydrate-specific immune response, devoid of any unnecessary component. Accordingly, there is a growing interest towards new kind of vaccines where the protein carrier is replaced by a synthetic short peptide able to act as a T cell epitope and to enhance the immunogenicity of the carbohydrate antigen. These new constructs include also fully synthetic vaccines exploiting the multivalency effect, where a significant enhancement of the immunogenicity is achieved by display of multiple copies of the oligosaccharide antigen on a suitable polyfunctional scaffold. A variety of scaffolds with different molecular architectures and chemical properties have been developed and applied during the last years, giving rise to new and very promising vaccine candidates that could open a new front line in vaccinology.

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Plant Polysaccharides and Their Effects on Cell Adhesion

69

Xianxiang Xu

Contents

Abstract

Cell adhesion mediated by cell adhesion molecules (CAMs) constitutes essential life phenomenon. In inflammation, immunity, infection, tumor, cardiovascular injury, and wound healing, cell adhesion comes into being the basic physiological and pathological process. Intervening with cell adhesion has been the important therapeutic and prophylactic strategies for diseases. Accumulated evidence has indicated that plant polysaccharides especially those exacted from Chinese traditional and herbal medicine (most of plant medicine) displayed various pharmacological effects such as anti-inflammation, antitumor, anti-infection, immunomodulation, cardiovascular protective effects, and so on.

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Focusing on cell adhesion, further research is expected to reveal the therapeutic mechanism of plant polysaccharides in disease processes.

Keywords
Polysaccharides • Plant medicine • Cell adhesion • Cell adhesion molecule • Inflammation • Immunity • Tumor • Infection • Cardiovascular injury • Wound healing

1 Introduction

Carbohydrates make up important components of biological organisms. They not only provide carbon source and energy for cells but also are a kind of important informational molecules in addition to proteins and nucleic acids. Most glycoproteins are made of N- or O-linked complex sugar residues. Glycosylated proteins were mainly localized extracellularly, including the cell surface and extracellular matrix (ECM) (Bektas and Rubenstein 2011). As for cell adhesion molecules (CAMs) which are glycoproteins (a few glycolipids), carbohydrate chains play key roles in CAM recognition. CAMs have been found to facilitate cell–cell interactions or interactions between cells and ECM. These interactions not only stick cells together in multicellular organisms but also enable CAMs to modulate intracellular signal transduction. Consequently, cell adhesion and signaling by CAMs are involved in a variety of biological processes such as cell proliferation, gene expression, differentiation, apoptosis, and migration. Not surprisingly, CAMs are therefore also implicated in numerous diseases.

Plant medicine can intervene with cell adhesion and regulate CAMs, with different pharmacological effects and modest risk (Spelman et al. 2011). As a kind of active ingredient in plant medicine especially Chinese herbal medicine, polysaccharides have wide sources and low toxicity. Plant polysaccharides exhibit various activities including anti-inflammation, antitumor, immune regulation, anti-infection, cardiovascular protection, etc. Some plant polysaccharides have been developed as drugs applied in clinic, such as Lentinan, *Astragalus* polysaccharide, *Polyporus* polysaccharide, and *Coriolus versicolor* polysaccharide. Polysaccharides are the important material basis of herbal medicine, while their intervention with cell adhesion may be an important function basis of their bioactivities (Figs. 1 and 2).

2 What Is Cell Adhesion?

Cell adhesion is the binding of a cell to another cell, ECM, or a specific surface, which is essential for cell growth and survival and also its communication with other cells. The ability of cells to interact with each other and their surroundings in a coordinated manner depends on multiple adhesive interactions between neighboring cells and their extracellular environment. The process of cell adhesion involves

Fig. 1 *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao

Fig. 2 *Radix astragali*, a vital qi-reinforcing herb medicine in China

a range of biological events such as three-dimensional reorganization of the cytoskeleton, biochemical reactions in the cell, and changes in molecules on the surface of the cell. The formation and remodeling of cell contacts are basic to cell and tissue structure (Braga and Harwood 2001). As an example, consider the epithelial cells that line the inner and outer surfaces of the human body, including the skin, intestines, airway, and reproductive tract. These cells provide a dramatic example of the different kinds of cell-to-cell junctions. The side surfaces of epithelial cells are tightly linked to those of neighboring cells, forming a sheet that acts as a barrier. Metazoans clearly need cell adhesion to hold cells together, but adhesion does much more than that. Adhesion receptors make transmembrane connections, linking ECM and adjacent cells to the intracellular cytoskeleton, and they also serve as signal transducers. All cells rely on cell signaling to detect and respond to cues in their environment. This process not only promotes the proper functioning of individual cells, but it also allows communication and coordination among groups of cells. Because of cell signaling, tissues have the ability to carry out tasks. Many physiological processes including cell activation, migration, proliferation, and differentiation require direct contact between cells or ECM. Cell adhesion participates in normal biological processes and disease states such as immune processes, leukocyte recirculation, wound healing, infection, tumor, thrombosis, and neurodegenerative disorders.

3 Cell Adhesion Molecules

Most cell adhesion events rely on members of CAMs. The term "CAM" can be traced back to the mid-1970s where it was used to describe molecules forming bonds between cells. According to their structural and functional features, CAMs have been classified into at least four major families: selectins E, P, and L; integrins; cadherins; and members of the immunoglobulin superfamily. More recently, several proteins with enzymatic activity have also been demonstrated to mediate cell adhesion, for instance, receptor protein tyrosine phosphatases, and an increasing number of CAMs have been found not to belong to any of the four protein families mentioned above, for example, neurexins/neuroligins and members of the family of proteins with extracellular leucine-rich repeats.

In addition to targeting cell adhesion to specific extracellular matrix proteins and ligands on adjacent cells, CAMs also act as receptors that interact via their cytoplasmic domain with numerous signaling molecules such as protein kinases and phosphatases. Unlike other adhesion molecules which bind to other proteins, the selectins interact with carbohydrate ligands. Recent work has demonstrated that adhesion molecules play a role in signaling as well. For example, integrins are cell adhesion molecules that have both direct and indirect roles in signaling (Arnaout et al. 2007). CAMs are key mediators in different disease states such as inflammatory disorders, cancer, cardiovascular diseases, autoimmune diseases (e.g., rheumatoid arthritis and type 1 diabetes), and even in the cold (Krieglstein and Granger 2001). In addition, CAMs have been implicated in pathogenic (i.e., bacteria and

virus) infections. The various isoforms of neural cell adhesion molecules (NCAM) play an important role in Alzheimer's disease (AD), which is characterized by a progressive loss of neurons and disturbances in neuronal plasticity and synaptogenesis. Interestingly, senile plaques containing accumulations of amyloid also contain NCAM and other adhesion molecules and may be able to stimulate neuritogenesis in the same microenvironment as degeneration. β-Amyloid may act via the NCAM–FAK/Fyn signal transduction pathway, which is important in apoptosis and inappropriately inducing neuronal death. β-Amyloid has also been shown to disrupt the biosynthesis of HNK-1, a glycoepitope conjugated to NCAM which modulates synaptic plasticity and memory consolidation (Thomas et al. 2005).

4 Cell Adhesion in Disease State

Considering the complicated interactions of blood cells and endothelium involved in many disease processes, abnormality of cell adhesion can lead to both acute and chronic disease states such as inflammation, thrombosis, angiogenesis, atherosclerosis, infection, and cancer metastasis. Within the vascular system, cell adhesion is important for the correct formation, networking, and remodeling of vessels. The semipermeable barrier property of microvascular endothelium is maintained by a layer of endothelial cells. On stimulation, endothelial cells express adhesion molecules that serve as keys that selectively open gateways to platelets, leukocytes, and sickle erythrocytes. Although adhesive junctions in vascular endothelial cells account for the integrity of the vessel wall, they are not to be considered as static molecular structures that function as intercellular glue (Liebner et al. 2006). The adhesive complexes at the endothelium not only provide an anchor point for blood cells to adhere but also transmit forces and biochemical signals (Wu 2005). A common theme in the processes is the need for rapid integrin activation, often initiated by binding of ligands to their cognate G protein-coupled receptors, followed by adhesion strengthening associated with integrin redistribution and outside-in signaling (Fig. 3).

4.1 Platelet Adhesion

Platelet adhesive properties play a well-defined role in hemostasis and thrombosis but also lead to other pathophysiological processes, including inflammation, immune-mediated responses to microbial and viral pathogens, and metastasis. Platelet adhesion and activation is a multistep process involving multiple platelet receptor–ligand interactions. Upon vessel wall injury, circulating platelets are rapidly decelerated by transient interactions between the glycoprotein (GP) Ib-V-IX complex and von Willebrand factor (VWF) bound to collagen. This interaction retains platelets close to the vessel wall and facilitates the contact between GPVI and collagen. The GPVI–collagen interaction induces intracellular

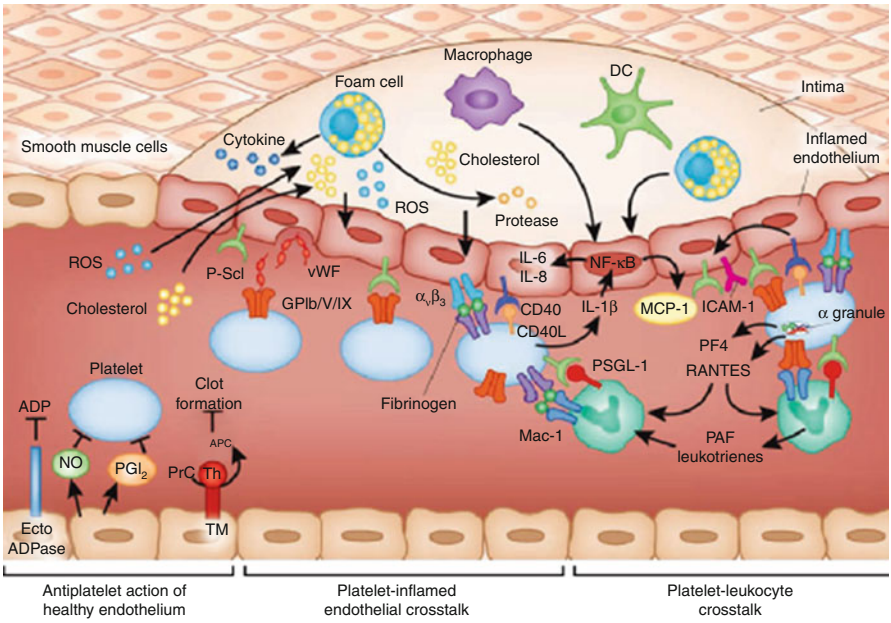

Fig. 3 The complicated interactions of blood cells and endothelium (Jackson 2011)

signaling leading to cellular activation and subsequent synthesis and release of secondary platelet agonists. Together, these events promote a shift of β_1 and β_3 integrins from a low to a high affinity state for their ligands through "inside-out" signaling allowing firm platelet adhesion and aggregation, further contributing to platelet activation through G protein-coupled receptors, inducing complex cellular responses such as release of granule contents and coagulant activity (Nieswandt et al. 2011).

4.2 Leukocyte Adhesion

Leukocytes are an integral component of the host defense system. However, in some disease states, their action may instead be deleterious to the host, and they may cause a significant amount of tissue damage. In inflammatory diseases, such as rheumatoid arthritis, leukocytes cause tissue damage without an apparent initial insult. In order for leukocytes to cause vascular or tissue damage, they must first adhere to the endothelium and then exit the bloodstream to gain access to extravascular tissue. Leukocyte emigration involves adhesion molecules both on the leukocyte and on the endothelial cell surface. Endothelial cells regulate mucosal immune homeostasis, acting as "gatekeepers," controlling leukocyte accumulation in the interstitial compartment. Leukocytes are recruited to the inflamed tissue by sequential adhesive interactions between leukocytes and the endothelium.

Table 1 Leukocyte–endothelial cell adhesion molecules

Adhesion molecule	Primary expression	Major ligands
P-selectin	Endothelial cells, platelets	PSGL-1
E-selectin	Endothelial cells	PSGL-1, ESL-1
L-selectin	Polymorphonuclear leukocytes, monocytes, majority of lymphocytes	CD34, GLYCAM-1
ICAM-1	Endothelial cells, lymphocytes, fibroblasts, other	LFA-1, Mac-1
ICAM-2	Endothelial cells, lymphocytes	LFA-1
ICAM-3	Polymorphonuclear leukocytes, monocytes, lymphocytes	LFA-1, CD11d/CD18
VCAM-1	Endothelial cells	VLA-4
MAdCAM-1	Endothelial cells	α4β7
PECAM-1	Endothelial cells, platelets, polymorphonuclear leukocytes, lymphocytes	PECAM-1
LFA-1	Polymorphonuclear leukocytes, monocytes, lymphocytes, other	ICAM-1, ICAM-2, ICAM-3
Mac-1	Polymorphonuclear leukocytes, monocytes, other	ICAM-1, Factor X, iC3b, fibrinogen
p150/95	Monocytes, polymorphonuclear leukocytes, dendritic cells	ICAM-1, iC3b, fibrinogen
CD11d	Polymorphonuclear leukocytes, lymphocytes, monocytes, specialized macrophages	ICAM-3, VCAM-1
VLA-4	Lymphocytes, monocytes	VCAM-1, fibronectin
VAP-1	Endothelial cells	Unknown
CD44	Lymphocytes, monocytes	Hyaluronan

Under certain conditions they can contribute to host destruction by participation in both acute and chronic immune/inflammatory responses. Leukocyte–endothelial cell adhesion molecules are essential mediators of both immune and inflammatory responses (Table 1) (Bullard 2002).

Much progress has been made in delineating the role of leukocyte–endothelial cell adhesion in the pathogenesis of cardiovascular disease. Adhesive interactions are involved in the pathogenesis of cardiovascular disease, regulating thrombus formation, facilitating leukocyte infiltration, mediating the migration and proliferation of smooth muscle cells, and enabling the deposition of fibrotic tissue. The magnitude of leukocyte–endothelial cell adhesion is determined by an array of factors including: the expression of adhesion molecules on the surfaces of leukocytes and endothelial cells, the inflammatory mediators which are secreted by activated leukocytes and endothelial cells, and the homodynamic forces resident within the blood vessels. They form a complex communication network (Eppihimer 1998). The multistep process of leukocyte recruitment illustrates how the coordinated and regulated expression of structurally and functionally distinct families of CAM can elicit a highly reproducible vascular response to inflammation. Selectins mediate the initial, low-affinity leukocyte–endothelial cell interaction that is

manifested as leukocyte rolling. This transient binding results in further leukocyte activation and subsequent firm adhesion and transendothelial migration of leukocytes, both of which are mediated by interactions between members of the integrin and immunoglobulin superfamily of CAM. This CAM-regulated process of leukocyte recruitment often results in endothelial cell dysfunction, which can be manifested as either impaired endothelium-dependent vasorelaxation in arterioles, excess fluid filtration in capillaries, and enhanced protein extravasation in venules. Consequently, CAMs have been implicated in a variety of vascular disorders (e.g., ischemia/reperfusion, atherosclerosis, allograft dysfunction, and vasculitis), and an enhanced expression of these CAMs has been invoked to explain the exaggerated microvascular dysfunction associated with some of the risk factors (hypertension, hypercholesterolemia, diabetes) for cardiovascular disease (Krieglstein and Granger 2001). The prevalence of cardiovascular disease in diabetic nephropathy patients is high and is a major cause of morbidity and mortality. The cell adhesion molecules, ICAM-1 and VCAM-1, are raised in both cardiovascular disease and diabetic nephropathy, with levels increasing in stepwise fashion with increasing kidney disease (Wu et al. 2005).

Leukocyte adhesion and platelet adhesion may be influenced by each other. For instance, adherent platelets, platelet-derived microparticles, and platelet-released substances, such as platelet-derived growth factor (PDGF), platelet factor 4, and thromboxane A_2 (TXA_2), may enhance leukocyte rolling and adhesion to the vessel wall. Conversely, platelet activation may be influenced by leukocyte adhesion and leukocyte-released mediators (Li et al. 2000). A key adhesion molecule implicated in platelet interaction with leukocytes is P-selectin, of which counterpart on leukocytes is known as P-selectin glycoprotein ligand-1 (PSGL-1) (McGregor et al. 2006).

4.3 Cancer Cell Adhesion

Cancer progression is a multistep process in which some adhesion molecules play a pivotal role in the development of recurrent, invasive, and distant metastasis. One critical step in tumor metastasis is tumor cell adhesion to the endothelium forming the microvessel wall. Cancer cells, especially the highly metastatic types, are believed to have enhanced adhesion ability that often facilitates the migration of the cells to establish new tumors in the body. Circulating cancer cells have been shown to extravasate to a secondary site using a process similar to inflammatory cells. The interaction of cancer and endothelial cells is considered not only to determine the physical site of metastasis but also to provide the necessary anchorage to facilitate tumor cell extravasation (Iiizumi et al. 2007). This process, shared by inflammatory cells and cancer cells, may partially explain the link between inflammation and tumorigenesis.

Multiple and diverse cell adhesion molecules take part in intercellular and cell–extracellular matrix interactions of cancer. During cancer metastasis, the formation of platelet–tumor cell aggregates in the circulation facilitates immune

evasion and the microvascular arrest of cancer cells at distant sites (Borsig 2008). Cancer cells can escape T-cell immunity by overexpressing the vascular cell adhesion molecule-1 (VCAM-1) (Wu 2007). The most studied ligands for CAMs expressed on cancer cells, sialyl Lewis (a/x) antigens, are shown to be involved in adhesion to endothelial cells by binding to E-selectin. In addition to participating in tumor invasiveness and metastasis, adhesion molecules regulate or significantly contribute to a variety of functions including signal transduction, cell growth, differentiation, site-specific gene expression, morphogenesis, immunologic function, cell motility, wound healing, and inflammation (Okegawa et al. 2004).

4.4 Red Cell Adhesion

Red cells express a large number of cell adhesion molecules at their surface. A subset of these molecules has been reported to play a role in pathological situations such as sickle cell disease, malaria, diabetes mellitus, and other vascular disorders and contribute to the clinical manifestations of these diseases by mediating abnormal RBC adhesion to the vascular endothelium (El Nemer et al. 2014). Among multiple pathologies associated with sickle cell disease, sickle red cell–endothelial cell interaction has been implicated as a potential initiating mechanism in vaso-occlusive events that characterize this disease. Sickle erythrocytes strongly adhere to endothelium by specific interactions between cell receptors and plasma proteins, such as thrombospondin, von Willebrand factor, fibrinogen, and fibronectin (Kaul 2008).

4.5 Bacteria and Virus Adhesion

Adhesion of enteric, oral, and respiratory bacteria is required for colonization and for subsequent development of disease. Moreover, bacteria assume a significantly greater resistance to clearance by normal cleansing mechanisms and to killing by normal immune factors, bacteriolytic enzymes, and antibiotics when they are adherent to surfaces and subsequent biofilm formation. Thus, the adherent state is advantageous for bacterial survival (Gu and Ren 2014). The alarming increase in drug-resistant bacteria makes a search for novel means of fighting bacterial infections imperative. Therefore, prevention of adhesion at an early stage following the exposure of the host to pathogens should prevent the disease. An attractive approach is the use of agents that interfere with the ability of the bacteria to adhere to tissues of the host, since such adhesion is one of the initial stages of the infectious process. Because anti-adhesive agents are not bactericidal, the propagation and spread of resistant strains is much less likely to occur than as a result of exposure to bactericidal agents, such as antibiotics (Ofek et al. 2003).

Viruses also have adhesion molecules required for viral binding to host cells. For example, influenza virus has a hemagglutinin on its surface that is required for recognition of the sugar sialic acid on host cell surface molecules. Since the

beginning of the HIV epidemic, endothelial function has been investigated in HIV-positive patients and was seen to be impaired to varying degrees. ECs lining liver sinusoids, human umbilical veins, bone marrow stroma, or brain microvessels have been shown to be variably permissive for HIV infection. Entry of virus into ECs may occur in different ways, such as via CD4 antigen, galactosylceramide receptors, or chemokine receptors. HIV has an adhesion molecule termed gp120 that binds to its ligand CD4, which is expressed on lymphocytes. The adhesive molecule-mediated interactions between ECs and leukocytes have been interpreted as a "Trojan horse" mechanism by which infected leukocytes cross the endothelial barrier and gain entry into extravascular tissue, where HIV replication may occur. Endothelial activation may also occur either by cytokines secreted in response to mononuclear or adventitial cell activation by HIV virus or by the effects of gp120 and HIV-associated protein Tat on endothelium. Endothelium could be activated either directly by HIV virus or by a leukocyte-mediated inflammatory cascade triggered by HIV infection (de Gaetano et al. 2004). Circulating (c) forms of the molecules have been used as monitors of disease progression. The levels of cICAMs and cVCAM-1 are increased during HIV infection. Serum cCAM-1 and cICAM-2 in HIV infection could be additional markers to discriminate between asymptomatic and progressor patients (Galea et al. 1997).

5 Anti-adhesion Therapies

Advances in our knowledge of cell adhesion mechanisms point to new therapeutic drugs capable of interfering with abnormality of cell adhesion. Much of the attention has been focused on inhibitors of adhesion molecules due to their crucial roles in pathological processes involving cell adhesion. The effects of many anti-inflammatory drugs can be ascribed, in part, to inhibition of the expression of CAMs. However, in the search for more selective and potent drugs for clinically important diseases such as multiple sclerosis, asthma, rheumatoid arthritis, inflammatory bowel disease, allergies, and atherosclerosis, direct inhibition of the function of CAMs has attracted increasing interest (Ulbrich et al. 2003). The first adhesion antagonists, anti-glycoprotein IIb/IIIa monoclonal antibody, have been tested in randomized trials as an adjunct to percutaneous transluminal coronary angioplasty. While considerably, progress in the development of orally available antagonists has been slower and more problematic than envisioned (Simmons 2005).

In fact, some old agents like aspirin and heparin have been used in treating diseases correlated with cell adhesion for many years in clinical practices. Experimental researches indicated they processed evident anti-adhesion activities. Aspirin can inhibit T-cell adhesion to and transmigration through endothelium by preventing integrin activation induced by contact with endothelial cells. The infusion of aspirin into healthy volunteers induced downregulation of L-selectin on circulating T cells (Gerli et al. 2001). Aspirin also inhibits the adhesion of T cells to IL-1beta-activated smooth muscle cells by reducing NF-kappaB activity

and decreasing expression of ICAM-1 and VCAM-1 and may prevent the development of atherosclerosis (Yotsui et al. 2007). The polysaccharides of animal origin, heparin, have been shown to interfere with selectins. Parnaparin, a low molecular weight heparin (LMWH), prevents P-selectin-dependent platelet-polymorphonuclear (PMN) aggregate formation and PMN leukocyte activation (Maugeri et al. 2007). Non-anticoagulant heparin prepared by chemical modifications exhibited strong anti-P-selectin and anti-L-selectin activity and more and inhibited cell adhesion in vivo (Xie et al. 2000).

6 The Polysaccharides Intervening with Cell Adhesion in Inflammation

Selectin-mediated leukocyte initial attachment and rolling over vessel endothelial surface are crucial steps for inflammatory responses. P-selectin is a promising target for anti-inflammation therapeutic strategy. Fucoidan is a natural polysaccharide extracted from brown seaweeds with anti-inflammatory and anti-oxidative effects. The anti-inflammatory effect of fucoidan related to its capacity to interact with the selectin or scavenger receptor on the cell membrane (Cui et al. 2008). Three water-soluble polysaccharide fractions (PPS-1, PPS-2, and PPS-3) were isolated from the roots of *Physalis alkekengi*. Among the polysaccharide fractions, PPS-2 could effectively block the interaction between P-selectin and its native ligand (Tong et al. 2011). The purified polysaccharides of ginkgo biloba leaves (PGBL) could effectively inhibit the acute inflammation in mice and interfere with the adhesion of HL-60 cells or neutrophils to P-selectin in static conditions, as well as the adhesion of neutrophils to Chinese hamster ovary cells expressing human P-selectin and human umbilical vein endothelial cells in flow conditions (Fei et al. 2008).

A pectic polysaccharide named comaruman (CP) was extracted from the aerial part of *Comarum palustre*. CP fractions obtained with acidic hydrolysis inhibit spontaneous and phorbol-12-myristate-13-acetate-activated adhesion of peritoneal leukocytes in vitro (Popov et al. 2005a). The polysaccharides isolated from Golden needle (GNP) mushroom possessed strong anti-inflammatory activity in burned rats and could significantly decreased ICAM-1 in serum and colon of normal and burned rats (Wu et al. 2010). The anti-adhesive activity of polysaccharides from the red alga *Lithothamnion muelleri* (Hapalidiaceae) was assayed by visualizing lipopolysaccharide-induced leukocyte rolling. The intravenous injection of fractions B1a and B1b in mice reduced leukocyte rolling by approximately 90 % (Soares et al. 2012). *Porphyra vietnamensis* possesses potential immunomodulatory activity. An isolated polysaccharide fraction evoked an increase in the percent of neutrophil adhesion to nylon fibers (Bhatia et al. 2013). Extracts of the vine-like plant *Tripterygium wilfordii* (TW) have been widely used in China as an immunosuppressant and anti-inflammatory drug for the treatments of rheumatoid arthritis, lupus erythematosus, and other inflammatory disorders. The lipopolysaccharide (LPS)-mediated stimulatory effects of tumor necrosis factor-alpha (TNF-alpha) cytokine production and cell adhesion molecule (CD11c, CD18, CD14, CD54)

expression in human monocytic THP-1 cells were modulated by treatments of the TW extracts. The TW polysaccharide moiety exhibited more profound immunosuppressive properties than the aqueous and ethanol extract (Luk et al. 2000). The pectic polysaccharide named bergenan BC was obtained using extraction of the green leaves of Siberian tea *Bergenia crassifolia* (L.) Fritsch. Bergenan BC was found to increase the spontaneous adhesion of peritoneal leukocytes and failed to influence adhesion stimulated by PMA or adhesion of peritoneal leukocytes incubated in the presence of EDTA. Thus, bergenan was shown to possess immunostimulating activity in relation to DTH response in vivo and phagocytic activity in vitro (Popov et al. 2005b). EA.hy926 cell cultures were used as an established bronchopulmonary dysplasia (BPD) cell model. *Astragalus* polysaccharide (APS) retards the inflammatory response by hyperoxia, as shown by the reduced expression of ICAM-1 (Huang et al. 2013).

7 The Polysaccharides Intervening with Cell Adhesion in Immunity

Tricholoma matsutake is well-known mushroom in Asian country. The immunomodulatory effect of *T. matsutake*-derived polysaccharide fraction (TmC-2) was evaluated using functional activation models of macrophages, monocytes, and splenic lymphocytes. TmC-2 stimulated CD29-mediated cell–cell or cell–fibronectin adhesions in monocytes, while CD43-mediated cell adhesion was downregulated (Byeon et al. 2009). *Coriolus versicolor* polysaccharide (PSK) is a plant polysaccharide widely used for cancer immunotherapy. It is considered that its antitumor effect is derived from its immunomodulating activity on the tumor-bearing host. ICAM-1 was expressed on human KATO-3 gastric cells but not on Colo205. The expression of ICAM-1 was enhanced by treatment with PSK (Iguchi et al. 2001). *Astragalus* heteropolysaccharides (AHPS) is obtained from the dried roots of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao. Its therapeutic effects were observed on erythrocyte immune adherence function in mice with adjuvant-induced arthritis (AA). AHPS significantly improved the primary and secondary local or systemic symptoms of the mice with AA. AHPS could significantly increase the number of complement receptor 1 (CR1, CD35) on erythrocyte, improve the elimination of circulation immune complex (CIC) in the peripheral blood, and reduce the deposition of IC in joint synovium (Yang et al. 2009).

8 The Polysaccharides Intervening with Cell Adhesion in Tumor

Ganoderma lucidum has been widely used as a miraculous herb for health promotion, especially by cancer patients. It has been known that *G. lucidum* affects cancer cell activities. Polysaccharides isolated from *G. lucidum* fruiting bodies grown on

logs of wood exhibited the greatest inhibitory activity on cell adhesion. Purified polysaccharides also inhibited cell adhesion to various matrix molecules. The polysaccharide interacted with cell surface proteins. Specially, β_1-integrin expression was greatly reduced (Wu et al. 2006). *Ganoderma lucidum* polysaccharides peptide (Gl-PP) could suppress invasion of human lung carcinoma cells in vitro (PG cell). Pretreated with Gl-PP, PG cell motility was inhibited significantly. PG cell adhesion was also inhibited (Cao et al. 2007).

One low molecular weight polysaccharide (LMW-ABP) was isolated from the fruiting bodies of *Agaricus blazei* Murill. LMW-ABP could effectively inhibit adhesion of HT-29 cells to human umbilical vein endothelial cells (HUVECs) in static conditions, as well as downregulating the expression of both α-1,3-fucosyltransferase-VII (FucT-VII) and sialyl Lewis X (sLex). The results suggested that LMW-ABP could suppress the metastasizing capacity of cancer cells through interfering with the interaction between E-selectin and sLex (Liu et al. 2010). The acidic polysaccharide (PL) from *Phellinus linteus* markedly inhibit melanoma cell metastasis in mice and directly inhibit cancer cell adhesion to and invasion through the extracellular matrix, but it has no direct effect on cancer cell growth (Han et al. 2006a). The pectic polysaccharide (angelan) of *Angelica gigas* Nakai directly inhibited cancer cell adhesion and invasion through the extracellular matrix, in addition to activating the immune functions of B cells and macrophages. Angelan can inhibit tumor growth and metastasis by stimulating host immunity and directly inhibiting cancer cell adhesion (Han et al. 2006b). An acidic polysaccharide (CPPA) from the roots of *Codonopsis pilosula* induced a potent inhibitory effect on the invasion and migration potential of human epithelial ovarian cancer HO-8910 cells in vitro. Moreover, the CD44 expression on the HO-8910 cells was also attenuated by CPPA treatment (Xin et al. 2012). T739 cells were treated with a combination of *Polyporus* polysaccharide (PPS) and Bacillus Calmette–Guerin (BCG). The expression of genes associated with the NF-κB signaling pathway such as ICAM-1 was downregulated (Wei et al. 2011).

9 The Polysaccharides Intervening with Cell Adhesion in Infection

Anti-adhesive compounds are potential prophylactic tools in alternative treatment regimes against bacterial infection, as bacterial adhesion is commonly mediated by carbohydrate–protein interactions between surface adhesions of microorganisms and the host cell. The use of exogenous polyvalent, high-molecular carbohydrates, and tannin-like plant-derived compounds should antagonize the adhesive interaction. High molecular weight polysaccharides from black currant seeds (*Ribes nigrum* L.) inhibit adhesion of *Helicobacter pylori* to human gastric mucosa (Lengsfeld et al. 2004). High-molecular glycosylated compounds (polysaccharides and glycoproteins) from the immature fruits of the okra plant, *Abelmoschus esculentus* (L.) Moench,, were shown to have a strong in vitro anti-adhesive activity against *Helicobacter pylori*, leading to an inhibition of the binding to mucosal

epithelia from human stomach in situ. In an in vitro adhesion model with fluorescent-labeled *C. jejuni*, the isolate okra polysaccharides strongly inhibited the microbial adhesion to colonic tissue (Lengsfeld et al. 2007). The acidic polysaccharide fraction APS-F2 from Aloe vera did not reveal an inhibitory effect on the growth of *H. pylori* or cell viability but could reduce the count of *H. pylori* attached to MKN45 cells. In addition, APS-F2 was shown to have a potent anti-adhesive effect against *Escherichia coli* (Xu et al. 2010). Aqueous extract of *Glycyrrhiza glabra* L. (Fabaceae) significantly inhibited the adhesion of *Helicobacter pylori* to human stomach tissue. This effect was related to the polysaccharides isolated from the extract, with one purified acidic fraction as main active polymer. Purified polysaccharides did not exhibit direct cytotoxic effects against *Helicobacter pylori* and did not influence hemagglutination. Additionally raw polysaccharides from *Glycyrrhiza glabra* were shown to have strong anti-adhesive effects against *Porphyromonas gingivalis* (Wittschier and Faller 2009).

A range of carbohydrates and carbohydrate- and proanthocyanidin-enriched plant extracts were screened for potential anti-adhesive effects against *Helicobacter pylori*, *Campylobacter jejuni*, *Porphyromonas gingivalis*, and *Candida albicans* in different in situ assays on primary tissue. The adhesion of *H. pylori* on human stomach tissue was effectively blocked by glucuronic acid-enriched polysaccharides from immature okra fruits (*Abelmoschus esculentus*). These compounds also had strong in vitro effects against *C. jejuni* but were ineffective in an in vivo study in infected chicken broilers due to metabolism in the gastrointestinal system. Polysaccharides from *Glycyrrhiza glabra*, also enriched with glucuronic acid, showed strong anti-adhesive properties against *H. pylori* and *P. gingivalis* (Wittschier et al. 2007). An acidic polysaccharide CS-F2 from *Camellia sinensis* might exert a selective anti-adhesive effect against certain pathogenic bacteria, most notably *Helicobacter pylori*, *Propionibacterium acnes*, and Staphylococcus aureus, while exerting no effects against beneficial and commensal bacteria including *Lactobacillus acidophilus*, *Escherichia coli*, or *Staphylococcus epidermidis*. CS-F2 showed marked inhibitory activity against the pathogen-mediated hemagglutination (Lee et al. 2006).

A polysaccharide with high uronic acid content from the roots of *Panax ginseng* was found to inhibit the ability of *Porphyromonas gingivalis* to agglutinate erythrocytes. This polysaccharide showed a strong inhibitory activity, but treatment with pectinase resulted in non-inhibitory hydrolyzed products. In contrast, the inhibition by the acidic polysaccharide from the leaves of *Artemisia capillaris* was negligible. The carbohydrate composition of the two polysaccharides indicated that the anti-adhesive activity may be correlated with glucuronic acid content, one of the components of glycosaminoglycans. Low molecular weight heparin and sucrose octasulfate revealed stronger inhibitory effects on bacterial binding than the acidic polysaccharide from *P. ginseng* (Lee et al. 2004). Lentinan, a mushroom polysaccharide, isolated from *Lentinus edodes* (shiitake mushroom) was sulfated in dimethyl sulfoxide to obtain a water-soluble derivative coded as LS. Then, two polysaccharide-based polyelectrolytes, polyanionic lentinan sulfate (LS) and polycationic chitosan (CS), were alternatively deposited onto the surfaces of

polyurethane (PU) via layer-by-layer (LbL) assembly technique. Polyurethane (PU) modified by polysaccharide-based multilayers showed antibacterial activity against *Pseudomonas aeruginosa*. The number of adherent platelets on the surface modified by five bilayers (LS as topmost layer) was reduced. The tests of L-929 cells indicated that LbL-modified PU surfaces had better cytocompatibility than unmodified PU (Wang et al. 2012).

10 The Polysaccharides Intervening with Cell Adhesion in Cardiovascular Injury

A polysaccharide from brown algae decreased polymorphonucleated leukocytes (PMNs) sticking to autologous rabbit aortae. The fucan sulfate could be a heparin substitute endowed with antithrombotic and anti-inflammatory activities, devoid of the problems caused to heparin by its animal origin, i.e., possible prion protein contamination (Trento et al. 2001). *Astragalus* polysaccharide effectively alleviates ischemia–reperfusion injury (IRI) of cardiac muscle. Human cardiac microvascular endothelial cells (HCMECs) were used to validate the protective effects of astragalus under an IRI scheme simulated through hypoxia/reoxygenation in vitro. *Astragalus* polysaccharide inhibited the cohesion between HCMECs and polymorphonuclear leukocyte (PMN) during IRI through the downregulation of p38 MAPK signaling and the reduction of cohesive molecule expression in HCMECs (Zhu et al. 2013).

11 The Polysaccharides Intervening with Cell Adhesion in Wound Healing

The fresh leaves and juice of *Sedum telephium* L. are used as wound-healing promoters. Cell adhesion represents a primary event in wound repair and in tissue homeostasis. Total *Sedum* juice strongly inhibited human fibroblast (MRC5) cell adhesion to laminin and fibronectin (EC50 = 1.03 +/− 0.12 mg/mL). This anti-adhesive feature was concentrated mainly in the two polysaccharide fractions (EC50 values comprised between 0.09 and 0.44 mg/mL). The flavonol fractions did not seem to contribute to this effect. The results confirmed that natural polysaccharides, with chemical structures different from heparin, were able to interfere with integrin-mediated cell behavior, and they contributed to the outstanding effects of *Sedum* juice and to the role of polysaccharides in cell–matrix interaction (Raimondi et al. 2000). The natural polysaccharide extracted from tamarind seed (xyloglucan or tamarind seed polysaccharide, TSP) exerted a positive influence on human conjunctival cell adhesion to laminin. TSP slightly but significantly increased corneal wound healing rate. The ability of the polysaccharide to promote corneal wound healing might depend on its influence on the integrin recognition system (Burgalassi et al. 2000). Xylan, a natural polysaccharide, was electrospun along with polyvinyl alcohol (PVA) to produce xylan/PVA nanofibers for skin

tissue engineering. The natural biodegradable xylan/PVA nanofibrous scaffolds have good potential for fibroblast adhesion, proliferation, and cell–matrix interactions relevant for skin tissue regeneration (Krishnan et al. 2012).

12 Discussion

With the increasing awareness of the roles of cell adhesion in the pathological process, anti-adhesion become an effective strategy for the prevention and treatment of diseases. Compared with protein–protein interactions, the knowledge on interactions between proteins and carbohydrates is still inadequate. How heparin effect on cell adhesion was previously reviewed (Xu and Dai 2010). Although as a classic anticoagulant drugs, heparin shows more and more its non-anticoagulant effects such as anti-inflammation, antitumor, and anti-infection in recent years. Additionally, other non-anticoagulant heparins and heparin derivatives can interfere with cell adhesion among various cells. The anti-adhesion characteristics of heparin may be many of its pharmacological action mechanism. Notably, the chemical components of heparin are impure polysaccharides extracted from animals, with "multiple components and multiple targets" which are similar to plant polysaccharides. Therefore, focusing on cell adhesion, application of glycobiology in polysaccharide research is expected to reveal the therapeutic mechanism of plant polysaccharides in disease processes.

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Polysaccharides from *Aloe vera* and Oral Ulcerations

70

Kanokporn Bhalang and Kevin Tompkins

Contents

Abstract

Aloe vera is a locally grown herbal plant. *Aloe vera* has historically been used to treat skin wounds. Investigations have discovered that *Aloe vera* can increase the number of fibroblasts, which play a key role in wound healing. The active ingredient in *Aloe vera* gel is acemannan. Acemannan is a polysaccharide located within the protoplast of the *Aloe vera* parenchyma cells. Acemannan is composed of β-(1 → 4)-acetylated polymannose chains. Acemannan has been reported to accelerate oral wound healing in animal studies. Recurrent aphthous ulcerations are common painful oral mucosal ulcers that affect the quality of life of patients. Clinically, acemannan is effective in reducing both ulcer size and associated pain. Patients reported that they were mostly satisfied with acemannan treatment compared to standard topical steroid treatment. Thus, acemannan can be used for the treatment of recurrent aphthous ulceration.

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Aloe vera • Acemannan • Oral ulcer • Aphthous

1 Introduction

Recurrent aphthous ulceration (RAU) is a lesion commonly found in the oral cavity, causing pain and discomfort in patients. Currently, there is no curative management of RAU available. The treatment of choice for these lesions is topical steroid application (Porter et al. 1998; Barrons 2001). Because of the possible adverse effects from steroid use, herbal medicines are very attractive as an alternative for use in treating RAU.

Several natural products have been investigated for the promotion of wound healing. *Aloe vera (Aloe barbadensis* Miller*)* has long been used to treat various skin conditions such as cuts and burns (Reynolds and Dweck 1999). *Aloe vera* has been demonstrated to stimulate dermal wound healing in rats by increasing collagen and glycosaminoglycan synthesis (Chithra et al. 1998a, b). *Aloe vera*'s beneficial properties may be attributed to the polysaccharides present in the inner gel of the leaf, especially β-(1 → 4)-acetylated polymannose, also known as acemannan (Reynolds and Dweck 1999; Femenia et al. 1993). Acemannan has been reported to increase oral epithelial and fibroblast cell proliferation through the activation of growth factors (Jettanacheawchankit et al. 2009). Animal experiments also confirmed the effect of acemannan in accelerating oral wound healing (Jettanacheawchankit et al. 2009). In a clinical study, it was found that acemannan had an immediate effect in relieving pain symptoms from RAU (Bhalang et al. 2013).

1.1 *Aloe vera*

There have been many studies investigating herbal plants used in traditional medicine. *Aloe vera (Aloe barbadensis* Miller*)* (Fig. 1) is a succulent plant that develops a compartment for water storage in the leaves to survive in the dry season. *Aloe vera* has been used since ancient Rome for treating burns and has been utilized widely in the cosmetic and medical industry in many types of products such as shampoo and herbal drinks. Because it can be easily grown in several climates, it is a good candidate to be commercially produced (Reynolds and Dweck 1999; Hamman 2008).

Extracts from *Aloe vera* can be divided into two parts, *Aloe vera* sap and *Aloe vera* gel. The bitter yellow sap is from the leaf, while the clear and colorless gel extract is from the inner leaf. The sap consists of anthraquinones such as emodin and aloe-emodin. Because its phenol content can irritate the skin, the sap has not commonly been developed for human use. The inner part of the leaf in which water is held in the form of a viscous mucin is a clear tissue that consists of large thin-walled parenchyma cells (Hamman 2008; Vogler and Ernst 1999).

Fig. 1 *Aloe vera* plants at the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

Aloe vera gel extract is composed of water, carbohydrates, proteins, vitamins, minerals, and sterols. Water is the main component (99 %) of the gel. Carbohydrates in the form of polysaccharides are the second most prevalent component of the gel. The carbohydrates can be divided into two main types: glucomannan and acemannan. Other sugars such as galactose, xylose, arabinose, rhamnose, cellulose, and pentose are also found in the gel. Protein is mostly found as glycoproteins, such as lectins, aloctin A, and aloctin B. The vitamins present in the gel are provitamin A, riboflavin, thiamine, niacin, and ascorbic acid. Potassium, sodium, and manganese are the most commonly found minerals in the gel extracts (Hamman 2008). The evidence for the medicinal effects of *Aloe vera* has historically been anecdotal. However, a number of studies investigating the use of *Aloe vera* in wound healing and other processes have been conducted, and the results are summarized as follows:

(a) *Aloe vera* has been demonstrated to stimulate dermal wound healing in rats by increasing collagen and glycosaminoglycan synthesis (Chithra et al. 1998a, b). *Aloe vera* can also help reduce skin irritation from ultraviolet rays (Strickland et al. 1994). Wound healing both on the skin and on the oral mucosa was accelerated by *Aloe vera*. *Aloe vera* accelerated the migration of epithelial cells into the wound area, increased the number of endothelial cells and new blood vessels, and stimulated the number of fibroblasts and collagen fibers in forming granulation tissue to fill the wound (Jettanacheawchankit et al. 2009).
(b) *Aloe vera* has immunomodulatory activity (Im et al. 2010; Seongwon and Myung-Hee 2003) and anti-inflammatory effects (Vazquez et al. 1996). These effects occur through the activation of macrophages and the suppression of mast cell migration to the injury and by inhibiting the enzyme cyclooxygenase, which controls the synthesis of prostaglandin E2, an important mediator in the occurrence of pain and inflammation.

(c) *Aloe vera* has antimicrobial effects against several bacteria strains and fungi (Im et al. 2010; Nejatzadeh-Barandozi 2013). These effects are exerted by enhancing the release of cytokines, including interleukin (IL), interferon (IFN), granulocyte/monocyte-colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF) (Talmadge et al. 2004).
(d) *Aloe vera* solution reduced the blood sugar level of diabetic rats (Okyar et al. 2001). This solution also significantly lowered the levels of blood glucose, cholesterol, triglycerides, and blood pressure of diabetic patients (Choudhary et al. 2014). Other reported effects of *Aloe vera* are the reduction of gastric ulcers (Mahattanadul 1996) and thyroid hormone levels (Kar et al. 2002).
(e) *Aloe vera* has also been studied for its use in dentistry. *Aloe vera* has been investigated as supplemental root canal filling material in primary teeth (Kriplani et al. 2013) and recommended as a suitable storage media for avulsed teeth (Badakhsh et al. 2014).

2 Acemannan: A Polysaccharide from *Aloe vera*

Polysaccharides are readily found in nature, plants, animals, and bacteria. The diversity and complexity of polysaccharides is based on the multiple monosaccharide linkage forms available (1 → 2, →3, →4, →5, or → 6), being in either the α or β confirmation, and the presence of chains branching from the main backbone (Hamman 2008). The *Aloe vera* leaf pulp contains multiple polysaccharides that differ in their location in the pulp (Fig. 2).

The major polysaccharide present in the inner gel of the *Aloe vera* leaf is acemannan (β-(1 → 4)-acetylated polymannose). Acemannan, also known commercially as carrysin, is produced by specialized cells known as leucoplasts (Hamman 2008; Lex 2012). Acemannan has a backbone chain of mannose that is acetylated at the C-2 and C-3 positions with a 1:1 mannose monomer/acetyl ratio. Acemannan contains side chains that are mainly composed of galactose attached at the mannose C-6 position (Hamman 2008). The molecular weight of acemannan ranges from 30 to 40 kDa (Lex 2012). A ratio of 1:3 (glucose/mannose) is commonly found in the repeating units. However, other ratios have also been reported, and these discrepancies could stem from differences among species or from sample processing (Hamman 2008).

When the linkage between the monomers in acemannan was analyzed, it was demonstrated that acemannan has a single-chain backbone of β-(1 → 4) mannose with β-(1 → 4) glucose inserted into the backbone with α-(1 → 6) galactose branching from the backbone (Fig. 3) (Talmadge et al. 2004). The manner in which acemannan is processed and handled can affect its structure and possibly its bioactivity. Acemannan loses mannosyl residues when dehydration was performed. Deacetylation and reduction of galactosyl residues were also detected (Femenia et al. 2003). The loss of galactosyl and acetyl residues may influence the interactions between mannose chains and change their binding capacity.

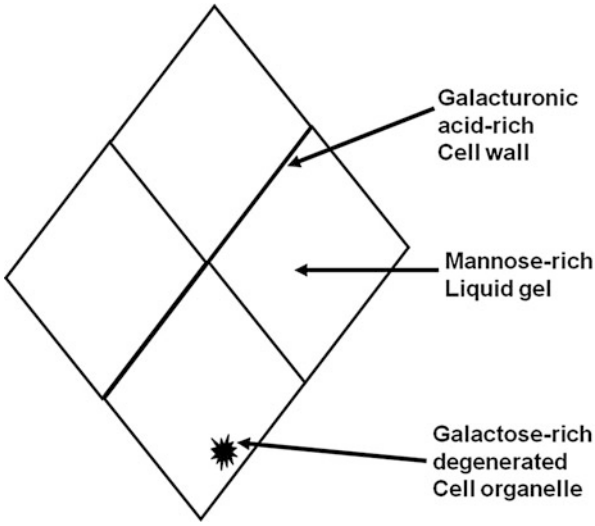

Fig. 2 Schematic of the distribution of the polysaccharides in the *Aloe vera* pulp

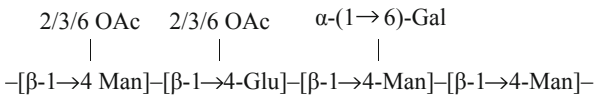

Fig. 3 Diagram of the molecular structure of acemannan

Because acemannan consists of long polymer chains, it must be broken down to facilitate cellular uptake while passing through the gastrointestinal system. The epithelial cells lining the gastrointestinal system have mannose-specific receptors, and when taken orally, acemannan can be detected in the blood within 90 min (Moreira and Filho 2008; Lex 2012). A study using orally administered 14C-labeled acemannan showed that 72 h after administration, 86.4 % of the radioactivity could be found in CO_2 and 2.2 % was found in the urine and feces, suggesting that the majority of orally administered acemannan is absorbed and metabolized. Thus after 72 h, 11.4 % of acemannan is stored in the mannose pool or efficiently used in the body. Detoxification and synthesis of glycoproteins and oligosaccharides occurs mainly in the liver. Reflecting acemannan's role in immunomodulation, the spleen and thymus are other sites of acemannan uptake (Lex 2012).

Many of the beneficial effects of *Aloe vera* derive from the effects of acemannan. Acemannan is known to have immunomodulatory activity both in vivo and in vitro. The immunoaugmenting activity of acemannan appears to be mediated through macrophage activation. Acemannan induces multiple macrophage functions including recognition of foreign antigens and removal of microorganisms. Part of the macrophage inducing effect of acemannan may be because mannose is rarely present as the terminal moiety in polysaccharides in humans, although it is

commonly so on those of microorganisms (Lex 2012). Thus, human macrophages will recognize the terminal mannose of acemannan as foreign and activate the immune system. Macrophage activation also results in the production of inflammatory cytokines such as IL-6 and tumor necrosis factor α (TNF-α) (Zhang and Tizard 1996).

Acemannan's anti-inflammatory effects occur by blocking the generation of histamine and bradykinin and inhibiting bradykinin activity, eicosanoid formation, and PMM leukocyte infiltration (Lex 2012). Acemannan has also been shown to have anticancer activity and has received approval for use in the treatment of fibrosarcoma in cats and dogs. The anticancer effects of acemannan occur by activating local macrophages and stimulating an immune response to the cancer cells (Lex 2012).

Acemannan was investigated for its use in treating dental pathologies such as periodontal disease or dental pulp exposure. It was found that acemannan induced the expression of bone-formation-associated proteins in both the periodontal ligament and dental pulp fibroblasts (Boonyagul et al. 2014). Acemannan also stimulated the mRNA expression of dentin sialophosphoprotein and dentin matrix protein 1, which are involved in the differentiation of the odontoblast cells that produce the dentin of the tooth (Jittapiromsak et al. 2010). When used on exposed pulp tissue, acemannan increased pulpal fibroblast number, alkaline phosphatase activity, and expression of dentin sialoprotein (Lardungdee et al. 2008). A retrospective study in the use of acemannan-containing patches on the reduction of alveolar osteitis following tooth extraction indicated that the acemannan-treated patients had a significantly reduced incidence of dry socket compared to the typical gelfoam treatment (Poor et al. 2002). In that study, smokers had twice the rate of dry socket compared to nonsmokers; however, no acemannan-treated smokers experienced dry socket. Furthermore, Acemannan has also been used as denture adhesive (Tello et al. 1998).

3 Toxicity and Side Effects

The direct cytotoxicity of acemannan to target cells was investigated, and acemannan was not found to be cytotoxic to these cells at any dose evaluated (Womble and Helderman 1992). When acemannan was administered as a solution to mice, rats, or dogs, there were no significant signs of toxicity and none of the animals died. Because of the immune-stimulating activity of acemannan, an accumulation of macrophages and monocytes without subsequent inflammatory reaction was found in the lungs, liver, and spleen of the animals. These findings were not considered adverse effects (Fogleman et al. 1992a). Investigations using rats or dogs determined that none of the animals had any adverse effects when administered up to 2,000 mg/kg for up to 6 months (Fogleman et al. 1992b). Importantly, no human subjects experienced undesirable reactions to the use

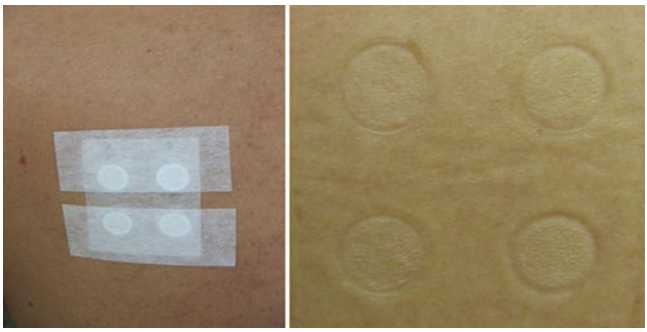

Fig. 4 Skin patch test of 0.5 % acemannan in Carbopol at the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. (*left*) Upper back of a patient with test and control materials in Finn Chambers. (*right*) After 24 h, the patch was removed revealing no allergic reaction

of acemannan either extraorally (Fig. 4) or intraorally (Bhalang et al. 2013; Poor et al. 2002). In addition, there were no local or systemic side effects of acemannan when used on normal oral mucosa (Bhalang et al. 2013).

4 In Vitro Studies

Early investigations of acemannan concentrated on its effects on cells in vitro. It was found that acemannan acts as an important immunoenhancer by increasing the lymphocytic response to alloantigens (Womble and Helderman 1998). Furthermore, the immunomodulatory activities of acemannan were effected through macrophages by increasing nitric oxide synthase mRNA expression in these cells (Ramamoorthy et al. 1996). Acemannan was also found to stimulate macrophage cytokine production and nitric oxide release (Zhang and Tizard 1996).

Acemannan significantly increased periodontal ligament cell proliferation; expression of vascular endothelial growth factor, bone morphogenetic protein 2, and type I collagen mRNA; alkaline phosphatase activity; and mineral deposition when compared with the untreated control group in vitro (Boonyagul et al. 2014). In addition, a study has established the antiviral potential of acemannan in vitro (Kahlon et al. 1991a), and it was shown that acemannan combined with azidothymidine and acyclovir acts synergistically to inhibit the replication of human immunodeficiency virus and herpes simplex virus, respectively (Kahlon et al. 1991b).

A study investigating the ability of acemannan in inhibiting adherence of *Pseudomonas aeruginosa* to cultures of human lung epithelial cells found that the binding of either mucoid or non-mucoid bacteria was inhibited by acemannan (Azghani et al. 1995). In addition, the preexposure of macrophages to acemannan resulted in 38 % killing of *Candida albicans* by these cells compared with 0–5 % killing in the control group (Stuart et al. 1997).

5 Animal Studies

The majority of the studies of the in vivo effects of acemannan were mostly performed in animals, with the results summarized as follows:

(a) Animal experiments on wound healing showed that acemannan accelerated oral wound healing. When used on rat oral wounds, increased levels of proliferating cell nuclear antigen, keratinocyte growth factor-1, vascular endothelial growth factor, and type I collagen were found (Jettanacheawchankit et al. 2009). Furthermore, when used as a wound dressing gel, acemannan reduced acute radiation-induced skin reactions in mice (Roberts and Travis 1995).
(b) It was hypothesized that acemannan could function as a bioactive molecule inducing bone formation by stimulating stem cell proliferation, differentiation into osteoblasts, and extracellular matrix synthesis. Thus, acemannan could be a candidate natural material for bone regeneration (Boonyagul et al. 2014). Indeed, in a study using canines, acemannan significantly accelerated new alveolar bone, cementum, and periodontal ligament formation in class II furcation defects (Chantarawaratit et al. 2014).
(c) Studies of feline virus infection found that administration of acemannan to clinically symptomatic cats significantly improved both the quality of life and the survival rate, demonstrating its antiviral effect (Sheets et al. 1991; Yates et al. 1992).
(d) Acemannan has demonstrated anticancer effects. When acemannan was administered intraperitoneally to mice into which murine sarcoma cells had been subcutaneously implanted, it was found that 40 % of the animals treated with acemannan at the time of tumor cell implantation survived. The tumors in the acemannan-treated animals showed vascular congestion, polymorphonuclear leukocyte infiltration, and central necrosing foci with peripheral fibrosis. The results suggested that acemannan initiated an immune attack on the cancer cells, necrosis, and regression of implanted sarcomas in mice (Peng et al. 1991). Studies in canines and felines with fibrosarcomas and other neoplasms also revealed comparable results (King et al. 1995; Harris et al. 1991).
(e) For the treatment of oral ulcers, an animal study has shown that intentional palatal wounds in rats treated with Carbopol® containing 0.5 % acemannan had significantly better healing compared to that of animals receiving normal saline and 0.1 % triamcinolone acetonide. Thus, acemannan can have a marked effect on the oral wound healing process, possibly through the induction of fibroblast proliferation (Jettanacheawchankit et al. 2009).

6 Oral Ulcerations

There are several kinds of oral ulcerations. The most commonly found ulceration in the oral cavity is recurrent aphthous ulceration (RAU) (Graykowski et al. 1966; Vincent and Lilly 1992). The name “aphthous” is derived from the Greek “aptha”

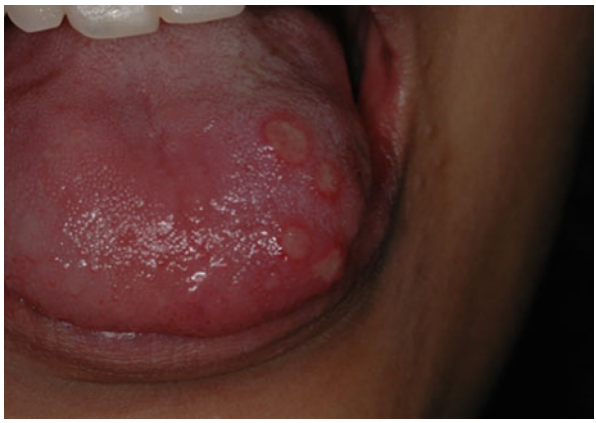

Fig. 5 Minor recurrent aphthous ulcerations on a patient's left lateral tongue

meaning ulcer. RAU is mostly found on nonkeratinized oral mucosa. RAU is present most commonly in young adults and the frequency of developing the ulcers reduces with age. RAU is painful and affects the quality of life of patients experiencing the ulcers. The incidence rate of RAU is 46.7 % and is detected more frequently in females than in males (Scully et al. 2003; Krisdapong et al. 2012).

RAU is a small, round or oval, well-defined ulcer with a yellowish gray center consisting of necrotic tissue. The ulcer commonly has an erythematous border and is found on the oral mucosa of movable tissues such the tongue and the lining mucosa on the cheek and inner side of the lip (Cooke 1969).

RAU is classified into three subtypes: minor aphthous ulcers, major aphthous ulcers, and herpetiform ulcers, based on the size and number of ulcerations. Minor RAU (MiRAU) (Fig. 5) is the most common type affecting approximately 80 % of RAU patients. Patients with MiRAU typically present with 1–5 ulcers per episode, with each ulcer varying in size from 3 to 10 mm. These ulcers normally resolve in 4–14 days without scarring (Femiano et al. 2007). Major aphthous and herpetiform ulcers are less common. Major aphthous ulcers are larger than 1 cm in diameter and quite painful, lasting up to 6 weeks with scarring. Herpetiform ulcers are very small in size (2–3 mm) and can number up to one hundred. Many of these small ulcers commonly coalesce to form larger irregularly shaped ulcers. These lesions differ from herpetic ulcers in that they do not contain virus and are not preceded by vesicle formation (Preeti et al. 2011).

The etiology of RAU has not been clearly identified. However, associations between RAU and immunological disorders have been reported (Lewkowicz et al. 2011). It was proposed that there is a relationship between cell-immediate immune response and RAU. Patients with RAU have been described as having increased numbers of blood CD8+ T lymphocytes and decreased CD4+ T lymphocytes (Pedersen et al. 1989). Furthermore, RAU patients demonstrated higher levels of T-cell receptor γδ cells, which are involved in antibody-dependent cell-mediated cytotoxicity (Pedersen and Ryder 1994). Cytotoxic T lymphocytes play a major role

in destroying epithelial cells, resulting in oral ulceration. The ulcerative phase is marked by an increase of CD8+ cytotoxic cells; however, during healing, these are replaced by CD4+ cells (Savage et al. 1985).

There are also associations between the occurrence of RAU and the levels of several cytokines such as tumor necrosis factor alpha and interleukin 2. There appears to be a genetic component to RAU, with 40 % of patients reporting a family history and a high correlation in identical twins (Sircus et al. 1957; Miller et al. 1977). In addition, there are several predisposing factors suspected of stimulating the onset of RAU. These factors include anxiety; hormonal imbalance; nutritional deficiency such as iron, folate, vitamin B, and zinc; gastrointestinal disorders such as Crohn's disease, ulcerative colitis, and celiac disease; and systemic diseases such as Behcet's disease, Reiter's syndrome, Sweet's syndrome, and cyclic neutrophilia (Jurge et al. 2006). Furthermore, sensitivity to certain medications, diets, or substances such as beta blockers, nonsteroidal anti-inflammatory medicines, alendronate, chocolate, coffee, peanuts, grains, cheese, strawberry, sodium lauryl sulfate, food dye, and preservatives may be involved in RAU development (Jurge et al. 2006). Interestingly, another stimulant for the development of RAU is smoking cessation. RAU develops on nonkeratinized oral mucosa, and smoking results in increased mucosal keratinization, which may serve as a barrier against both trauma and bacteria (Shapiro et al. 1970). Furthermore, nicotine reduces the expression of TNF-α and IL-1 and IL-6 (Floto and Smith 2003).

There is presently no known curative management of RAU available. Most current treatments are palliative and aim to reduce the pain and inflammation resulting from the ulcers. The treatments of choice for RAU are steroid medications in the forms of pastes or gels to be used topically on the lesion. Commonly used steroid medications are triamcinolone acetonide, betamethasone valerate, fluocinonide, fluocinolone acetonide, clobetasol propionate, and dexamethasone elixir (Barrons 2001). Topical steroids are very effective in the treatment of RAU but do not prevent the reoccurrence of the ulcers. The most important side effect of topical steroids is local fungal infection. Other treatments for RAU include antimicrobial agents, topical analgesics, immunosuppressive agents, anti-inflammatory agents, and laser therapy (Barrons 2001). Because steroid treatment has the potential for the development of secondary oral candidiasis over long-term use, herbal medicines have been considered as an alternative treatment.

7 Acemannan and Oral Ulcerations

To evaluate the use of acemannan in the treatment of oral ulcerations, a double-blind clinical study compared three groups of subjects with MiRAU treated with 0.1 % triamcinolone acetonide (topical steroid), 0.5 % acemannan in Carbopol® 934P NF, or pure Carbopol® 934P NF for 7 days (Bhalang et al. 2013).

Four aspects of treatment success were measured in this study: ulcer size reduction, pain control, symptom control, and satisfaction. Although acemannan

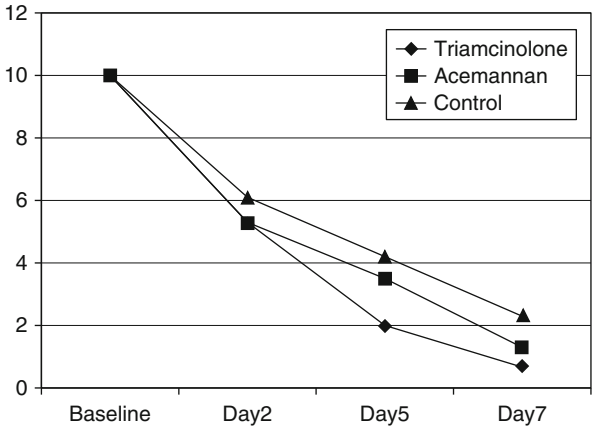

Fig. 6 Comparison of symptom relief rating between the groups receiving 0.1 % triamcinolone acetonide (topical steroid), 0.5 % acemannan in Carbopol® 934P NF, or pure Carbopol® 934P NF. Patients were asked to rate the symptom relief as compared to the level before treatment if the baseline level was 10

treatment did not result in a reduction in ulcer size to the same extent as did 0.1 % triamcinolone acetonide, the difference was not significant. Wound healing is composed of overlapping phases of inflammation, reepithelialization and dermal connective tissue growth, and tissue remodeling. An in vitro study indicated that acemannan stimulated gingival fibroblast proliferation, the expression of VEGF, an angiogenic factor, and collagen type I, all of which contribute to the formation of the granulation tissue in wound healing (Jettanacheawchankit et al. 2009). In addition, this study found increased fibroblast expression of keratinocyte growth factor, which is involved in wound reepithelialization. These findings likely explain the increased wound healing by acemannan found in the clinical RAU study.

When ulcer pain ratings were compared between the three groups, they found that acemannan had a more immediate effect in relieving pain symptoms. Acemannan has been shown to inhibit the formation of bradykinin and to have other anti-inflammatory effects (Lex 2012), which may account for this finding. The subjects were asked on follow-up if the medication used had relieved their symptoms, and at day 5, the subjects who received 0.1 % triamcinolone acetonide felt their symptoms were mostly relieved, followed by acemannan, and then control (Fig. 6). Post hoc tests revealed that the 0.1 % triamcinolone acetonide group experienced significantly greater aphthous ulcer symptom relief compared to the acemannan group at day 5 and the control group at days 5 and 7. Notably, the subjects were significantly more satisfied with 0.1 % triamcinolone acetonide and acemannan treatment compared to control, and there was no difference in satisfaction levels between subjects receiving 0.5 % acemannan and 0.1 % triamcinolone acetonide at day 7. Subjects who received all three treatments at different time points reported that they were mostly satisfied with acemannan treatment compared to standard topical steroid treatment. This indicates that acemannan is a suitable alternative to steroid medication in the treatment of RAU.

8 Conclusion

Acemannan is effective in reducing RAU ulcer size and pain. Patients were mostly satisfied mostly with acemannan treatment when compared to topical steroid and control. Thus, acemannan can be used for the treatment of RAU.

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Natural Polysaccharides from Mushrooms: Antinociceptive and Anti-inflammatory Properties

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Contents

Abstract

Mushrooms have been used in traditional Chinese medicine or as functional foods in Japan and other Asian countries. They have received increasing attention from the researchers for the use in food and pharmaceutical fields. Nowadays there is an increasing public interest in the mushroom constituents for discovering new drugs or lead compounds. A number of bioactive compounds, especially polysaccharides, have been isolated from mushrooms. These bioactive components have become popular sources of natural antioxidative, antitumor, antiviral, antimicrobial, and immunomodulatory agents, and more recently polysaccharides have been explored about their effects against nociception and inflammation. Although there are many studies showing interesting results, more research is necessary to explore such potential. From the results obtained till now, the present chapter explores the antinociceptive and anti-inflammatory bioactivity of natural polysaccharides from mushrooms and

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reviews their mechanism of action, toxicity, bioavailability, and metabolism, besides describing other biological activities and the already done clinical trials.

Keywords

Mushroom • Polysaccharides • Biological activities • Antinociceptive • Anti-inflammatory • Mechanism of action • Toxicity • Clinical trials

1 Introduction

For millennia, mushrooms have been valued as edible and medicinal, especially taking into account their use in the folk medicine of the Western world and in traditional medicine of the Orient. Consequently, nowadays mushrooms have been widely evaluated for their nutritional value and acceptability as well as for their pharmacological properties. Although there are limited direct human intervention trials, there is a rapidly growing volume of in vitro and in vivo animal trials describing a range of possible health benefits attributed to mushrooms, including immunomodulatory, antitumor, and antimicrobial properties, among others (Wasser 2002; Zhang et al. 2007). Nevertheless, mushrooms represent a vast and currently untapped source for researchers, only 10 % (approximately 15,000–22,000 named species) are known by scientists (Hawksworth 2001; Kirk et al. 2008; Mueller and Schmit 2007).

A great variety of active molecules have been obtained from mushrooms and identified; among them, the polysaccharides have been the subject of intense research, taking into view its high potential for application in different sectors. Polysaccharides can be isolated from fruiting bodies, mycelia, and culture media, released as exopolysaccharides (Smith et al. 2003; Wasser 2002, 2010; Zhang et al. 2007), which deserve attention, since this class of molecules make up a vast and yet largely unexploited source of powerfully new pharmaceutical products.

The fungal polysaccharides and polysaccharide-protein complexes have been mainly investigated by presenting a variety of biological responses, such as antitumor, antioxidative, and immune stimulatory activities and more recently antinociceptive and anti-inflammatory effects (Baggio et al. 2010; Carbonero et al. 2008a; Novak and Vetvicka 2008; Ruthes et al. 2013a; Smiderle et al. 2008a). The therapeutic application seems to depend on chemical structure and spatial conformation of each macromolecule, and small structural differences of each polymer can result in peculiar features for new biotechnological applications (Wasser 2010).

Several polysaccharides have been isolated from mushrooms, the most studied are structurally different β-D-glucans (Carbonero et al. 2006; Rowan et al. 2003; Ruthes et al. 2013b; Smiderle et al. 2008a), besides xyloglucan (Moradali et al. 2007), xylomannan (Smiderle et al. 2006), and heterogalactans, which may contain *O*-methyl groups or a variety of side chains (Wasser 2002; Zhang et al. 2007) including manogalactans (Jakovljevic et al. 1998; Rosado et al. 2003; Smiderle et al. 2008b; Zhang et al. 2013), fucogalactans (Fan et al. 2006; Komura et al. 2010; Mizuno et al. 2000; Ruthes et al. 2012, 2013a; Zhang et al. 2006), and

fucomannogalactans (Alquini et al. 2004; Carbonero et al. 2008; Cho et al. 1998, 2011; Smiderle et al. 2006; Ruthes et al. 2013c).

Recent studies concerned to anti-inflammatory and antinociceptive effects evaluated the potential of crude extracts or isolated polysaccharides such as glucans, fucogalactans, fucomannogalactans, and mannogalactans isolated from different mushroom species such as *Agaricus brasiliensis* and *Agaricus bisporus* var. *hortensis* (Komura et al. 2010), *Agaricus bisporus* (Ruthes et al. 2012, 2013a), *Amanita muscaria* (Ruthes et al. 2013c), *Caripia montagnei* (Castro et al. 2014; Queiroz et al. 2010), *Flammulina velutipes* (Wu et al. 2010), *Geastrum saccatum* (Dore et al. 2007), *Inonotus obliquus* (Ma et al. 2013), *Lactarius rufus* (Ruthes et al. 2013b), *Lentinus edodes* (Carbonero et al. 2008), *Pleurotus pulmonarius* (Smiderle et al. 2008b), and *Rhodotorula mucilaginosa* (Valasques-Junior et al. 2014).

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It typically involves a noxious stimulus or experience that activate nociceptors in the body's tissues, which deliver signals to the central nervous system, where they are processed and generate multiple responses. It could be divided into two main categories: acute and chronic pain. Acute or nociceptive pain involves the motor neurons of the central nervous system. It is mediated by nociceptors on A-δ and C fibers. Chronic pain, however, is considered a disease process itself. There are two types of chronic pain, the inflammatory nociceptive pain and the neuropathic pain. Inflammatory nociceptive pain is associated with tissue damage and the resulting inflammatory process, while neuropathic pain is produced by damage to the neurons in the peripheral and central nervous systems and involves sensitization of these systems (Campbell and Meyer 2006).

On the other hand, inflammation itself is a host defense mechanism to eliminate invading pathogens and to initiate healing process. Uncontrolled or overproduction of inflammatory products can lead to injury of host cells, chronic inflammation, chronic diseases, and also neoplastic transformation (Mantovani et al. 2008). During inflammatory process, the inflammatory biomarkers are highly produced such as reactive oxygen species (ROS), reactive nitrogen species (RNS), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and cyclooxygenase (COX)-2.

Therefore, taking steps to ease inflammation is an effective means of interfering with the process of pain sensitization. This is why drugs like acetaminophen and ibuprofen, which are anti-inflammatory, relieve pain. Unfortunately, though these drugs and others like them are very effective for reducing inflammation and pain, they often cause side effects, which compromise their long-term risk versus benefit profile.

A variety of natural anti-inflammatory compounds are able to target inflammation by reducing the synthesis of inflammatory mediators or modulating inflammatory pathways. Natural polysaccharides from mushrooms represent a source of natural compounds able to target inflammation and alleviate nociception showing promising results in recent studies.

Thus, the aim of the present text is to explore the antinociceptive and anti-inflammatory bioactivity of natural polysaccharides from mushrooms and review their mechanism of action, toxicity, bioavailability, and metabolism, besides describing other biological activities and the already done clinical trials.

2 Bioactivity

Regarding the anti-inflammatory and antinociceptive properties of mushroom polysaccharides, it is possible to infer that such polymers have shown pronounced therapeutic effects by experiments performed in vitro and in vivo (Chang et al. 2013; Kim et al. 2007; Komura et al. 2010; Queiroz et al. 2010; Ruthes et al. 2013c).

Although inflammation is a normal host response to repair tissue damage provoked by external stimuli, chronic inflammation can induce the host to develop various diseases, including cancer (Li et al. 2014; Wu et al. 2014). The assessment of anti-inflammatory compounds is in continuous update, and besides the evident immunomodulatory properties of mushroom polysaccharides, it was observed that they can also act against inflammation (Castro et al. 2014; Fangkrathok et al. 2013; Wu et al. 2010). In vitro analysis showed that neutral and sulfated polysaccharides extracted from *Armillariella mellea* inhibited the secretion of TNF-α and IL-6 by RAW 264.7 macrophages when stimulated with lipopolysaccharide (Chang et al. 2013). As some in vivo examples, an extract from *Caripia montagnei* containing 98.7 ± 3.3 % of carbohydrates, which was 63.3 ± 4.1 % composed of β-D-glucans, was tested (Castro et al. 2014; Queiroz et al. 2010). The extract reduced the inflammatory infiltrate produced by thioglycolate-induced peritonitis by 75.5 ± 5.2 % when administered to mice; furthermore, a decrease in cytokine production was observed (Queiroz et al. 2010). In rats, the same extract inhibited the carrageenan-induced paw edema by 90.7 ± 3.5 % (Castro et al. 2014). Another polysaccharide extract containing glucose, mannose, and xylose, obtained from *Flammulina velutipes*, was administered to burned rats. The treatment showed an anti-inflammatory effect by the reduction of ICAM-1 concentrations and MPO activity and by the increase of the anti-inflammatory cytokine IL-10 in the serum (Wu et al. 2010).

Purified polysaccharides have also demonstrated strong anti-inflammatory activity. A fucomannogalactan and a β-D-glucan isolated from *Amanita muscaria* were effective against the inflammatory pain of formalin-induced nociception in mice (Ruthes et al. 2013c). The nociception and inflammation can be evaluated in mice/rats by a well-established approach which consists in the injection of formalin, and the nociception can be observed in two distinct phases: neurogenic pain (early phase) and inflammatory pain (late phase) (Tjolsen et al. 1992). This method is an alternative for the problems encountered to evaluate the nociception because there are several limitations in the use of human patients to assess pain mechanisms and pathways. Therefore, in vitro studies and research on animals are more frequently used; however, they do not eliminate another limitation: the investigator can only

speculate on the experience of the animal and must instead use secondary behavioral indicators to infer pain (Hogan 2002).

Homopolysaccharides, as β-D-glucans isolated from *L. rufus* and *P. pulmonarius*, were tested in mice using the formalin-induced nociception method (Tjolsen et al. 1992). In both studies, a slight inhibition of the neurogenic pain (early phase) at the higher doses (30 mg kg^{-1}) was observed , while the greater analgesic effect was observed on the late phase (inflammatory pain) at the same or lower doses (Ruthes et al. 2013b; Smiderle et al. 2008a). These studies showed that the analgesic effect demonstrated by the mushroom β-D-glucans is probably related to an anti-inflammatory action, being similar to that of nonsteroidal anti-inflammatory drugs. The strong analgesic effect of the β-D-glucan isolated from *P. pulmonarius* was confirmed by other methods as nociception is induced by glutamate, capsaicin, cinnamaldehyde, menthol, and acidified saline (Baggio et al. 2010, 2012).

Heteropolysaccharides isolated from *L. edodes*, *Agaricus* spp., and *P. pulmonarius* have also demonstrated an analgesic effect. The abdominal constriction caused by the intraperitoneal injection of acetic acid was inhibited in mice that received the heteropolysaccharide injections 30 min before. A fucomannogalactan from *L. edodes* (Carbonero et al. 2008), two different fucogalactans from *Agaricus* spp. (Komura et al. 2010), and a mannogalactan from *P. pulmonarius* (Smiderle et al. 2008b) were tested. Comparing these studies, it can be suggested that the bioactivity of the polysaccharide is closely related to its chemical structure, because these four compounds exhibited different effects, and although these polymers present the same main chain structure [α-D-Gal*p*-(1→6)-linked], they presented different branching degrees and substitutions, as fucose, mannose, galactose, and methyl groups. Furthermore, two of them have also diminished the leukocyte migration and the peritoneal capillary permeability, showing a pronounced anti-inflammatory activity (Carbonero et al. 2008; Komura et al. 2010).

There is a variety of methods to study nociception responses in animals, although, as cited previously, the results may be subjective and based on observations on the animal behavior. Taking into account that the analgesic properties of mushroom polysaccharides are closely related to anti-inflammatory effects, it is strongly recommended to evaluate both bioactivities to determine their mechanism of action.

3 Mechanism of Action

It is well established that a lot of known and tested polysaccharides isolated from various natural sources, particularly from mushrooms, occupy a notorious position in turn of their biological potential. This fact should possibly be due to their very low or absent toxicity.

The benefits of mushroom compounds on different clinical conditions have attracted the interest of the scientific community in the last decade in order to understand the molecular mechanisms responsible for their actions.

β-D-Glucans are undoubtedly the most exploited polysaccharide structures by researchers, regarding the elucidation of their chemical structure and their probable biological potential and mechanisms of action. Despite long-term interest and research, the mechanism of how β-D-glucans affect health remained a mystery for a long time. Only in the last decade, extensive research has helped to reveal the effects that β-D-glucans have on the immune system. However, little is known about the mechanisms of action of β-D-glucans or other polysaccharide structures in relation to the antinociceptive and anti-inflammatory effects. The probable mechanisms of action of mushroom natural polysaccharides on nociception and inflammation studied to date will be given in the following.

The pain sensation acts as a warning device that alerts an organism to the presence of damaging stimuli. Although acute pain has a protective role as a warning system (Julius and Basbaum 2001), chronic pain, as neuropathic pain, is produced by dysfunction or damage to the peripheral or central nervous system (Somers and Clemente 2009; Zimmermann 2001). Frequently, tissue damage leads to the activation of nociceptors through the release of diverse chemical mediators such as excitatory amino acids, protons, peptides, lipids, and cytokines, which act on specific receptors and ion channels to various signal transduction cascades (Sawynok 2003).

Smiderle et al. (2008a) demonstrated that GL, a (1→3, 1→6)-linked β-D-glucan isolated from *P. pulmonarius*, inhibits abdominal contractions induced by acetic acid with a concomitant reduction of leukocyte migration to the peritoneal cavity and plasmatic extravasation. It was also shown that GL inhibits both the first and second phases of formalin-induced licking (Smiderle et al. 2008a). Acetic acid and formalin are thought to act through the release of endogenous inflammatory mediators such as bradykinins, glutamate, SP, prostanoids, and cytokines (TNF-α, IL-1β, and IL-8), which stimulate primary sensory neurons (Collier et al. 1968; Ikeda et al. 2001; Ribeiro et al. 2000; Vinegar et al. 1979). Thus, these previous findings suggest that the possible antinociceptive action of GL could be due to the inhibition of the release of endogenous mediators (i.e., glutamate, TNF-α, or IL-1β).

Baggio et al. (2010), extending their findings on GL possible mechanisms of action, investigated more evidence concerning the participation of the glutamatergic system on antinociception caused by GL. Glutamate is a major excitatory neurotransmitter involved in the transmission of nociceptive signals. Furthermore, the nociceptive neurons activated by glutamate may release several inflammatory mediators and neuropeptides that could also be involved on nociceptive transmission both in the central and peripheral nervous system (Fundytus 2001; Millan 1999). GL effectively inhibits the nociceptive response induced by the intraplantar injection of glutamate into the mouse hind paw. The nociceptive response induced by glutamate, which acts through the glutamate receptors, is present in the peripheral, spinal, and supraspinal sites of action (Beirith et al. 2002). So, GL antinociception may arise from the inhibition of ionotropic or metabotropic glutamate receptors. Furthermore, animals were intraperitoneally treated, and GL showed to inhibit the nociceptive response caused by NMDA, AMPA, and kainate. However, GL did not inhibit the nociceptive response induced

by intrathecal injection of trans-ACPD, a nonselective metabotropic glutamate receptor agonist. The potency of GL to inhibit ionotropic glutamate receptor-induced nociception is important because ionotropic glutamate receptors are involved in several pain conditions (Bleakman et al. 2006). Moreover, there is evidence indicating the interaction of substance P (SP) and glutamate at the spinal level. Behavioral changes observed after spinal administration of SP are potentiated by the coadministration of NMDA and kainite (Mjellem-Joly et al. 1991, 1992). In turn GL does not reduce the nociceptive response induced by the intrathecal injection of SP. These observations suggest that the antinociceptive action of GL could be regulated by ionotropic glutamate receptor pathways that modulate nociceptive transmission at the spine (Baggio et al. 2010).

The proinflammatory cytokines are also involved in the modulation of nociceptive processes, and the ability of cytokines, such as TNF-α and IL-1β, to induce nociceptive behavior when administered intrathecally has been attributed to the induction of glutamate and SP release from nerve terminals (Choi et al. 2003; Tadano et al. 1999). For this reason, the effect of GL against the TNF-α- and IL-1β-induced nociceptive responses in mice was evaluated. Authors demonstrated that the treatment of animals with GL inhibits the nociceptive response induced by IL-1β, suggesting the inhibitory activity of GL on events triggered by IL-1β (Baggio et al. 2010). The glutamatergic system and proinflammatory cytokine pathways play important roles in acute pain. Likewise, the glutamatergic system and proinflammatory cytokines, such as IL-1β and TNF-α, participate in the establishment or maintenance of chronic pain (Sawynok 2003; Zimmermann 2001). The next step of the study was to use an experimental model of mononeuropathy produced by partial sciatic nerve ligation (PSNL) in mice, which mimics important symptoms observed in patients with peripheral neuropathy and is used in behavioral research (Paszcuk et al. 2007).

Peripheral nerve injury is often accompanied by transient local inflammation, which probably contributes to the instigation of neuropathic pain. Several inflammatory mediators, such as prostaglandin E2 (PGE2), bradykinin, nerve growth factor, histamine, serotonin, IL-1β, TNF-α, ATP, glutamate, endothelin-1, and various chemokines, can be released from damaged axons or Schwann cells or from satellite cells, mast cells, and infiltrating leukocytes (Ji and Strichartz 2004). Interestingly, the results demonstrated that GL significantly reverses mechanical allodynia caused by PSNL (Baggio et al. 2010). Moreover, the anti-allodynic response caused by GL was not susceptible to tolerance. This conclusion is based on data showing that (1) the withdrawal of GL was followed by complete return to baseline allodynia and (2) the intraperitoneal treatment with GL twice a day produced very similar and pronounced anti-allodynic effects. This finding allowed authors to infer that the anti-allodynic effect of GL involves the inhibition of the glutamatergic system, ionotropic glutamate receptors, and the proinflammatory cytokine IL-1β (Baggio et al. 2010).

Finally, the open-field test was used to exclude the possibility that the antinociceptive action of GL could be related to nonspecific disturbances in the locomotor activity of the animals. Authors observed that at doses that have

antinociceptive action, GL did not alter the motor performance of mice (Baggio et al. 2010). In this regard, it has been reported that several drugs that antagonize glutamate receptors produce significant disturbances in locomotor activity (Jordan et al. 2008).

Collectively, the results of Baggio et al.'s (2010) research confirm the data about the antinociceptive properties of GL on acute or chronic pain. It was demonstrated that systemic treatment with GL provides a pronounced inhibition of acute nociceptive responses induced by chemicals (EEAs, SP, and proinflammatory cytokines). Furthermore, prolonged systemic treatment of animals with GL is effective in preventing persistent mechanical allodynia caused by partial sciatic nerve ligation in mice.

A better understanding of the mode of action of GL would enhance the potential therapeutic interest on this naturally occurring β-D-glucan. Several ion channels play an essential role in the ability of nociceptors (C and A fibers) to detect and discriminate noxious thermal, mechanical, and chemical stimuli and convey this sensory information to second-order nociceptive neurons of the dorsal horn of the spinal cord (McCleskey and Gold 1999). GL effectively inhibited nociceptive responses induced by intraplantar injections of capsaicin or cinnamaldehyde, which are highly selective activators/agonists of TRPV1 and TRPA1 channels, respectively, as well as menthol (a preferential TRPM8 channel agonist that can also activate TRPA1 channels at higher concentrations) or acidified saline (which activates TRPV1 and ASIC channels) (Baggio et al. 2012). This study confirmed that the nociceptive responses elicited by capsaicin, cinnamaldehyde, and acidified saline were extensively inhibited by prior treatment with antagonists of their respective specific or preferential targets, i.e., ruthenium red (TRPV1), camphor (TRPA1), and amiloride (ASIC). Due to the lack of a suitable selective antagonist, authors were unable to determine if responses to menthol resulted from activation of TRPM8 channels (Baggio et al. 2012). In addition, the potency of GL in inhibiting cinnamaldehyde-induced nociception is in excellent agreement with that previously observed in the model of formalin-induced hind paw nociception (Smiderle et al. 2008a), especially considering that formalin is an activator of TRPA1 channels (McNamara et al. 2007). These results strengthen considerably the evidence that GL is an effective analgesic in rodents (Baggio et al. 2012). However, it appears most unlikely that GL induces antinociception by selectively antagonizing the binding of capsaicin, cinnamaldehyde, menthol, or protons to their respective target channels, which was supported by the no alteration of nociceptive responses when the GL was coadministered with these activators/agonists (Baggio et al. 2012). It was demonstrated that capsaicin, stimulating TRPV1 receptor, mediates the release of several neurotransmitters, including glutamate; besides, menthol (TRPM8 agonist) and allyl isothiocyanate (TRPA1 agonist) enhance spontaneous glutamatergic synaptic transmission onto lamina II neurons in spinal cord slices (Baccei et al. 2003; Kosugi et al. 2007; Medvedeva et al. 2008). Indeed, it was previously demonstrated by the same research group that GL inhibited the nociceptive responses induced by injection of intrathecal excitatory amino acids (Baggio et al. 2010), suggesting that the GL effect seems to be partially related to

the inhibition of glutamate release in the dorsal horn after peripheral stimulation of TRPs (Baggio et al. 2012). It was also shown that GL effectively inhibited nociceptive responses to intraplantar injection of PMA, a direct activator of PKC (Ferreira et al. 2008). Given the extensive evidence implicating PKC in signaling mechanisms leading to nociception and hyperalgesia (Souza et al. 2002), Baggio et al. (2012) hypothesized that this could constitute a potentially relevant target for the antinociceptive action of GL. In this regard, using Western blot analysis, they obtained ex vivo evidence that intraplantar PMA injection clearly promoted the translocation of PKC from the cytosolic to the membrane-enriched fractions of hind paw skin homogenates, as would be expected of a PKC activator (Ferreira et al. 2008). More importantly, prior i.p. treatment of the animal with GL, at a dose promoting effective analgesia, fully prevented this PMA-induced PKC translocation (Baggio et al. 2012). In addition, authors found that GF109203X, a selective PKC inhibitor (Ferreira et al. 2008), inhibited nociceptive responses induced by i.pl. injection of capsaicin and menthol (Baggio et al. 2012). However, unlike GL, GF109203X did not influence responses induced by either cinnamaldehyde or acidified saline (Baggio et al. 2012). As the phosphorylation of TRPV1 by PKC sensitizes this channel to activation by capsaicin (Bhave et al. 2003), blockade of this process by GL might account for the analgesic effect of GL (and GF109203X) against capsaicin-induced nociception (Baggio et al. 2012). Nonetheless, this does not appear to be a plausible explanation for its inhibitory effects on nociception induced by menthol, cinnamaldehyde, or protons (i.e., acidified saline), as PKC actually dephosphorylates and downregulates TRPM8 channels (Premkumar et al. 2005), does not contribute to the sensitization of TRPA1 channels (Wang et al. 2008), and appears to phosphorylate and sensitize only ASIC channels containing ASIC2 subunits (Baron et al. 2002; Deval et al. 2004), which are poorly expressed by DRG neurons (Ugawa et al. 2004). It is interesting to note, however, that the antinociceptive profile of action of GF109203X (i.e., more effective against responses induced by menthol than capsaicin and inactive against cinnamaldehyde or acidified saline) was remarkably similar to that of GL, which displayed an ID_{50} against nociception triggered by menthol that was 42-, 72-, and 156-fold lower than those obtained against responses induced by capsaicin, cinnamaldehyde, and acidified saline, respectively (Baggio et al. 2012). Therefore, the collected evidence strongly suggests that the antinociceptive effects of GL are associated, at least to a significant extent, to the inhibition of PKC activation (Baggio et al. 2012).

In conclusion, Baggio et al. (2012) demonstrated that GL, a naturally occurring β-D-glucan from *P. pulmonarius*, displays pronounced systemic antinociceptive properties in chemical models of nociception models in mice. The mechanisms underlying GL-induced antinociception appear to involve inhibition of PKC. It remains to be determined if GL also has other potentially relevant targets for its antinociceptive actions and if it is a selective inhibitor of PKC or can also block activity of other PKC isoforms.

The research using GL showed to be the deeper study regarding the mechanism of action of a natural mushroom polysaccharide on nociception and inflammation

published till now (Baggio et al. 2010, 2012). Other researches evaluating the potential of D-glucans or heteropolysaccharides, especially heterogalactans as antinociceptive and anti-inflammatory compounds, just make suggestions for possible mechanisms of actions.

Smiderle et al. (2008b) demonstrated that a mannogalactan (MG) from *P. pulmonarius* and indomethacin gave rise to a similar analgesic effect, while the inflammatory response, characterized by leukocyte infiltration and peritoneal capillary permeability, was inhibited by dexamethasone and indomethacin, but not MG. Such results could indicate that MG showed an antinociceptive action, independent of an anti-inflammatory activity, in the acetic acid-induced writhing test. The mechanism by which MG produces antinociception still remains unclear, but the inhibition of cytokine proinflammatory release or glutamate in the cerebrospinal fluid increase seems to explain, at least in part, its analgesic effects.

Studying some structural different heterogalactans from *L. edodes* (Carbonero et al. 2008), *A. brasiliensis* and *A. bisporus* var. *hortensis* (Komura et al. 2010), *A. bisporus* (Ruthes et al. 2012, 2013a), *Lactarius rufus* (Ruthes et al. 2012), and *Amanita muscaria* (Ruthes et al. 2013c), it was demonstrated that tested polysaccharide fractions have significant antinociceptive and anti-inflammatory properties using the model of abdominal constrictions induced by acetic acid (Carbonero et al. 2008; Komura et al. 2010) which, additionally, involves endogenous inflammatory mediators or the formalin-induced pain model in mice (Ruthes et al. 2012, 2013a).

The formalin test is a satisfactory and comprehensive model for evaluating the antinociceptive activity of drugs. The intraplantar injection of formalin activates nociceptive nerve terminals and produces neurogenic pain, whereas inflammatory pain is mediated by a combination of peripheral input and spinal cord sensitization (Hunskaar and Hole 1987; Tjolsen et al. 1992). Moreover, it has been demonstrated that the intraplantar injection of formalin in rodents increases the spinal levels of excitatory amino acids, PGE2, nitric oxide, tachykinin, and kinins, among other peptides (Malmberg and Yaksh 1995; Santos and Calixto 1997; Santos et al. 1998; Tjolsen et al. 1992). Experimental data indicate that formalin predominantly evokes activity in C-fiber peroxidase (Tjolsen et al. 1992), although A fibers are thought to be responsible for fast nociceptive transmission in the first phase of the pain response (Julius and Basbaum 2001). It is notable that the nociception produced by formalin (neurogenic phase) is quite resistant to the majority of NSAIDs, such as acetylsalicylic acid, indomethacin, paracetamol, and diclofenac. However, these drugs can dose-dependently attenuate the inflammatory phase of formalin-induced licking (Hunskaar and Hole 1987; Malmberg and Yaksh 1992; Santos et al. 1998).

Ruthes et al. (2013a) also examined the effects of *A. bisporus* fucogalactan (EFP-*Ab*) on iNOS and COX-2 expression in the ileum of septic mice by immunoblotting. EFP-*Ab* decreased both iNOS and COX-2 expression by 53 % and 54 %, respectively. Dexamethasone also affected both iNOS and COX-2 expression, reducing by 74.5 % and 71.4 %, respectively. These results strongly confirmed the anti-inflammatory activity of EFP-*Ab*.

The enzymes iNOS and COX-2 are both upper expressed during proinflammatory events including sepsis. The iNOS (inducible nitric oxide

synthase), once expressed, produces high amounts of NO over long periods of time, which causes cellular damage. It is also associated with the septic shock, considered the main cause of mortality among the septic patients (Landry and Oliver 2001). Compounds that inhibit iNOS expression or iNOS activity have anti-inflammatory properties (Tinker and Wallace 2006). In turn, COX-2 is the inducible isoform of the cyclooxygenase enzyme that catalyzes the production of inflammatory prostanoids. Systemic COX-2 is increasingly recognized as an important player in sepsis-induced inflammation. In fact, COX-2-deficient mice are protected from sepsis-induced inflammation and death (Ejima et al. 2003).

The inflammatory response is induced by various agents, resulting in tissue damage and triggering immune activities (Castro et al. 2014). These responses can be enhanced by a cyclic process, promoting angiogenesis-inducing effects, such as facilitation of tumor growth and metastasis. The inflammatory process exposes the body to prolonged levels of ROS, leading to the accumulation of oxidative damage in tissues (Ferguson 2010). A class of receptors linked to the regulation of inflammation and angiogenesis are peroxisome proliferator-activated receptors (PPAR), which regulate expression in many genes, including COX-2, nitric oxide synthase, and vascular endothelial growth factor (VEGF), among others (Dupont et al. 2008). Angiogenesis also stimulates the growth and development of tumor cells. This is considered a step in cancer therapy, where the study of angiogenesis inhibitors is of great importance (Pitt et al. 2004). Inflammation also exposes the body to prolonged levels of ROS, causing an accumulation of oxidative damage in tissues. Prooxidant compounds are part of the immune response against pathogens (Costantini and Møller 2009).

Nitric oxide, a short-lived free radical produced by enzyme nitric oxide synthase (NOS), acts as an effector of vasodilatation in the inflammatory process. It is converted to a reactive nitrogen species, such as peroxynitrite, by reacting with superoxide and/or oxygen (Luca and Olefsky 2008). Inhibition of these reactive oxygen species is important in reducing inflammation. Several authors suggest that receptors activated by peroxisome proliferators (PPARs) act as modulators of inflammation both in vivo and in vitro (Castro et al. 2014). However, the mechanisms of this process are still unclear. Moreover, research on paw edema inhibition has demonstrated that the activation of PPAR receptors may also contribute to antiangiogenic action of its ligands (agonists), which, once activated, inhibit angiogenesis through the modulation of cytokines (Keshamouni et al. 2005; Queiroz et al. 2010). The search for antioxidant compounds, inhibitors of inflammation and angiogenesis, is of great medical importance due to their potential as potent suppressors of tumor proliferation, among others (Dong and Yao 2008).

Inflammation and angiogenesis are two dependent processes regulated by macrophages. These produce a series of potent angiogenic factors, such as the vascular endothelial growth factor (Farges et al. 2006; Wang et al. 2009). Although inflammation and the action of free radicals are correlated, studies remain scarce in the literature regarding mechanisms of action combining the processes of oxidation, inflammation, angiogenesis, and tumorigenesis. Castro et al. (2014) demonstrated that polysaccharides from *Caripia montagnei*, rich in D-glucans, have important

anti-inflammatory properties, indicating strong pharmacological potential for these polysaccharides. The findings suggest that these polysaccharides can act by a COX pathway and through PPAR to inhibit MPO and iNOS. The antiangiogenic extract of *C. montagnei*, rich in β-D-glucans, does not occur as demonstrated via VEGF, but by a different pathway. Authors affirm in their previous research which showed that this polysaccharide inhibits NF-κB (Queiroz et al. 2010). Furthermore, they suggest that this polysaccharide's potential antitumor, antiagiogenic, and COX-2 inhibitor effects are due to its inhibitory actions on COX-2 and free radicals (Castro et al. 2014).

Other way to evaluate the anti-inflammatory potential of polysaccharides is the intrapleural administration of carrageenan, which implies the induction of an inflammatory process with increased neutrophil and mononuclear leukocyte number (Ke et al. 2009). The inflammation in the respiratory pathway induces histamine, thromboxane A2, leukotrienes, cytokines, and nitric oxide release (Wang et al. 2009).

The anti-inflammatory effect of D-glucans from *Geastrum saccatum* extract on carrageenan-induced pleurisy was studied by Dore et al. (2007). The results have revealed synergistic interaction between iNOS or COX inhibitors and glucan extract, which have led the authors to suggest that the anti-inflammatory effect of glucan extract from *G. saccatum* is mediated by inhibition of both NOS and COX (Dore et al. 2007).

Up to date, there is a lack of information regarding the mechanisms by which the mushroom polysaccharides exhibit anti-inflammatory and antinociceptive effects. More research on this field is required to clarify how these macromolecules act on the living organisms.

4 Toxicity

Considering that mushrooms are consumed as food, medicine, and, more recently, dietary supplements, it is important to determine their toxicity (Cheung 2013; Hardy 2008). Usually, the commercially available mushrooms present no or low toxicity; however, the great number of species requires a careful evaluation on their edibility and poisonous characteristics, considering that some of them were not described yet (Wasser 2011).

In vitro assays are frequently performed to determine the cytotoxicity of mushroom extracts, before continuing with further investigations on their medicinal properties. It was observed that aqueous extracts from mushrooms *Ganoderma lucidum*, *Lignosus rhinocerotis*, *Pleurotus giganteus*, and *Grifola frondosa* showed absence of embryotoxic or neurotoxic effects when incubated with mouse embryonic fibroblast (BALB/3T3) and mouse neuroblastoma (N2a) cells. The same was observed for ethanol extracts obtained from *Cordyceps militaris* (Phan et al. 2013). Furthermore, a species from Taiwan, *Antrodia cinnamomea*, presented hepatoprotective effect, including anti-hepatitis, anti-hepatocarcinoma, and anti-alcoholism both in vitro and in vivo, showing that no hepatotoxicity was exhibited (Yue et al. 2013).

Regarding the consumption of mushrooms by humans, some adverse effects were observed on patients that received a treatment containing chemotherapy (tegafur/uracil) plus mushroom PSK, a protein-bound polysaccharide extracted from the basidiomycete *Coriolus versicolor*. It was observed that 15.1 % of patients had grade 1 or 2 hematological or gastrointestinal toxicity, although no grade 3 or 4 toxicity was observed. Symptoms of gastrointestinal toxicity were loss of appetite, nausea, vomiting, diarrhea, and stomatitis, while the hematological problems were hepatotoxic, neurotoxic, and dermatological effects. However, it was not proven that the PSK was the responsible for such dysfunctions (Ohwada et al. 2004).

A case of a 17-year-old male that suffered from a systemic anaphylactic episode was reported. The patient presented the anaphylactic reaction during exercise 50 min after the ingestion of mushrooms cooked with wine in a restaurant. Investigations on this case showed that an immunologic cross-reaction between the mushroom and other fungus from environment might have occurred, although it was not proven that the edible mushroom presented any toxicity by itself (Carrapatoso and Bartolome 2013).

The main problems concerning mushroom toxicity rely on their ability of absorbing pollutants from the environment. Mushrooms can be efficiently used as mycoremediation tools because they are able to degrade or bioconvert wastes/pollutants by their enzymatic machinery; however, they can also absorb these compounds, such as arsenic, lead, cadmium, mercury, copper, zinc, iron, nickel, and even ^{137}Cs (Firenzuoli et al. 2008; Hardy 2008; Kulshreshtha et al. 2014). Therefore, high levels of toxic compounds may offset whatever health benefits that a diet rich in mushrooms or mushroom supplements can provide. This shows the importance of cultivate mushrooms in a "clean" environment free of heavy metals and other pollutants.

5 Bioavailability and Metabolism

Although there are a lot of studies pointing to the beneficial health effects of mushrooms, there are few studies evaluating the absorption and pharmacokinetics of mushroom compounds (Rice and Adams 2005). Besides, the β-D-glucans are not the only bioactive compounds of mushrooms, and there are other nutrients that should be considered as possible biological active agents, such as proteins, triterpenes, phenolics, sterols, nucleosides, and mannitol (Cheung 2013; Ng and Wang 2005; Roupas et al. 2012). The main studies performed in vitro or in vivo use the whole milled mushrooms or hot water and/or alcoholic extracts, which are composed by hundreds of compounds in different proportions, making impossible to study their benefit individually. The main protocols required to obtain claims from national and international food agencies (e.g., ANSES and ESFA) are strongly modeled on the pharmacological approaches developed for drugs that aim to test the effect of a single molecule. These approaches are not compatible to assess supplements and nutraceutical foods such as mushroom extracts, considering that it

is not known whether the bioactive effects are caused by a single component or are a result of a synergistic impact from several ingredients (Fardet and Rock 2014; Wasser 2011).

From the published data, it is possible to infer that the oral administration of mushroom β-D-glucan extracts may be the best via, because these polysaccharides are not digested by the animal or human enzymes, passing almost intact through the digestive tract. In the intestines, the β-D-glucans can be absorbed by the M cells located among the epithelial cells and get in contact with macrophages of the gut-associated lymphatic tissue (GALT) (Aouadi et al. 2009; Bouike et al. 2011; Rice and Adams 2005). Once in contact with immune cells, the β-D-glucans bind to membrane receptors as dectin-1, complement receptor 3, scavenger receptor, and Toll-like receptors, initiating the immunological responses (Aouadi et al. 2009; Chen and Seviour 2007; Rice and Adams 2005; Underhill 2003).

The main concern is that mushrooms are composed by different β-D-glucans, which present linear or branched structures, and they are not completely purified for the analysis of biological effects or even bioavailability. It is required to have more studies on this field to determine which component(s) is (are) responsible for the therapeutic effects of mushrooms and also to evaluate if the mushroom capsules/extracts on the food supplement market are really effective, considering their bioavailability.

6 Other Biological Activities

In addition to the antinociceptive and anti-inflammatory effects already mentioned, mushroom polysaccharides, specially β-D-glucans, have been shown to have other numerous biological effects. The most studied are surely their effects as immunomodulators and in cancer therapy.

Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunomodulating properties. Although the process of isolation, structural characterization, and antitumor activity of mushroom polysaccharides have been extensively investigated in the past three decades, the relationship between the antitumor activity and the chemical composition as well as the high-order structure of their active components is still not well established.

Antitumor effects, primarily in human cell lines, have been reported from polysaccharides extracted from various mushrooms. The polysaccharides generally belong to the β-D-glucan family and appear to exert their anti-tumorigenic effects via enhancement of cellular immunity. The antitumor action of polysaccharides is mediated through a thymus-dependent immune mechanism, stimulating the immune system via effects on NK cells, macrophages, monocytes, neutrophils, and dendritic cells and via T cells and their cytokine production.

Cytokines in turn activate adaptive immunity through the promotion of B cells for antibodies production and stimulation of T-cell differentiation to T helper (Th) 1

and Th2 cells, which mediate cell and humoral immunities, respectively (Borchers et al. 2008). Based on their high molecular weight, mushroom polysaccharides are not able to penetrate the immune cells to activate them directly. The stimulation mechanism of polysaccharides involves different cell receptors such as dectin-1, complement receptor 3 (CR3), lactosylceramide (LacCer), and Toll-like receptor (TLR) 2. Thus, the effectiveness of polysaccharide immunomodulatory effects is governed by their binding affinity to immune cell receptors (Chen and Seviour 2007).

Immune-active compounds isolated from more than 30 medicinal mushroom species have demonstrated antitumor activity in animal treatments. However, only a few have been tested for their anticancer potential in humans, mainly β-D-glucans or β-D-glucan-protein complexes. The main clinical studies proving the cancer inhibitory effects have been done showing the inhibitory effects caused by polysaccharides isolated from *Lentinus edodes* (Jeff et al. 2013a, b; Mizuno 1997; Yu et al. 2009), *Grifola frondosa* (Adachi et al. 1994; Cun et al. 1994; Mizuno 1997; Zhuang et al. 1994), *Schizophyllum commune* (Zhang et al. 2013), *Ganoderma lucidum* (Mizuno 1997; Wang et al. 2002), *Trametes versicolor* (Chow et al. 2003), *Inonotus obliquus* (Fan et al. 2012; Kim et al. 2005a, 2006; Mizuno et al. 1999), *Phellinus linteus* (Han et al. 1999; Kim et al. 2004; Li et al. 2004, 2013), *Flammulina velutipes* (Leung et al. 1997; Yang et al. 2012; Zeng 1990), and *Cordyceps sinensis* (Leung et al. 2009; Nie et al. 2013), among others (Zhang et al. 2007; Zong et al. 2012).

Five polysaccharide constituents from mushrooms have shown significant anticancer efficacy against several human cancers in clinical trials as biological response modifiers, including lentinan from *Lentinus edodes*, D-fraction from *Grifola frondosa*, schizophyllan from *Schizophyllum commune*, and polysaccharide-K (PSK) and polysaccharide peptide (PSP) from *Trametes versicolor* (Wasser 2002). Such polymers have been developed with clinical and commercial purposes.

Other biological activities presented by natural polysaccharides from mushrooms are the antioxidant, radical scavenging, cardiovascular, antihypercholesterolemic, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective, and antidiabetic effects (Dai et al. 2009; Gao et al. 2002, 2003, 2004; Ichinohe et al. 2010; Rowan et al. 2003; Sullivan et al. 2006; Wasser 2010; Zhang et al. 2007).

The antioxidative activity of polysaccharides is attributed to their ability to scavenge free radicals, their reduction property and ability to chelate Fe^{2+} ions, lipid peroxidation inhibition, erythrocyte hemolysis, and the increase of activities in eukaryotic as well as in prokaryotic cells of enzymes that take part in antioxidative processes, such as SOD, CAT, and GPx (Guo et al. 2009; Liu et al. 2010; Lo et al. 2011; Ping et al. 2009; Tseng et al. 2008). In some researches, besides the increase in the enzyme activity, antioxidative properties are also attributed to the property of polysaccharides to induce an increase expression of antioxidative enzymes, such as SOD and GPx (Jia et al. 2009).

The ability of polysaccharides to scavenge free radicals depends on its size and may be conditioned by the presence of hydrogen from specific, certain monosaccharide units, and the type of their binding in side branches of the main chain.

The most studied mushroom regarding the antioxidant potential of its polysaccharides was *L. edodes*, especially lentinan (Liu et al. 2014). Polysaccharide fractions from *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum*, and *Phellinus linteus* also presented antioxidant effects and are shown to be composed mainly by D-glucans (Kozarski et al. 2011).

Some animal studies, using both normal and diabetic animals, have demonstrated a hypoglycemic effect of mushrooms and mushroom components. This effect appears to be mediated via mushroom polysaccharides, possibly both α- and β-D-glucans, via a direct interaction with insulin receptors on target tissues, although the mechanism remains to be confirmed (Hsu et al. 2007; Kim et al. 2010; Roupas et al. 2012; Vetvicka and Novak 2011).

Aqueous extracts of various mushrooms – *Agaricus blazei* (Kim et al. 2005), *G. lucidum* (Seto et al. 2009), *P. pulmonarius* (Badole et al. 2006), *G. frondosa* (Cui et al. 2009), and *Coprinus comatus* (Han and Liu 2009) – have been shown to possess hypoglycemic activity and antihyperglycemic activity against diabetes-inducing compounds in obese and diabetic animal models. However, the consistency between the effects of the mushroom extracts in diabetic animal models and the preliminary data from human trials, which mirror decreases in plasma glucose, blood pressure, and cholesterol and triglyceride concentrations, strengthen the level of evidence for anti-diabetogenic effects of the studied mushrooms and their extracts.

Mushrooms and mushroom components have been reported to have an innumerable positive health benefits, mainly on the basis of in vitro and in vivo animal trials. However, the majority of these effects are indirect, being caused probably due to a stimulation or modulation of natural cellular immunity. Mushrooms and mushroom components exert many of their positive effects on health via a balance of T helper cells, the induction of interferon-gamma and certain interleukins, or NO-mediated mechanisms. Many of these immunomodulating effects are due to the polysaccharide content of mushrooms, either from β-D-glucans or from polysaccharide-protein complexes (Roupas et al. 2012).

Unfortunately, most studies are made with crude polysaccharide extracts, thus not allowing the knowledge of the component responsible for such effects. There is a lack of information regarding the relation between the polysaccharide structure and its biological effect.

A wide range of biologically active polysaccharides could be found among mushrooms, and their practical application is dependent not only on their unique properties but also on biotechnological availability. Isolation and purification of polysaccharides from mushroom material are relatively simple and straightforward. Once a molecule of interest is found and characterized and have its biological application confirmed, mycelia formed by growing pure cultures in submerged conditions are of constant composition and appear as the best technique for obtaining consistent and safe mushroom products (Reshetnikov et al. 2001; Wasser 2011).

7 Clinical Trials

The human clinical trials performed with mushrooms and mushroom extracts up to date evaluated mainly their anticancer properties, considering that mushroom compounds are immunoceuticals. β-D-Glucan extracts from *L. edodes* and *S. commune* are used in traditional medicine for cancer treatment in Japan since 1980, although little was published about their effects in humans (Akramien et al. 2007; Kidd 2000). The human trials carried out to date have primarily been smaller observational studies, or studies without appropriate placebo or other matched controls, and therefore larger, double-blind, placebo controlled human studies are required before clear effects on human health outcomes can be substantiated (Roupas et al. 2012).

Scientific publications about some clinical trials were released from 1976 to 1999 and the more recent publications date from 2004 to 2011. The investigators carried out their studies mainly in hospitals of Japan, China, and Korea, where the mushrooms have been used for many years as traditional medicine for treating various diseases (Abrams et al. 2011; Ahn et al. 2004; Kidd 2000; Ohwada et al. 2006). However, these fungi are not recognized as medicine in Occidental countries yet, and significant questions still arise about their safety, standardization, regulation, efficacy, and mechanism of action (Deng et al. 2009; Wasser 2011).

One of the trials evaluated a mushroom extract of *A. blazei Murill* Kyowa (ABMK) that was administered (three packs/day) to gynecological cancer patients undergoing chemotherapy for 3 weeks. The natural killer cell activity was significantly higher in the ABMK-treated group as compared with non-treated placebo group. Besides, the chemotherapy-associated side effects (appetite, alopecia, emotional stability, and general weakness) were reduced by the ABMK treatment, and the welfare of patients was improved. However, no significant difference in lymphokine-activated killer cell and monocyte activities was observed (Ahn et al. 2004).

Patients that presented advanced lung cancer received 1,800 mg of Ganopoly, three times daily before meals for 12 weeks, totalizing 5,400 mg day^{-1}. Each capsule of Ganopoly contained 600 mg of hot water extract of *G. lucidum*, with 25 % (wt/wt) crude polysaccharides. In the 30 cancer patients who completed the trial, treatment with Ganopoly did not significantly alter the mean mitogenic reactivity to phytohemagglutinin; mean counts of CD3, CD4, CD8, and CD56; mean plasma concentrations of IL-2, IL-6, and interferon (IFN)-γ; or natural killer cell activity in the patients, but the results were significantly variable. The data obtained of this study suggested that subgroups of cancer patients might be responsive to Ganopoly in combination with chemotherapy/radiotherapy; therefore, this trial was not conclusive (Gao et al. 2005).

P. ostreatus was also used, although, in this trial, the aim was to assess the safety and efficacy of the mushroom in reducing the lipid levels of patients with HIV undergoing antiretroviral treatment (ART). During the study, patients received packets of freeze-dried *P. ostreatus* (15 g day^{-1}) to be administered orally

for 8 weeks. Lipid levels of the patients were drawn every 2 weeks, and it was concluded that the mushroom did not lower non-HDL cholesterol in that patients (Abrams et al. 2011).

One of the most studied mushrooms as human therapeutics is *C. versicolor* from which was isolated two polysaccharide peptides or proteoglycans, named PSK and PSP. The latter was coadministered with *Salvia miltiorrhiza* to treat nasopharyngeal carcinoma and breast cancer patients. For the nasopharyngeal carcinoma (NPC) study, 27 patients histologically proven NPC were recruited to take PSP (3.6 g day^{-1}) plus *S. miltiorrhiza* (1.4 g day^{-1}) in the form of 12 combination capsules or placebo for 16 weeks. The normal decrease of T lymphocyte count in NPC patients was alleviated by the treatment with the PSP + *S. miltiorrhiza* (Bao et al. 2006). On the other trial, 82 patients with breast cancer were recruited to take PSP (50 mg kg^{-1} body weight) plus *S. miltiorrhiza* (20 mg kg^{-1} body weight) capsules every day for a total of 6 months. The investigators observed that the absolute counts of T helper lymphocytes (CD4+), the ratio of T helper (CD4+)/T suppressor and cytotoxic lymphocytes (CD8+), and the counts of B lymphocytes were significantly elevated in patients with breast cancer after receiving the treatment (Wong et al. 2005).

PSK was also evaluated about its effects on patients at stage II or III colorectal cancer, and the authors observed that the administration of 3 g of PSK plus chemotherapy reduced the recurrence of cancer, decreased the mean serum immunosuppressive acidic protein (IAP) level, and increased the mean population of natural killer cells (Ohwada et al. 2004, 2006).

There are plenty of studies using milled mushroom capsules and others evaluating the effects of mushroom extracts, although none of them investigated the isolated compounds as the bioactive β-D-glucans, for example. More standardization of the production of these extracts is required, and also the clinical trials should be carefully designed to guarantee the analysis of the data, the efficacy of the treatment, and the mechanism of action of the bioactive compound(s). The clinical trials available up to date are not conclusive to develop new natural drugs. Besides, other parameters such as anti-inflammatory and antinociceptive effects were not assessed in humans yet.

8 Conclusion

The above information, compiled after careful revision of the research on mushroom polysaccharides, has shown the importance of such macromolecules for the treatment of many health problems, including inflammation and nociception.

It was observed that fungal polysaccharides and polysaccharide-protein complexes have been mainly investigated by presenting a variety of biological responses, such as antitumor, antioxidative, and immune stimulatory activities and more recently antinociceptive and anti-inflammatory effects. The therapeutic application of these polymers seems to be closely related to their chemical structures and tridimensional conformations, showing the great importance of the

chemical analysis to determine the fine structure of each polysaccharide. Some researchers noticed that small differences in the polymer structure may exhibit different effects, when the antinociceptive and anti-inflammatory properties were tested. Besides, it was observed that the analgesic effect of some polysaccharides may be related to their capacity of blocking the production or release of proinflammatory molecules and PKC activity. However, the mechanism of action behind the antinociceptive and anti-inflammatory effects of these polymers is still under discussion and requires further studies.

The development of the research on this field on the last decades has provided plenty of data regarding the medicinal benefits of mushroom polysaccharides, although there are some limitations that must be transcended. The main studies are still based on the crude polysaccharide extracts, making difficult to define which compound is the responsible for the bioactivity. There are no standard procedures on the preparation of the extracts, and there are fewer authors who isolated the molecules for their assessments. Furthermore, the clinical trials and toxicity assays have primarily been smaller observational studies or studies without appropriate placebo or other matched controls, and therefore, larger, double-blind, placebo controlled human studies are required before clear effects on human health outcomes can be substantiated.

With the advances of biotechnological tools, the fungus polysaccharides can be obtained not only from the fruiting bodies, but from the cultured mycelia or culture medium. Therefore, the research on this field has a promising future, with the possibility of finding and producing natural medicines with lower or no side effects, and great benefits. But the first step is to improve the clinical analysis and provide clear information about the medicinal properties of mushroom polysaccharides.

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Polysaccharides in Cancer Prevention: From Bench to Bedside

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Contents

Abstract

Polysaccharides (PS) are one of the major classes of carbohydrate biomolecule found within living system. Current knowledge built on scientific rationale and anecdotal evidence over the years acknowledge therapeutic and health benefit effects of bioactive polysaccharides and PS- protein complex from medicinal mushrooms and botanical sources. Medicinal PS is also acknowledged as "biological response modifiers" – substances that stimulate the body's response to infection and disease. In related context of cancer therapy, efficacy of bioactive PS has been documented in preclinical models and found to reduce tumor growth and prolong survival by immune stimulation, apoptosis, and cell

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cycle arrest. The immunomodulating activity by PS includes activation of macrophage(s), natural killer (NK) cells, lymphocyte activated killer (LAK) cells, dendritic cells, tumor-infiltrating lymphocytes, and stimulated release of various cytokines including interferons, tumor necrosis factor, interleukins, and colony-stimulating factors by specific receptor-mediated (Toll-like receptor, dectin-1 and CR3) induction of gene expression. Many of the investigated PS from mushrooms and botanicals have proceeded to pharmaceutical-grade GMP products such as lentinan, schizophyllan/sizofiran/sonifilan, Krestin (polysaccharide-K), GanoPoly, astragalan, GCS-100, and PectaSol, with low toxicity and potent antitumor activity. In countries such as Japan, China, and Korea, pharmaceutical-grade bioactive PS have been introduced as an adjuvant alongside standard radio- and chemotherapy in cancer treatment. This chapter presents a succinct overview of some select bioactive PS derived from Basidiomycetes class of mushrooms (lentinan from *Lentinus edodes*, Krestin from *Coriolus versicolor*, schizophyllan from *Schizophyllum commune*, and *Ganoderma* sps) and few other botanical herbs (*Angelica sinensis, Astragalus membranaceus, Panax ginseng*, pectins, and modified citrus pectin) citing instances of preclinical test results and clinical efficacy outcome signifying their anticancer perspective either as monotherapy or combination therapy for future implementation either as chemopreventive or therapeutic regimen in clinic.

Keywords

Polysaccharide • β-glucan • Lentinan • *Schizophyllan* • Krestin • Astragalan • Modified citrus pectin • *Angelica* • Ginsan • *Ganoderma*

1 Introduction

Cancer singularly represents a class of disease characterized by feature of uncontrolled division of abnormal cells and their dissemination to other parts of body through blood and lymphatic systems. Despite innovations in early detection and prevention strategies, cancer continues as a threat to public health in developing and developed countries. According to American Cancer Society, a total of 1,658,370 new cancer cases and 589,430 cancer deaths are projected to occur in the USA in 2015 (Siegel et al. 2015). Further, complying to International Agency of Cancer (IARC) and World Health Organization (WHO) estimates, by 2030 above 25 million new incidences of cancer cases with estimated 11 million deaths worldwide are anticipated. This underscores current efforts in existing cancer control knowledge and strategies across all segments of population in finding a cure for this deadly menace. Research has shown that up to two-thirds of cancer deaths are caused by smoking, physical inactivity, and poor dietary choices and can be prevented by adopting smart lifestyle and early detection and prevention.

In recent years, non-starchy polysaccharide (PS) macromolecules and PS-protein complexes from botanical and microbiological sources have attracted the attention of researchers as an important class of bioactive products.

Numerous studies based on pharmacology have demonstrated their potency in preventive and therapeutic effects alongside chemotherapy towards a variety of diseases including cancer (Zong et al. 2012; Lemieszek and Rzeski 2012). Emerging evidence indicates that PS fractions are associated with modulation of a variety of biological effects including hematopoietic, antitumor, antioxidant, antidiabetic, radioprotective and as immune stimulant signifying a wide spectrum of clinical applications of PS along with relatively low toxicity (Schepetkin and Quinn 2006; Ramberg et al. 2010). Unlike human and mammalian cells, botanical PS occur as structural constituent of cell wall and accordingly basic procedure for their extraction involves breaking the cell wall under controlled conditions such as pH and temperature thereby avoiding undesirable alterations in their configuration and structural features. Thus, the simple and economical hot water extraction (with short period of heating temperature) followed by concentration under decompression conditions continues as a preferential scientifically validated and classical method for the extraction of PS from botanical sources. Further purification prior to preclinical testing usually involves a combination of techniques such as ethanol precipitation, fractional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography (Wasser 2002). Additional characterization of the purified polysaccharides product for physiochemical and structural features is pursued based on molecular weight, monosaccharide composition and their sequence, configuration and position of glycosidic linkages, type and polymerization degree of branch, spatial configuration, particle size, solubility, rheological properties, etc. (Jin et al. 2011; Nie and Xie 2011). This chapter presents a succinct overview and objective appraisal of evidences focusing primarily on published preclinical data and ongoing clinical trials in context of cancer prevention and therapy. Figure 1 depicts the chemical structures of various PS from different sources included in the text.

2 General Mechanism of Antitumor Action of Polysaccharides

Evidences collected till date for beneficial activity of polysaccharides from different biological origins reveal antitumor action mainly by two mechanisms against tumor cells: (a) a broad spectrum immune-enhancing activity predominantly by activation of the immune system response of host with relative low accompanying toxicity and (b) indirect inhibition of tumor growth and apoptosis of tumor cells.

2.1 Indirect Actions of Polysaccharides in Immunological Functioning

Even though few PS have been scrupulously investigated, results thus far point to noteworthy effect of PS in the regulation of immune response. Essentially, plant polysaccharides are considered as biological response modifiers and being not

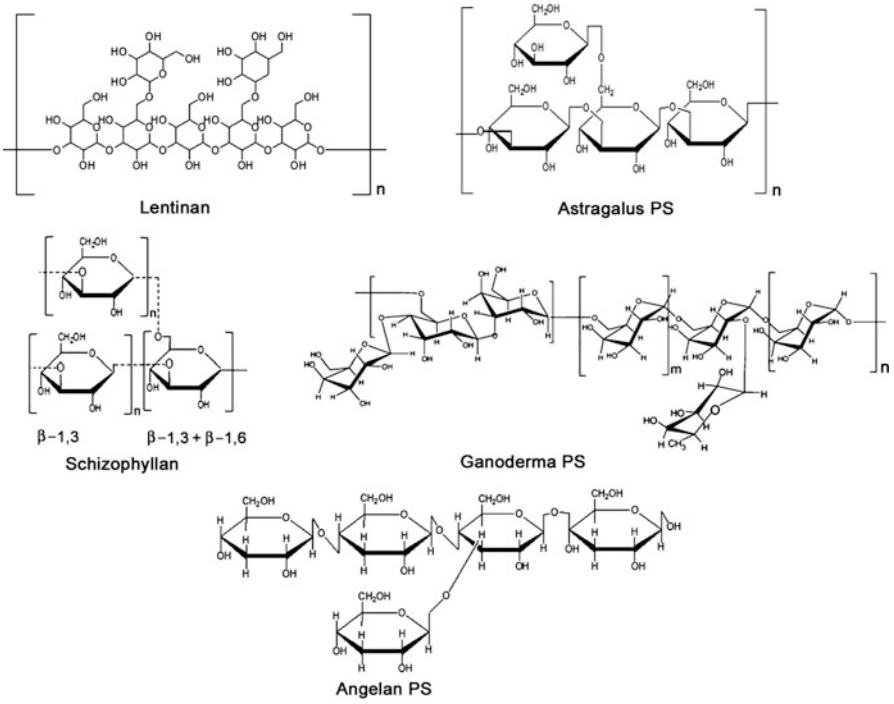

Fig. 1 Chemical structures of various PS from different botanical sources as included in text

synthesized by humans are recognized by the immune system as foreign molecules eliciting immunological response addressed by the innate and the adaptive immune system (Fig. 2).

Emerging evidence from studies on immune modulation have led to an important conclusion that PS with evolutionary conserved structural features do not attack cancer cells directly, but invoke antitumor effects mainly by potentiating the cellular and humoral component of the host immune system and repressing the production of immunosuppressive cytokines (Volman et al. 2008). A broad spectrum possibility of immune-potentiation by PS include the ability to stimulate T-cell generation and potentiate the induction of different types of antitumor effector cells such as cytotoxic T cells, NK cells, macrophages, and dendritic cells (DC). NK cells are frontline defenses in cancer prevention and circulate in blood to lyse cancer and virus-infected cells. Macrophages together with neutrophils are key participants in innate immune response and produce cytokines and local immunomodulators which act to activate adaptive immunity which involves both B and T cells. B cells produce antibodies to mediate humoral immunity, whereas T cells induce cell-mediated immunity. The activation of macrophages by PS is thought to be mediated primarily by the recognition of PS polymers by specific receptors as discussed in the following section. Another element of the adaptive immune system reactive to PS is dendritic cells (DCs) derived from monocytes; these cells present antigens to T cells and therefore

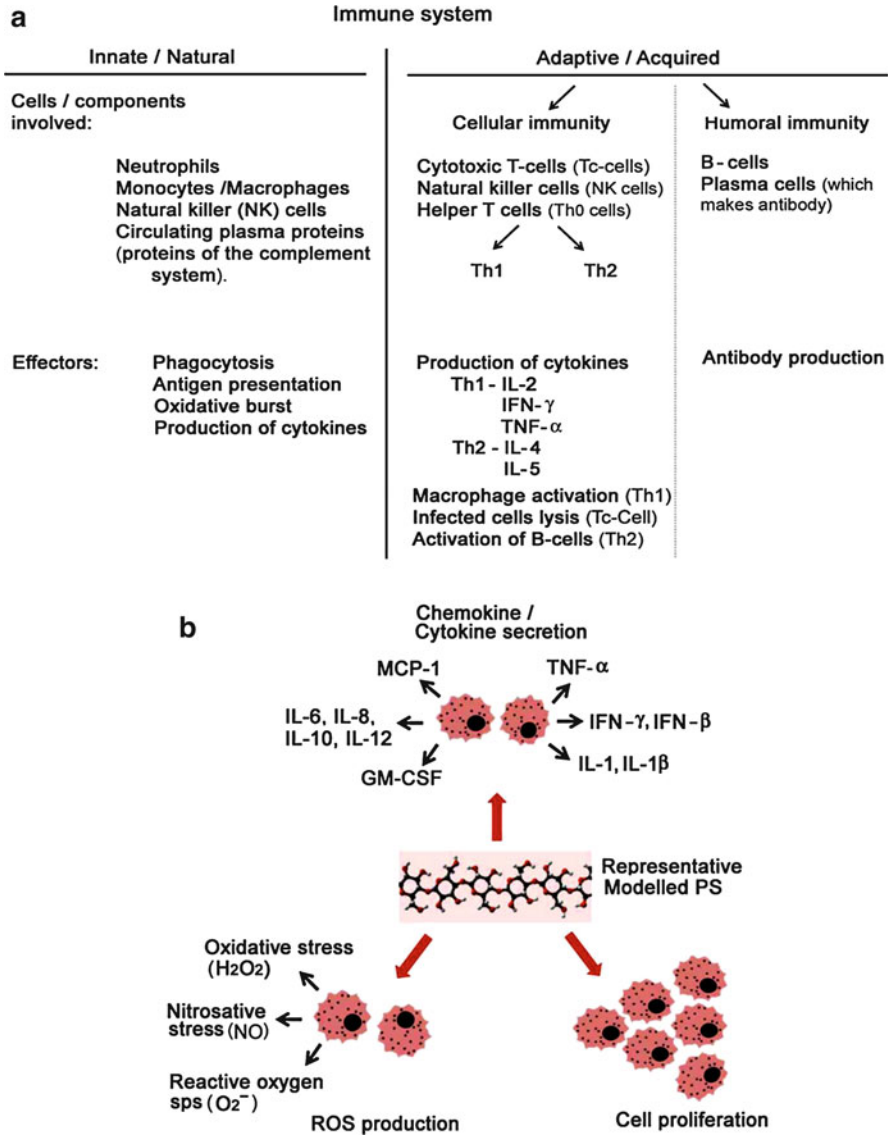

Fig. 2 Overview of immunological response (**a**), and macrophage response to polysaccharides (**b**)

are considered important in the initiation of antitumor T cell response. Crude Ps isolated from *Angelica dahurica* activates DC to produce cytokines and costimulatory molecules to increase all-T cell activation (Kim et al. 2013). Accordingly, an intact thymus (T-cell component) is a precondition for antitumor action of polysaccharides; this has been verified in studies indicating abrogation of antitumor effects of PS in neonatally thymectomized mice and also after administration of

antilymphocyte serum (Maeda et al. 1971). In phase II and phase III clinical trial in China, mushroom polysaccharides PSK and PSP significantly enhanced the immune status in 70–97 % of patients with cancers of the stomach, esophagus, lung, ovary, and cervix. In these studies reported, the investigated mushroom polysaccharides were well tolerated and compatible with chemotherapy and radiotherapy and concur with an increase number of immune cells and infiltration of dendritic and cytotoxic T cell into tumors. In preclinical animal model, the antitumor dose of polysaccharide SNL-P1a from *Solanum nigrum* showed a protective effect on thymus tissue of tumor-bearing mice, whereas the thymus of control mice was damaged seriously because of the tumor ambush (Li et al. 2007). It has been reported that the addition of PS into cell cultures increases proliferation of T cells augmenting the cytotoxic activity of T lymphocytes. Furthermore, PS from 35 different plant sps have shown to increase macrophage cytotoxic activity against tumor cells following in vitro and in vivo treatment of PS by increasing reactive oxygen species (ROS) and nitric oxide (NO) production and enhancing secretion of cytokines and chemokines, such as TNF-α, interleukin (IL)-1β, IL-6, IL-8, IL12, and IFN-γ (Schepetkin and Quinn 2006). Additionally, several lines of evidence suggest that besides macrophage activating effects of Ps, they also affect macrophage hematopoiesis. A high MW polysaccharide isolated from *Salicornia herbacea* and polysaccharide fraction from fu-ling (*Poria cocos*) used in traditional oriental remedies against cancer induce differentiation of monocytic cells into macrophages (Chen and Chang 2004; Im et al. 2006).

2.1.1 Polysaccharides and Cell Signaling in Immunological Context

Noteworthy insight from mechanistic analysis reveals that several well-researched plant PS signals a distinct signal transduction pathway resulting in induction and secretion of cytokines and other paracrine factors supposedly critical for potentiation of innate immunity. Accumulating evidence point that many effect of PS are at least in part mediated through NF-κB and IRF (interferon regulatory factor) family of transcription factors and MAPK signaling. Polysaccharide polymers are primarily recognized during the initial phase of the immune response by specific receptors collectively referred to as pattern recognition receptors (PRRs). These receptors have a carbohydrate recognition domain (CRD) in their extracellular carboxy-terminal domain which recognizes PS as a pathogen-associated molecular pattern (PAMP) during the initial phase of immune activation. Specifically, the major receptors to which botanical PS and glycoproteins bind and implicated in signaling are Toll-like receptor 4 (TLR4), complement receptor 3 (CR3), dectin-1, and CD14 (Fig. 3).

Dectin-1 Receptor and Signaling

Dectin-1 is the major functional receptor for fungal β-1,3-glucans on cells of the myeloid lineage, namely, macrophage and dendritic cells (DC), initiating cytokine-related antitumor biological effects of β-glucans. It is a transmembrane protein with lectin-like carbohydrate recognition domain (CRD) connected by a stalk to the transmembrane region, followed by a cytoplasmic tail containing an immunoreceptor tyrosine-based activation motif (ITAM). After ligand binding, receptor engagement

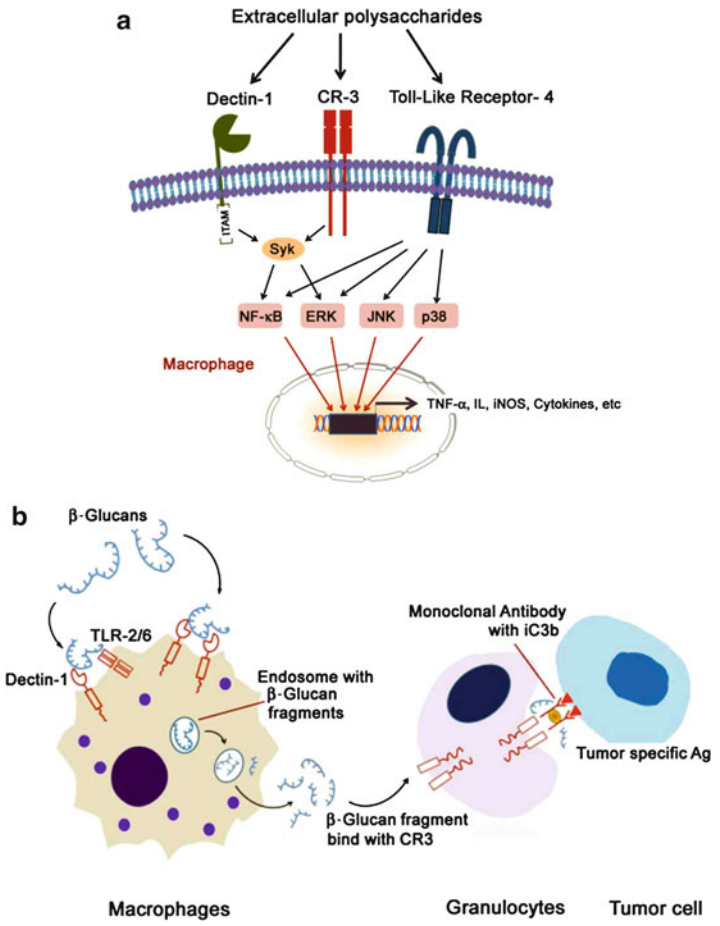

Fig. 3 Schematic model illustrating signaling pathways activated by polysaccharides (**a**) and CR-3 receptor mediated tumor cell lysis (**b**)

leads to tyrosine phosphorylation by Src family kinases and the phosphorylated sequences provide docking sites for Syk kinases. Syk is then activated through intervening phosphorylated substrates (SLP76 and PKC isoform) and induces the CARD9-Bcl10-MALT1 complex. This complex mediates the downstream activation of NF-κB and production of pro-inflammatory cytokines (IL-2, IL-10, and TNFα) (Grunebach et al. 2002). It is known that MAPK-ERK also participates in dectin-1 downstream signaling. In addition to activation of immune response, binding of dectin-1 with ligand induces its own signaling including response such as ROS production (Dennehy and Brown 2007). Dectin-1-mediated signaling is involved among many in SPG-mediated antitumor activity for sarcoma 180 cell growth in mice (Ikeda et al. 2007) and benefit to enhance immunological surveillance system during treatment in combination with irradiation.

Toll-Like Receptor-4 (TLR-4) Signaling

TLR-4 are critical signaling component of innate immune response and mainly expressed on monocytes, macrophages, dendritic cells, and B cells (Leung et al. 2006). These receptors are predominantly activated by BRM-associated molecular pattern and characterized by an extracellular N-terminal leucine-rich-repeat (LRR) domain, a single transmembrane domain, and a cytoplasmic C-terminal intracellular TIR (Toll/IL-1 receptor) domain (Tang and Wang 2010). PS from *Ganoderma lucidum*, *Sparassis crispa*, *Angelica gigas*, and *Phellinus linteus* activate dendritic cells through TLR4 (Kim et al. 2011a). TLR signaling is mediated by two types of pathways, the myeloid differentiation primary-response gene 88 (MyD88)-dependent and (MyD88)-independent (TRIF) pathway. Upon binding with ligand, TLR4 recruit adaptor proteins and activate both the MyD88- and TRIF-dependent signaling pathways leading to common coordinated downstream activation of MAP kinase and activation of interferon regulatory factors (IRF-3 and IRF-7) that leads to production of IFN-β and NF-κB. Together these acts to regulate phenotypic and functional maturation of DC upregulate costimulatory molecules and pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) as well as reactive nitrogen and oxygen products that drive events such as recruiting leucocytes, activating APCs, and initiating adaptive immune response (Kawai and Akira 2006).

CR3 Receptor and Signaling

The CR3 receptor is a transmembrane heterodimer protein of integrin superfamily and composed of a α-subunit (CD11b) and β-chain (CD18) domains having binding site for β-glucans located within the C-terminus of CD11b domain. It is expressed mainly on neutrophils, monocytes, and NK cells, but not macrophages (Ross 2000), and associated with adhesion to the extracellular matrix and functions in addition to phagocytosis. As no β-glucan is synthesized by humans, binding of β-glucan to ligand-binding domain of the receptor prime activates the iC3b pathway causing tumor cytotoxicity as depicted in Fig. 3.

2.2 Direct Action of Polysaccharides: Apoptosis and Cell Cycle Arrest

In addition to the aforementioned accepted dogma of antitumor effect of PS resulting from enhancement of host immunity, emerging evidence citing induction of cancer cell death by activation of apoptotic pathways as another mechanism for inhibition of tumor growth has gained attention. An increasing number of reports confirm that PS or their complexes can induce apoptosis with likelihood of therapeutic implication in the future for use in cancer regression as well as in prevention strategies. In general terms, apoptotic pathways have been divided into two categories: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway begins outside the cell being initiated by

proapoptotic ligands (FasL, TNF, and TRAIL) binding to cell surface receptors (Fas, TNF receptor, and TRAIL receptor); ligand binding causes receptors to cluster and form death-inducing signaling complex (DISC) which next adopt and activate membrane-proximal caspases (caspase-8 and caspase-3). The intrinsic pathway initiated from within the cell requires disruption of the mitochondrial membrane and efflux of small mitochondrial proteins, such as cytochrome c (cyt c) and calcium into the cytosol. Once cyt c is in the cytosol, it binds to Apaf-1 and gives rise to activated caspase-9, and caspase-3. Subsequently, active caspase-3 mediates inhibition of caspase-activated DNase (ICAD) and cleaves the substrate protein poly(ADP-ribose) polymerase (PARP) to cause DNA fragmentation, cell dysfunction, and elimination of cancer cells. Further evidence suggests that Bcl-2 family proteins composed of both antiapoptotic (Bcl-2 and Bcl-xL) and proapoptotic members (Bax, Bak, and Bim) are important regulators of the mitochondrial apoptotic pathway and ensure cell survival. Thus, reduction in expression of Bcl-2 and conversely upregulation of Bax encourages apoptotic response to antitumor PS. In addition, tumor suppressor p53 has been identified as an effector of apoptosis signals, and upregulation of p53 has been recorded as suppressing the survival of tumor cells. The antitumor effect of most natural chemopreventive agents has been reported to be associated with induction of apoptosis.

Bioactive PS such as lentinan from *Lentinus edodes* and PS-K/PS-P from *Coriolus versicolor* and *Ganoderma lucidum* PS from Basidiomycetes family are associated with apoptotic efficacies, in addition to boosting of the immune system as basis for their cytotoxic effect against different types of tumor cells in vitro and in vivo. PSG-1, a novel bioactive PS from *Ganoderma atrum*, exhibit no significant effect on cell proliferation but markedly suppresses tumor growth of colon cancer cells on tumor-bearing mice by apoptosis induction via mitochondria-mediated apoptotic pathway (Zhang et al. 2014). Intriguingly, PS from another *Ganoderma* sps (*G lucidum*) reportedly induces apoptosis in colon cancer cells by activating Fas-mediated caspase-dependent apoptosis (Liang et al. 2014). The antitumor effects of fucoidan, a sulfated PS found in brown algae, are mediated via both the death receptor- and mitochondria-mediated apoptotic pathways (Kim et al. 2010). Furthermore, fucoidan PS increases the levels of activated caspase-8, caspase-9, and caspase-3 and cleaves PARP along with Bak and truncate Bid proteins and additionally increases the levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), Fas, and death receptor-5 protein suggesting its usefulness in cancer prevention and use as a therapeutic adjuvant (Kim et al. 2010). Another instance is novel PS isolated from *Angelica sinensis* named ASP-1d, which inhibits tumor growth in nude mice and induces apoptosis of cervical cancer HeLa cells through mechanism involving intrinsic apoptotic pathway (Cao et al. 2010a). The antitumor effects of ulvan PS in EAC-bearing mice have been recorded as being mediated by downregulation of antiapoptotic protein Bcl-2 and upregulation of proapoptotic and cell cycle arrest protein p53 (Ahmed and Ahmed 2014).

3 Antitumor Polysaccharides of Clinical Interest from Basidiomycetes Class

The Basidiomycetes class of fungal family compendium comprises edible mushrooms with dual medicinal and nutritional values. Conventionally, edible mushrooms have been categorized as macrofungus with a fleshy aerial umbrella-shaped fruiting body called carpophore/myocarp growing from spacious underground mycelia (hyphae). Unlike plants, both the fruiting body and mycelia lacks chlorophyll and contains low fat levels, but a wide range of beneficial compounds (high amounts of proteins and essential amino acids and dietary fibers) impacting human health such as anti-inflammatory, antitumor, cholesterol-lowering properties, and most importantly immunomodulatory effects have been reported (Wasser 2002; Patel and Goyal 2012). Of particular significance with regard to mushrooms of Basidiomycetes class is the presence of biologically active polysaccharide biomolecules in fruiting bodies, cultured mycelium, and culture broth affecting human health. Researchers in Japan found that enokitake mushroom producers had lower cancer rates than the rest of the population. A survey conducted among mushroom workers in the Nagao Prefecture in Japan inferred that regular eating of medicinal mushrooms was associated with lower death rate from cancer than for other people in the prefecture (Ikekawa 2001). Another case-control study published compared the diets of 1,009 women diagnosed with breast cancer with healthy women. Compared to nonconsumers of mushrooms, women who consumed at least 10 g of fresh mushrooms per day had a breast cancer risk of only 36 %. The risk for those who consumed at least 4 g of dried mushrooms per day was 53 % (Zhang et al. 2009). A similar case-control study involving 362 women with breast cancer also found a strong association between mushroom consumption and decreased risk of breast cancer in postmenopausal, but not premenopausal, women (Hong et al. 2008).

Notwithstanding much debate, mushroom polysaccharide and polysaccharide-protein complex are generally considered as biological response modifiers, so the immune system is stimulated by their presence in a way, leading to inhibition of tumor growth. At cellular and molecular level, these include the activation of macrophages, T lymphocytes, and natural killer cells (NK), which are able to secrete inflammatory mediators and cytokines such as the tumor necrosis factor α (TNF-α), γ interferon (IFN-γ), and interleukin (IL-1β), IL-6, IL-8, and IL-12, among others. However, some of the effects owing to direct cytotoxic action cannot be ruled out.

3.1 Lentinan

As a representative purified bioactive polysaccharide fraction derived from mycelium of one of the most widely edible Shitake mushroom – *Lentinus edodes*, lentinan has been approved for clinical use in cancer treatment in Far East oriental countries. First evidence of a mechanistically focused anticancer effects associated with lentinan intake was proposed by Chihara et al. confirming in vivo administration of lentinan with growth inhibition of sarcoma 180 cancer cells transplanted in

CD-1/CD mice (Chihara et al. 1969, 1970). Subsequent studies captivated attention for use of lentinan as stimulant of host immune response by activating and increasing the number of various types of immune cells (such as T cells, natural killer cells, and macrophages). In multiple clinical trials, addition of lentinan to standard cancer therapies augmented sensitivity of tumor cells towards therapy resulting in reducing recurrence along with improved quality of life and patient's survival. The efficacy of lentinan has been investigated both in preclinical and in clinical subjects for its role mainly as therapeutic adjuvant for treatment of advanced gastrointestinal (GI) malignancies such as gastric, pancreatic, colorectal, and hepatocellular carcinoma as described in the following sections. Essentially, the addition of lentinan have been estimated to be safe, with results from multiple studies pointing to a profound reduction in recognizable effect of chemotherapeutic drug-related adverse effects in recurrent and metastatic conditions along with improvement in quality of life and patient's survival. Unfortunately, well designed, large-scale randomized studies in Western setup are lacking to establish the role of lentinan as a useful adjunct to cancer treatment. Large overdoses of lentinan, above the specified clinical dose, may cause immune suppression without any immune-potentiating effect. Otherwise, side effects are few, except for skin rashes that clear up shortly after discontinuing the extract.

As resolved from basic understanding of its chemical structure, lentinan contains a branched chain molecule with a backbone of 1,3β-D-glucan and side chains of β1,3- and β1,3-D-glucose residues and exists in form of triple helix structure in aqueous solution sustained by inter- and intramolecular hydrogen bonds. The retention of triple helix structure is vital for desired antitumor efficacy of lentinan since once the triple helix is broken into single random coils, it loses antitumor activity (Zhang et al. 2011). Additionally, chemical modifications such as sulfation reportedly enhance the efficacy of lentinan, especially against viral diseases including inhibition of HIV viral replication, and thus lentinan have also been exploited as vaccine (Yoshida et al. 1988; Guo et al. 2009).

A great deal of preclinical research linking animal models and human clinical studies confirms the antitumor efficacy of lentinan and its competency in preventing viral and chemical carcinogenesis. The antitumor and antimetastatic activity of lentinan has been reviewed by Chihara et al. and acknowledged to be host-mediated T cell-oriented adjuvant (Chihara et al. 1987). Lentinan elicits an increased production of various kinds of bioactive serum factors associated with immunity and inflammation such as IL-1, CSF, IL-3, vascular dilation inducer, and acute-phase protein induction by the direct action on macrophages or indirectly via lentinan-stimulated T cells resulting in the induction of many immunobiological changes in the host.

In 1985, lentinan injection was approved as therapeutic adjuvant for treatment of certain cancer in Japan and China, and the effect of lentinan in gastric cancer treatment has recently been reviewed (Ina et al. 2013). Lentinan has been evaluated in combination with other agents as well singularly for treatment of patients with advanced or recurrent gastric or colorectal cancer. Results of phase III study of lentinan in combination with chemotherapeutic agents such as 5FU + mitomycin C

or tegafur on patients with advanced or recurrent (GI) cancer reveal that lentinan confer a significant life-span prolongation effect and a higher survival rate especially in combination with tegafur in gastric cancer patients (Taguchi et al. 1985). In a multi-institutional randomized prospective protocol, comprising patients with advanced unresectable gastric cancer and recurrent diseases, the effect of lentinan on patients administered tegafur and cisplatin (control group), and patients administered lentinan, tegafur, and cisplatin (lentinan group), was performed. Addition of lentinan was found to prolong survival and improved quality of life in combination with chemotherapeutic agents (Nakano et al. 1999). Another multicenter chemoimmunotherapy trial using S-1 combined with lentinan benefited patients with unresectable or recurrent gastric cancer with high QOL and prolonged NC period within window of acceptable toxicity (Nimura et al. 2006). Trastuzumab, an antibody against HER2/neu growth factor receptor, has been used for the treatment of gastric and breast cancer in combination with cytotoxic chemotherapeutic agents. Lentinan has been appreciated exerting a synergistic action with anticancer monoclonal antibodies to activate complement systems through the mechanism of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (Ina et al. 2013). There are also reports mentioning higher overall response rates with addition of lentinan to conventional regimens in esophageal and hepatocellular carcinomas (Wang et al. 2012; Yang et al. 2008). Recently, superfine dispersed lentinan – an oral formulation – has become available with seemingly QOL improvement and prognosis in patients with advanced gastric, colorectal, pancreatic, and hepatocellular cancer, although many of the comparison arms in these trials did not utilize standard Western chemotherapy regimens (Yoshino et al. 2010; Hazama et al. 2009; Isoda et al. 2009; Shimizu et al. 2009).

The clinical safety of lentinan administration for patients with advanced or recurrent breast cancer with bilateral oophorectomy (Ox) and adrenalectomy (Ax) and its efficacy as judged by prolongation of life span and antitumor effect has been established. Improvement in prognosis was observed in lentinan-treated patients, compared with the control (Kosaka et al. 1982). Another related extension of this study evaluated lentinan posttreatment with endocrine therapy to patients undergoing surgical therapy (adrenalectomy and oophorectomy) for recurring breast tumor patients. It has been noted that lentinan injection following surgical therapy (Ax + Ox) resulted in a much greater regression of tumor growth than that obtained by surgery alone, but not after tamoxifen therapy. Immunohistochemistry of resected tumor tissue confirmed that lentinan-surgical therapy results in marked atrophy of tumors as well as intense infiltration of T cells, B cells, and macrophages into the stroma around the tumor. Blood prolactin level was also found greatly reduced by lentinan injection (Kosaka et al. 1987).

3.2 Schizophyllan

It is a natural nonionic, water-soluble exopolysaccharide produced using strains of the wood-rotting, filamentous basidiomycete *Schizophyllum commune* commonly

known as split gill mushroom. Schizophyllan (SPG) consists of a linear chain of β-D-(1,3)-glucopyranosyl backbone with single β-D-(1,6)-glucopyranosyl side chains at approximately every third monomer of the main β-1,3-chain (Fig. 1) and dissolves in water as a rigid rodlike triple helix molecule. SPG has been approved for clinical use in Japan and thus commercially produced by several Japanese pharmaceutical companies. It has been reported to be active as biological response modifier and nonspecific stimulator of immune system. Its physicochemical properties, bioactivities, and other developments regarding its application have recently been reviewed and accessible (Zhang et al. 2013). Scientific understanding in context for a mechanistic basis for SPG-associated antitumor effects conclude that aqueous solution of SPG potentiates host-mediated antitumor activity which corroborate with increased overall survival of patients with tumors.

Assembling the reported antitumor studies in preclinical models reveals antitumor activity of SPG against both the solid and ascites forms of sarcoma 180, as well as against the solid form only of sarcoma 37, Ehrlich sarcoma, Yoshida sarcoma, and Lewis lung carcinoma (Wasser 2002). The survival of sarcoma 180 xenografts was not affected by pretreatment with SPG, while pre- and posttreatment with SPG resulted in increased survival. The underlying antitumor events following SPG treatment in these models have been shown being associated with increased cellular immunity by enhanced generation of T lymphocytes and augmented increased production of cytokines – interleukin-2 and interferon-γ – by the peripheral blood mononuclear cells that results in ~95 % tumor inhibition (Matsuo et al. 1982); the antitumor activity of SPG diminishes in thymectomized mice indicating antitumor actions of SPG is T cell mediated (Borchers et al. 1999). In chemically induced breast carcinomas, SPG inhibits tumor incidence along with decrease cell proliferation in tumor tissue (Mansour et al. 2012), while in rat ovarian adenocarcinoma, SPG administration augmented the antitumor effect of cisplatin as inferred from reduced tumor size and improved survival rate in SPG alone and combination arm together with infiltrative increase in cytotoxic T lymphocytes; clinical antitumor effect was also amplified by induction of IL-2 receptor expression and increased NK cell activity (Sugiyama et al. 1995; Hoshino et al. 1993) In ovarian cancer patients undergoing chemotherapy, SPG has been found useful against myelosuppression by chemotherapy (Hoshino et al. 1996).

SPG potentiates photodynamic therapy of squamous cell carcinoma-bearing mice revealing three times increase in tumor cure rate when PDT was preceded by the SPG therapy (Krosl and Korbelik 1994). Schizophyllan pretreatment also prolonged the survival time of B16 melanoma-bearing mice when treated with fractionated X-rays and reestablished mitosis of bone-marrow cells previously suppressed by anticancer drug treatment (Takai et al. 1994). The effect of SPG on lung metastases, immune reactions in tumor tissue, and the survival time of mice bearing transplanted Lewis lung cancer cells subjected to X-rays has been promising (Inomata et al. 1996). SPG reduced the number of lung metastases and prolonged the life span of the mice, these effects being independent of radiation. The addition of SPG to radiation increases both the macrophage infiltration and T-lymphocyte infiltration in the local tumor and the lung nodules (Inomata et al. 1996).

In a previously reported randomized controlled clinical study of 367 patients with recurrent and inoperable gastric cancer, response to conventional chemotherapy – tegafur or mitomycin and 5-fluorouracil – was found increased by SPG, along with significant prolongation of life span without serious side effects but without any significant influence on tumor size (Furue et al. 1985). SPG has been shown to extend the survival time of patients with head and neck cancers by augmenting the accelerated recovery of cellular immunity compromised by radiation, chemotherapy, and surgical procedure. SPG enhances the immune functioning of the regional lymph nodes in patients, as revealed by increased IL-2 production and cytotoxic activities of effector cells (NK, LAK) and increased helper T lymphocytes (CD4+) in the tumor-uninvolved lymph nodes (Kimura et al. 1994; Kano et al. 1996). Subsequently, in a phase II randomized controlled trial, the clinical efficacy and tolerability of SPG in combination with standard chemotherapy in cohort of patients with locally advanced head and neck squamous cell carcinoma revealed no significant difference in overall response rate, although a higher number of complete responses (CR) was registered in Arm A (SPG associated with chemotherapy) verses Arm B (treatment with chemotherapy regimen alone) (Mantovani et al. 1997).

Sizofiran is a potent biologic response modifier in augmenting immune function of regional lymph nodes in patients with cervical cancer (Shimizu et al. 1991, 1992). Further, in one randomized controlled study of SPG in combination with radiotherapy, this polysaccharide molecule significantly prolonged the overall survival time and time to recurrence of stage II but not stage III cancer (Okamura et al. 1989). Later, another study reported higher complete response rate among stage II and III patients than the control group along with rapid significant recovery of decreased lymphocyte counts due to radiotherapy (Noda et al. 1992). In a prospective, randomized clinical trial involving 312 patients treated with surgery, radiotherapy, chemotherapy (fluorouracil), and SPG in various combinations, it is reported that patients treated with SPG survived significantly longer than those who did not receive SPG (Miyazaki et al. 1995). A prospective randomized control trial at https://clinicaltrials.gov is currently recruiting participants [T01926821] for a study evaluating the impact of sonifilan on the quality of life in patients with cervical cancer during radiation or chemoradiotherapy. The primary endpoint of this study is to evaluate QOL, while secondary endpoint relates to complications and treatment effect of sonifilan. Proposed in this study is a dose of 20 mg of sonifilan twice per week for 8 weeks.

3.3 Krestin

Is a protein-bound polysaccharide (PSK; Krestin®) isolated and purified from the cultured mycelium of a nonedible traditional medicinal mushroom *Coriolus versicolor* of Basidiomycetes family (strain: CM-101). It has an average MW of 9.4×10^4, and its major sugar moiety is glucan having a main chain β1-4 bond and a side chain β1-3 as well as β1-6 bond that binds to a protein moiety through *O*- or *N*-glycoside bond (Tsukagoshi et al. 1984). PSK has been shown to regress tumors and

nonspecific immunomodulatory activity is considered the principal mechanism of action of this agent (Tsukagoshi et al. 1984). Specifically, PSK improves host immune competence by either inhibiting the production or neutralizing immunosuppressive substances that are increased in cancer. Secondly, PSK activates immune cells such as lymphocytes, either directly or by regulating the production of various cytokines. Thirdly, PSK acts directly on cancer cells. These mechanisms support the clinical effectiveness of PSK in suppressing cancer relapse (Maehara et al. 2012).

Many basic studies reported in preclinical models show signs of preventive effect of PSK on chemical carcinogen-induced, radiation-induced, and spontaneously developing cancers, suppressing pulmonary metastasis of methylcholanthrene-induced sarcomas, human prostate cancer metastasis, and lymphatic metastasis of mouse leukemia P388 and prolonging survival period in various spontaneously developing metastasis models. In murine models of metastasis, PSK has been shown to suppress the metastasis of rat hepatoma AH60C, mouse colon cancer cells, and mouse leukemia RL (Kobayashi et al. 1995). Decades of numerous clinical trials have shown that the administration of PSK in conjunction with other pharmaceutical drugs escalates the effectiveness of chemotherapy and survival in patients suffering from lung, breast, gastric, colon, liver, and prostate cancer. PSK has been used both orally and intravenously, and in one reported clinical study with a median follow-up of 82 months, there were no significant differences in the 5-year disease-free survival (73 % vs. 55 %) and overall survival (77 % vs. 66 %) between oral chemo-immunotherapy (CITX) versus intravenous CITX in locally advanced gastric cancer patients (Ahn et al. 2013). Other reported clinical trials comprising PSK and chemotherapeutic agents, including fluoropyrimidines (Sakai et al. 2008), UFT (tegafur/uracil) (Ohwada et al. 2006; Yoshitani and Takashima 2009), S-1(tegafur/gimeracil/oteracil) (Kono et al. 2008), or FOLFOX4 (5-fluorouracil/folinic acid/oxaliplatin) (Shibata et al. 2011) all demonstrate favorable long-term prognosis along with reduction in risk of recurrence and extension in survival rates of patients with gastric and colorectal cancer. The effectiveness of PSK as an adjuvant therapy has been a subject to evaluation mainly during postoperative period, by comparing chemotherapy + PSK with chemotherapy alone, and validates that chemotherapy + PSK improve survival in patients with curatively resected gastric cancer (Oba et al. 2007). For advanced colorectal cancers prognosis of surgical treatment is poor and postoperative adjuvant therapy is considered essential. In Japan, multiple multicenter randomized control trial evaluated the efficacy of PSK as postoperative adjuvant therapy for colorectal cancer with oral 5-fluorouracil or oral UFT (tegafur/uracil) or intravenous mitomycin C, compared with surgery alone, and concluded significantly better overall survival (OS) and disease-free survival (DFS) (Torisu et al. 1990; Ito et al. 2004; Ohwada et al. 2004). Since aberrant Wnt-β-catenin signaling is a common phenomenon in colon cancer, a study comprising 44 % patients of colon cancer with diffuse nuclear accumulation of β-catenin activation underwent adjuvant immunochemotherapy protocol [5-fluorouracil plus PSK vs. chemotherapy with 5-fluorouracil alone]. The study outcome exposed significantly improved recurrence-free survival, cancer death survival, and overall survival rates compared with patients receiving chemotherapy alone (Yamashita et al. 2007).

It is not yet known whether vaccine therapy and trastuzumab is more effective when given with or without polysaccharide-K in treating breast cancer (https://clinicaltrials.gov). To address this, a joint collaborative study between University of Washington, National Cancer Inst. (NCI), and National Center for Complementary and Alternative Medicine (NCCAM) is currently recruiting participants for phase I/II randomized study of combination immunotherapy with or without polysaccharide Krestin (PSK®) concurrent with a HER2 ICD peptide-based vaccine and trastuzumab in patients with stage IV breast cancer [NCT Number NCT01922921 at https://clinicaltrials.gov].

Since radiotherapy is generally associated with reducing immune function, preoperative PSK therapy administered to patients reveals improved immune functioning as inferred from the significant increase of NK cell count in peripheral blood and cytotoxic T-cell counts in the peri-tumoral and normal mucosa, along with significant decrease of serum immunosuppressive acidic protein levels noted in the PSK group (Sadahiro et al. 2010). It is also interesting to note that in patients, PSK partially prevents apoptosis of circulating T cells induced by anticancer drug S-1 (Kono et al. 2008).

Other site-specific solid tumors including breast, esophageal and in lung cancer patients the benefit of PSK inclusion in adjuvant setting has been consistently demonstrated in clinical trials. A large trial on patients with breast cancer undergoing curative resection followed by adjuvant therapies with PSK had a better 10-year survival rate of 81 % compared to 64 % in the group using chemotherapy alone (Iino et al. 1995). Similarly, the beneficial effect of PSK supplementation was confirmed in prospective multicenter studies on patients diagnosed with esophageal cancer showing significantly better 5-year survival compared to patients without PSK supplementation (Ogoshi et al. 1995a, b). Patients diagnosed as stage III lung cancer and continuing with PSK treatment (cycle of 2 weeks on and 2 weeks off) after the cessation of radiotherapy had better prognosis than those with stage I and II of the disease but without access to PSK supplementation therapy (Ke et al. 1999).

3.4 *Ganoderma lucidum*

This woody basidiomycete rot fungus commonly referred as "linghzi" and touted in traditional Chinese medicine as a remedy for promoting longevity and preserving human vitality. It has also been overvalued for use as an antitumor medication and known to have anti-inflammatory and antidiabetic effects as well (Sliva 2004; Xu et al. 2011a). The anticancer effects of *Ganoderma* sps is associated with diversified chemical constituents extracted from the fruiting body, mycelium, and spores and includes a wide variety of bioactive compounds such as triterpenes and polysaccharides and other minor constituents such as amino acids, lignin, phenols, sterols, and vitamins (Yuen and Gohel 2005). Biologically active compound originally isolated and purified from *G. lucidum* was a fucose-containing polysaccharide fraction (F3) from water-soluble extract. Most linghzi isolated polysaccharides are heteropolymeric macromolecules having MW ranging between 4×10^5 and

1×10^6 Da containing xylose, mannose, galactose, and fucose in different conformations including 1 → 3-, 1 → 4-, and 1 → 6-linked β- and α-D (or L)-substitutions (Bao et al. 2002). Upon further characterization of different polysaccharide fractions, only branched glucan conformation consisting of (1 → 3)-, (1 → 4)-, and (1 → 6)-β-D linkages has been noted to have strong antitumor activity and better oral absorption compared to other polysaccharide fractions in *G. lucidum* (Miyazaki and Nishijima 1981). Further investigation uncovered that the antitumor activity of β-D-glucans is mainly localized at the branched (1 → 3) chain which binds to complement receptor type 3 and prompts a series of molecular pathways such as NF-κB, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC), which converge and activate the host immune system stimulating immune cell proliferation and cytokine production (Ooi and Liu 2000; Lee and Hong 2011; Ren et al. 2012). The polysaccharide compounds from *G. lucidum* has been suggested to be carcinostatic and reportedly highly suppressive to tumor cell proliferation by boosting host immune functions resulting in the production of cytokines that activate anticancer activities of immune cells rather than direct cytotoxic effect (Kao et al. 2013; Wang et al. 1997). This underlying pathway of cellular action invokes the stimulation of macrophages, maturation and function of dendritic cells, natural killer (NK) cells, cytotoxic T lymphocytes (CTL), and cytokine-induced killer cell (CIK) function and activities along with immune-enhancing secretory products, such as reactive nitrogen and oxygen intermediates and a range of cytokines, for instance, IL-1β, IL-6, TNF-α, and IFN-γ (Wang et al. 1997). Corollary to this hypothesis, *Ganoderma* polysaccharides have been reported to enhance the function of immunologic effector cells in immune-suppressed mice (Zhu et al. 2007). In Adriamycin (ADM)-resistant leukemic (K562/ADM) cell line, *Ganoderma* polysaccharide was effective in reversing multidrug resistance (MDR) through downregulation of the expression of MDR-1 and MDR-associated protein-1 (MRP-1) (Li et al. 2008a). In human lung cancer cells, the addition of this polysaccharide was found effective in inhibiting induction of vascular endothelial growth factor (VEGF) along with inhibition of growth of vascular endothelial cells and thus seems effective as an anti-angiogenic molecule as well (Cao and Lin 2006).

In clinical setup, two randomized trials investigated the effect of GanoPoly® (*G. lucidum* polysaccharide) on the immune functions in advanced-stage cancer patients (Gao et al. 2003a). A total of 34 advanced-stage cancer patients were included and treated with 1,800 mg of GanoPoly® three times daily orally before meals for 12 weeks. The tumors were mainly from lung, breast, liver colon, prostate, bladder, and brain and GanoPoly® was the only anticancer agent administered during the 12-week study period. Another study evaluated the effect of GanoPoly® on 68 lung cancer patients revealing improvement in quality of life in ~65 % of patients. Of clinical interest was a significant increase of total T cells and NK cells along with slight increase of CD4/CD8 ratios in treatment arm compared to placebo (Gao et al. 2003b). Recently, Sun et al. reported *G. lucidum* polysaccharide could antagonize and potentially reverse immune-suppressive effects of lung cancer patient derived plasma-induced suppression of lymphocyte function

and activation (Sun et al. 2014a). Such reversal of immunosuppression by *Ganoderma* polysaccharide treatment suggests the potential of this polysaccharide in cancer therapy (Sun et al. 2014a).

4 Polysaccharides of Clinical Interest from Botanicals in Preclinical and Clinical Models of Cancer

PS component of some medicinally important terrestrial plants with anecdotal anticancer effect has been isolated and characterized. A few select important ones are discussed below.

4.1 *Astragalus* Polysaccharide

Astragalus membranaceus (Fisch.) Bunge (AM), Maxim of the Leguminosae family, is a traditional Chinese medicinal herb. The dried root of AM (known as Huang qi in Chinese) with a sweet warm taste has been acknowledged as a promising complimentary medication for cardiovascular diseases, antiviral infection, diuretic, antidiabetic nephropathy, and antioxidative damage prevention. Using modern analytical techniques major bioactive constituents of the roots of AM have been identified as flavonoids, polysaccharides (astragalan), saponins, and amino acids. Among various component functional fractions, polysaccharides have emerged as an important class receiving much attention for a variety of biological activities such as antitumor, immune modulation, hypoglycemic, and other related beneficial effects of *Astragalus*. Chemical structure characterization by NMR spectroscopy of a homogenous bioactive polysaccharide (APS) obtained by hot water extraction of sliced root of *Astragalus* followed by purification on DEAE-cellulose and Sephacryl-S400 columns detected structure of the polysaccharide being composed of repeating (1 → 4)-linked dextran backbone with a (1 → 6)-linked branch every ten residues as shown in Fig. 1.

APS have been found associated with antioxidant activity including scavenging of hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) and chelating effect on transition metals such as ferrous iron (Fe^{2+}); the latter can facilitate the generation of ROS. The high chelating capacity of APS against Fe^{2+} could be an important approach in retarding metal-catalyzed oxidation since ferrous ions are the most effective prooxidants in biological systems. ROS such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical, and hydrogen peroxide are generated ubiquitously as part of normal cellular physiological process, and their involvement in the pathogenesis of certain human diseases including cancer, cardiovascular diseases, and other aging-associated health problems is well recognized. Thus, the antioxidant effect of APS as free radical scavenger suggests it might be a potentially useful agent to reduce adverse effects of oxidative stress. Recent reports have pointed association between *Astragalus* and symptom relief including improvement in organs function in severe heart and liver diseases (Ma and Guan 2005).

From cancer therapeutic viewpoint, application of *Astragalus* polysaccharide in cancer immunotherapy seems promising. An increasing number of experiments both in vivo and in vitro have shown that *Astragalus* polysaccharide can enhance the body's natural defense mechanism including immunorestorative effect by stimulating phagocytic activity of macrophages (Wang 1989; Yang et al. 2013). Mechanistically, *Astragalus* polysaccharide enforces anticancer effects at least partly via improving immune responses of host organism through modulation of cytokine production such as increase IL-1α, IL-2, IL-6, TNF-α, and the levels of IFN-gamma and significantly decreasing IL-10 and TGF-β levels as deduced from the analysis of tumor-bearing mice treated with *Astragalus* polysaccharide (Yang et al. 2013; Tian et al. 2012a; Sun et al. 2014b). *Astragalus* polysaccharide also downregulate the expression of cyclooxygenase-2 (Liu et al. 2014).

Rui et al. demonstrated antitumor efficacy of oral *Astragalus* polysaccharide treatment to mice bearing B16F10 xenograft tumors. Additionally, moderate to strong antitumor effect was observed following single intraperitoneal treatment with CD-40 gene siRNA plasmid to *Astragalus* polysaccharide-treated mice; the effect being confirmed with decreasing CD-40 cell surface marker (Zhu et al. 2011). *Astragalus* polysaccharides as a therapeutic adjunct along with Adriamycin (AMD) chemotherapy have shown promising results in tumor models along with restoration of AMD-induced immunosuppression (Li et al. 2008b); in another study, it was shown to enhance sensitivity of chemotherapy drugs such as cyclophosphamide, Adriamycin, 5-fluorouracil, cisplatin, etoposide, and vincristine in Adriamycin-resistant cell line by pharmacologically inhibiting P-glycoprotein efflux pump function (Tian et al. 2012b). A recent report reveals that *Astragalus* polysaccharides ameliorate doxorubicin-induced cardiac dysfunction in cancer patients by a mechanism involving suppression of oxidative stress and apoptosis by regulating the PI3k/Akt and p38MAPK pathways (Cao et al. 2014). In advanced small cell lung carcinoma patients undergoing vinorelbine and cisplatin chemotherapy, APS injection integrated with chemotherapy showed increased tumor response, stable improvement in performance status including QOL and survival, and reduced toxicity towards chemotherapy dose (Guo et al. 2012; McCulloch et al. 2006).

4.2 *Angelica*

The dried roots of genus *Angelica* of Umbelliferae family are a source of many oriental drugs in Korea, Japan, and China and include mainly all-purpose women's medicinal herbs such as *Angelica sinensis* Diels, *Angelica acutiloba* Kitagawa, *Angelica dahurica*, and *Angelica officinale* Koch. Phytochemical investigations point attention to polysaccharides as principle constituent associated with many of the reported bioactivities such as anti-inflammatory, antimutagenic, antimicrobial, antitumor, and antioxidant and as immunotherapeutic biological response modifier (Kim et al. 1991; Xu et al. 2011b; Sarker and Nahar 2004). Additional pharmacological activity of *Angelica* polysaccharides on hematopoiesis, blood coagulation, and platelet aggregation and its protective effect on hepatic injury

have also been documented (Ye et al. 2001; Yang et al. 2002). Based on chemical and instrumental analysis till date, 36 polysaccharides have been identified from the root of *A sinensis* (RAS); an overview of their primary structural features such as monosaccharide composition and MW has been compiled and published recently by Jin et.al. (2012). Most polysaccharides isolated from *Angelica* sps are heteropolysaccharides, with constituent units identified as comprising of fucose, galactose, glucose, arabinose, rhamnose, arabinose, mannose, and xylose. A neutral polysaccharide (ASP1) and two acidic polysaccharides (ASP2 and ASP3) have been separated from RAS by hot water extraction (Chen et al. 2013). A pectic polysaccharide – angelan (MW 10 kDa) – was purified from the root and cell culture of *A. gigas* Nakai and chemically characterized being composed of arabinose, galactose, and galacturonic acid and inorganic compounds (Ca^{2+} and Mg^{2+}) but does not contain glucose (Ahn et al. 1996, 1998).

From a therapeutic viewpoint, Cao et al. evaluated the antitumor activity of several different kinds of PS from *A sinensis*. A novel arabinoglucan heteropolysaccharide – APS-1d (MW 5.1×10^3 Da) – purified from *A. sinensis* significantly inhibited proliferation of two representative human solid cancer cell lines (cervical cancer HeLa cells and lung carcinoma A549 cells) by apoptosis primarily involving the activation of the intrinsic mitochondrial pathway. In addition, APS-1d inhibited in a dose-responsive manner the growth of HeLa cell xenograft tumors in nude mice through apoptosis, and also suppressed the growth of transplanted sarcoma 180 (S180) by a mechanism suggestive of activation of immune response of host (Cao et al. 2006, 2010a; Ooi and Liu 2000). Furthermore, three acidic homogenous polysaccharides (APS-3a, APS-3b, and APS-3c) from *A. sinensis* were successfully isolated and purified and their antitumor efficacy appraised (Cao et al. 2010b). Of the three APS, only two – APS-3b and APS-3c – could significantly inhibit the growth of S180 tumors and increase life spans of S180 tumor-bearing mice, whereas APS-3a had no significant effect. Mechanistically, the antitumor effects were found associated with enhancement of some immunological parameters as was evident from concentration-dependent proliferation of the splenocytes in concurrence with the upregulation of IFN-γ, IL-2, and IL-6 mRNA expressions and production of NO and TNF-α in peritoneal macrophages (Cao et al. 2010b).

Shang et al. previously isolated total polysaccharide (AP-0) and its subconstituents (AP-1, AP-2, and AP-3) from fresh roots of *A. sinensis* (Oliv.) by ethanol and multi-precipitation after boiling water extraction and carried an assessment of the antitumor efficacy of total polysaccharide (AP-0) on three in vivo murine tumor models (sarcoma 180, leukemia L1210, and Ehrlich ascites cancer (EAC)). Additionally, the in vitro inhibitory effects of AP-0 and its subconstituents (AP-1, AP-2, and AP-3) on invasion and metastasis of human hepatocellular carcinoma cells (HHCC) into Matrigel reconstituted basement membrane were also evaluated (Shang et al. 2003). From transplantation experiments it is worth to note the influence of AP-0 fraction on the thymus of mice, although the effect was not strong enough to inhibit the growth of S180 tumor, but instead this fraction was effective in inhibiting the production of ascitic fluid and prolonging life of mice bearing L1210 leukemic cells. Collectively, these results of in vivo experiments

suggest that AP-0 has antitumor effect on murine ascitic tumors (EAC and L1210) and affects the thymus of mice bearing S180 tumor. Reference to invasion and migration proficiency on HCC, AP-0, and its subconstituent AP-2 had significant inhibitory effect on the invasion of HHCC into the Matrigel with inhibition rate up to 68 %. Furthermore, AP-0, AP-1, and AP-3 could partially inhibit the chemotactic migration abilities of HHCC cells. Cumulatively, these results are suggestive that polysaccharides from *A. sinensis* exhibit antitumor effects on experimental tumor models in vivo and inhibitory effects on invasion and metastasis in vitro.

Of interest to human health, contemporary investigations by Jiang et al. on the modulatory effect of *A. sinensis* PS and Tai Chi exercise on oxidative injury in middle-aged women subjects revealed reduced oxidative stress and improved blood lipid metabolism; it was shown that antioxidant activities such as superoxide dismutase, catalase, and glutathione peroxidase in the *A. sinensis* PS-treated group were significantly elevated while lipid peroxidation products were found reduced compared to control group (Juan et al. 2009). Survey on the federal website https://clinicaltrials.gov/ flag two studies currently recruiting participants – NCT01720550 and NCT01720563 – using PG2® (PhytoHealth Corp; *Astragalus* polysaccharide 250 and *Astragalus* polysaccharide 500 mg in saline) in cancer patients. The objective of NCT01720550 study is to evaluate the efficacy and safety of different doses of PG2 treatment for relieving fatigue among advanced cancer patients who are under standard palliative care (SPC) at hospice setting and have no further curative options available. NCT01720563 is a phase II double-blind and randomized trial comparing concurrent chemoradiotherapy plus PG2 injection versus concurrent chemoradiotherapy (cisplatin/tegafur plus uracil (UFT)/leucovorin) every 2 weeks plus placebo in advanced pharyngeal or laryngeal squamous cell carcinoma. No study results of these trials have been posted yet.

Radiotherapy is an alternate treatment modality frequently used in cancer treatment but unfortunately is associated with non-targeted adverse effects especially in hematopoietic and reproductive organs. Contextually, pectic PS – ASP3 isolated from *A. sinensis* – exhibited significant radioprotection to mice exposed to a ^{60}Co source of irradiation (Sun et al. 2005, 2009). ASP-3 pretreatment for 7 days demonstrates a protective effect on peripheral lymphocytes against not only apoptotic effect of irradiation, but had significant impact on formation of micronucleus within polychromatic erythrocytes in bone-marrow and sperm aberration, enhances transformation rates of spleen lymphocyte, and improves endurance of the body against radiation. Other emerging studies reveal multimodal therapeutic effect of *A. sinensis* polysaccharide (ASP) combined with cytarabine (Ara-C) in leukemia treatment. ASP combined with Ara-C inhibited proliferation of human acute myeloid (AML) cell line KG1α in vitro by blocking cells in the Go-G1 phase of the cell cycle (Xu et al. 2014). Intraperitoneal injections of ASP + Ara-C in human leukemia NOD/SCID mouse model induced by implanting K562 human leukemic cells alleviate liver damage and pathological changes as shown by improved liver function test and improvement in antioxidant enzyme status in the liver of treated mice along with decreased inflammation of hepatocytes. Of greater interest was the observation that diffuse leukemic cell infiltration into the liver lobules was

significantly reduced along with remarkable increase in the number of apoptotic cells (Zhu et al. 2014). The effect of APS in inducing differentiation of erythroleukemia cells (K562) towards erythrocyte and granulocyte series has been documented (Zheng and Wang 2002). Other studies report the efficacy of ASP as representing an important agent in leukemia stem cell-targeted therapy by a mechanism inducing senescence of human AML stem and progenitor cells by telomere end attrition and by repressing telomerase activity (Liu et al. 2013). Collectively, these preclinical findings hold promise for ASP in hematological malignancies. Angelan exhibited features of tumor growth inhibition and metastasis by stimulating host immune functioning of B cells and macrophages and also directly inhibited cancer cell adhesion; it significantly prolonged the survival of melanoma B16F10 cell-implanted mice and reduced the frequency of pulmonary metastasis of B16F10 melanoma (Han et al. 2006). A further extension to this study incorporating the combined treatment of angelan and doxorubicin (a cytotoxic anticancer agent) shows greater effectiveness in the inhibition of tumor growth and metastasis than either compound alone by a mechanism involving direct inhibition of cancer cell adhesion and invasion through the extracellular matrix in addition to activating the immune functions of B cells and macrophages (Han et al. 2006).

4.3 *Ginseng*

The roots of *Panax* herb of Araliaceae family have been in use for centuries because of assumingly many therapeutic effects. It has been reported to have neuroprotective (Nah 2014), antidiabetic (Kim et al. 2011b; Uzayisenga et al. 2014), and cardiovascular effects (Lee and Kim 2014) and in addition perceived to improve cancer treatment (Chen et al. 2014). Although it is not approved by the Food and Drug Administration in the USA, it remains as one of the most frequently purchased herbal supplement in the USA, whereas in Asian countries and Western Europe, it is commonly used as adjuvant for cancer therapy (Qi et al. 2010; Vogler et al. 1999). The use and benefit of ginseng by cancer patients appears to be well accepted because of reported improvement in clinical outcome and its potential to reduce the adverse effects of chemo- and radiotherapy. Of greater interest in the use of this drug relates to its associated chemopreventive action as deduced from epidemiological studies revealing that subjects with ginseng intake had significantly lower risk of recurrence of cancer compared to nonusers of this supplement (Choi 2008; Shin et al. 2000; Yun and Choi 1995). Two species of ginseng with their roots as preferred source for multi-action compound isolation have been largely focused for anticancer properties, i.e., Asian (*Panax ginseng* CA Meyer) and American (*Panax quinquefolius*) ginseng. Their roots contain various bioactive components including 20 different ginsenosides, polysaccharides, flavonoids, and poly-acetylene alcohols, among which ginsenosides and polysaccharides appear to be responsible for a spectrum of biological activities including immunomodulation, antioxidation, and anticancer effects (Nag et al. 2012; King and Murphy 2010).

Ginseng polysaccharides naturally occur as admixture of structurally and functionally different compounds with yet unclear structure-activity relationship. According to published results, water-soluble ginseng root polysaccharides constitute ~10–15 % of dry weight and these hydrophilic macromolecules are heterogenous, being formed from complex chains of monosaccharides, such as L-arabinose, D-galactose, L-rhamnose, D-galacturonic acid, D-glucuronic acid, and D-galactosyl residues linked together through glycosidic bonds with molecular weights ranging from 3.5 to 2,000 kDa (Azike et al. 2015). The monosaccharide composition of American ginseng root polysaccharides (AGRPS) has been ascribed to contain glucose, galactose, arabinose, rhamnose, and galacturonic acid (Lui et al. 2012). Using a multistep procedure, Cheng et al. fractionated and characterized the heterogenous fractions of ginseng polysaccharides that enabled their group to study and compare the effects of individual components. Accordingly, eluting ginseng polysaccharides on a preparative DEAE-cellulose column yield an acidic population (20 %) and a neutral polysaccharide population (80 %) (Cheng et al. 2011). Neutral polysaccharide fraction of ginseng mainly contains starch-like glucans with no antiproliferative effect on cancer cell, while the acidic fraction is found composed mainly of two types of polysaccharides – the HG-rich pectins and the arabinogalactans. Further bioassay studies revealed that HG-rich pectins, but not arabinogalactans, contain the active component of ginseng polysaccharides that inhibits cell proliferation possibly by G2/M phase arrest of cell cycle. Further, high temperature treatment of HG-rich pectins revealed an improved growth inhibition of colon cancer cells by inducing apoptosis in addition to cell cycle arrest (Cheng et al. 2011). Conceivably, steam treatment might generate or enrich specific structural element that are responsible for the apoptotic activity akin to temperature-modified citrus pectin becoming effective in inducing apoptosis of human cancer cells, while its intact counterpart remains ineffective (Jackson et al. 2007). Ginsan, an acidic ginseng polysaccharide fraction, has been shown to reduce the incidence of benzo(a)pyrene-induced primary lung tumors in mice and the pulmonary metastasis of B16F10 melanoma cells (Lee et al. 1997; Shin et al. 2002). RGAP, another acidic polysaccharide isolated from red ginseng, inhibits the growth of lung carcinoma 3LL, sarcoma 180, and adenocarcinoma JC and enhances the antitumor effects of paclitaxel (Shin et al. 2004a, b). The results from a 14-week randomized, placebo-controlled, double-blind clinical trial evaluating efficacy and safety of ginseng polysaccharide extract (Y75®) registered at https://clinicaltrials.gov/ [NCT 02161198] have recently been published. Interestingly, Y75 significantly enhanced NK cell cytotoxic activity, phagocytic activity of peripheral blood cells, and serum level of TNF-α after treatment. Additionally, administration of Y75 is well tolerated without treatment-related adverse events or alteration of complete blood cell count or blood chemistry over the entire study period (Cho et al. 2014).

4.4 Pectins and Modified Citrus Pectin

Pectins are natural cell wall component representing a family of complex polysaccharides containing galacturonic acid residues and in addition containing galactose,

rhamnose, and arabinose with occasional glucose, mannose, and glucuronic acid residues whose content and linkages vary in pectin from different sources. Thus, centering on the content of sugar residues and linkages along with accompanying structural domains, pectins have been organized into few different types as homogalacturonan, type I rhamnogalacturonan, type II rhamnogalacturonan, xylogalacturonan, apiogalacturonan, arabino-galacturonan, and galactan (Perez et al. 2003). An important feature of pectins is their capability to form gels in the presence of Ca^{++} or sugar and acid and usually resistant to digestion by human intestinal enzymes. Typical levels of pectin as food additive are between 0.5 % and 1 % which is almost equivalent to the amount obtained from consumption of fresh fruit. Accumulating evidence suggests pectins play an important role in prevention of cancer and metastasis. Interestingly, pectin polysaccharides usually have low toxicity and are resistant to digestion by human intestinal enzymes and thus could be of significance in the inhibition of colon cancer in humans. The introduction of pectin into the diet of experimental animals considerably inhibited the development of chemical carcinogen-induced precancerous lesions in colonic mucosa of rats and significantly suppressed the growth of transplantable EMT6 breast carcinoma, TLT liver tumor, and Ehrlich adenocarcinoma (Taper et al. 1997; Taper and Roberfroid 1999; Khotimchenko et al. 2007) and prevented dissemination of B16F1 melanoma cells into the lungs of mice (Platt and Raz 1992). Apple pectins are reportedly effective in significantly reducing the frequency and the number of tumor foci induced by the chemical carcinogen azoxymethane and dimethylhydrazine in the colon of rats and transgenic mice bearing the human transgenes – *c-Ha-ras* (Ohno et al. 2000; Ohkami et al. 1995). The effect of rhamnogalacturonan I pectin from potato and ginseng pectin on HT-29 colon cancer cells inhibited the proliferation of tumor cells by inducing significant G2/M cell cycle arrest associated with downregulation of cyclin B1 and cyclin-dependent kinase 1 expression (Cheng et al. 2011, 2013).

Citrus pectins are acidic water-soluble polysaccharide obtained from the skin and pulp of citrus fruits. Citrus pectin modified under the effects of high pH and temperature – modified citrus pectin (MCP) – has emerged as a promising agent affecting metastatic dissemination of various cancers. It retains structural elements present in the citrus pectin, including the homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II domains and the side chains. MCP has been shown in laboratory to be effective either in vitro or in vivo or both against prostate carcinoma (Pienta et al. 1995; Hsieh and Wu 1995; Glinskii et al. 2005), colon carcinoma (Hayashi et al. 2000; Nangia-Makker et al. 2002), breast carcinoma (Glinskii et al. 2005; Nangia-Makker et al. 2002; Sathisha et al. 2007), melanoma (Platt and Raz 1992; Inohara and Raz 1994), multiple myeloma (Chauhan et al. 2005), and hemangiosarcoma (Johnson et al. 2007). MCP is speculated to affect metastasis in part by its ability to inhibit the prometastatic regulatory protein galectin-3, a β-galactoside-binding protein related to tumor cell adhesion, motility, and metastatic properties; it inhibits cell migration and metastatic spread (Inohara and Raz 1994). Citrus pectin is ineffective in controlling proliferation in cells that are deficient in the expression β-galactosidase (Bergman et al. 2010). MCP inhibits the development of metastatic deposits of human breast carcinoma cells in the lungs and prostate

carcinoma in the bones (Glinskii et al. 2005). The effect of citrus pectin on cytokine production by human peripheral blood mononuclear cells has been cited showing a dose-dependent inhibition of the pro-inflammatory cytokine IL-1β secretion and a dose-dependent increased secretion of the anti-inflammatory cytokines IL-1ra and IL-10 testifying immunomodulatory response in human PBMC (Salman et al. 2008). As further extension to these findings, the ability of MCP to induce activation of human blood lymphocyte subsets like T, B, and NK cells has been reported including the activation of functional NK cells against K562 leukemic cells (Ramachandran et al. 2011).

There are data indicating that addition of therapeutic regimens in Gal-3-expressing malignancies could increase the sensitivity of cancer cells to cytostatic and cytotoxic chemotherapeutic agents. Contextually, MCP has shown to significantly increase the sensitivity of multiple myeloma cells to dexamethasone (Chauhan et al. 2005) and hemangiosarcoma cells to doxorubicin-induced apoptosis (Johnson et al. 2007). Increased sensitivity of malignant cells to various other chemotherapeutic agents such as cisplatin, staurosporine, etoposide, and oxaliplatin enhancing response to apoptosis has also been recorded and published (Glinsky and Raz 2009).

GCS-100 is a polysaccharide derived from citrus pectin and currently undergoing evaluation in several disease conditions including chronic kidney disease, liver disease, and cancer immunotherapy. GCS-100 has shown to induce apoptosis in various multiple myeloma cell lines, including those resistant to dexamethasone, melphalan, or doxorubicin. The findings were also recapitulated in purified patient multiple myeloma cells. Specifically, GCS-100 decreased viability of bortezomib/PS-341-resistant multiple myeloma patient cells, and when combined with dexamethasone, GCS-100 induced an additive anti-multiple myeloma cytotoxicity (Chauhan et al. 2005).

Clinical investigation emerging from a phase II study report the tolerability and effect of modified citrus pectin (marketed under the commercial name "PectaSol" (Econugenics, Santa Rosa, CA)) in 13 men with prostate cancer. A statistically significant enhancement of PSA doubling time index was noted in seven out of ten patients after taking MCP for 12 months compared to the baseline before initiation of MCP therapy validating "proof of concept" theme indicative of slowing down of cancer progression with accompanying possible extension of the expected lifetime of patients (Guess et al. 2003).

5 Polysaccharides from Other Plants in Preclinical Models of Cancer Therapy

In recent years different parts of many other terrestrial plants are undergoing thorough investigation with a premise to identify promising PS components in disease curability including cancer. Accordingly, a rich collection of PS showing activity against cancer has emerged with limited clinical data. Due to limited constrain it is not possible to describe an in-depth detail of PS isolation and characterization. A preview of PS from other plant sources with anticancer effects has been summarized in Table 1 incorporating tumor cells investigated,

Table 1 Anticancer polysaccharides obtained from plants (Adapted from Zong et al. (2012))

Sources	Tumor models	Effects
Aconitum coreanum	H22 tumor cells in vitro and in vivo	Inhibited tumor growth, induced cell cycle arrest
Actinidia eriantha	S180- and H22-bearing mice	Inhibited tumor growth, improved immune responses
Anemone raddeana	H22-bearing mice	Inhibited tumor growth, improved immune responses
Asparagus officinalis	BEL-7404 and HeLa cells	Inhibited cell proliferation
Black currant	Ehrlich carcinoma in mice	Inhibited tumor growth
Brassica napus L.	S180- and B16-bearing mice	Inhibited tumor growth, improved immunofunction and leukogenic actions
Boschniakia rossica	S180-bearing mice	Inhibited tumor growth, presented synergistic effects with 5-FU
Buckwheat	THP-1 cells	Inhibited cell proliferation, induced cell differentiation and maturity
Cactus pear fruit	S180-bearing mice	Inhibited tumor growth, promoted apoptosis and immune responses
Camellia oleifera Abel.	S180-bearing mice	Inhibited tumor growth
Codonopsis pilosula	H22-bearing mice	Inhibited tumor growth, activated immune system
Curcuma kwangsiensis	CNE-2 cells	Inhibited cell proliferation, induced cell apoptosis
Dahlia tubers γ-inulin	B16BL6-, MCA205-, and FsaR-bearing mice	Enhanced effect of photodynamic therapy, reduced recurrence rates of tumor
Dendrobium nobile Lindl	HL-60 cells, S180-bearing mice	Inhibited tumor growth
Dimocarpus longan Lour.	S180-bearing mice	Inhibited tumor growth, stimulated immune system
Gastrodia elata Bl.	PANC-1 cells	Inhibited cell proliferation
Ginkgo biloba sarcotesta	U937 cells	Inhibited cell proliferation
Gynostemma pentaphyllum Makino	Hep-G2 cells and HeLa cells	Inhibited cell proliferation, induced cell cycle arrested
Hedysarum polybotrys	Hep-G2 and MGC-803 cells	Inhibited cell proliferation
Lycium barbarum	MGC-803, BIU87, SGC-7901, SW480, and Caco-2 cells	Inhibited cell proliferation, induced cell cycle arrested
Melia toosendan Sieb. Et Zucc	BGC-823 cells	Inhibited cell proliferation
Ornithogalum caudatum Ait	K562 cells, S180-bearing mice	Inhibited tumor growth
Orostachys japonicus	HT-29 cells	Inhibited cell growth, induced cell apoptosis and cell cycle arrested

(*continued*)

Table 1 (continued)

Sources	Tumor models	Effects
Passiflora edulis	S180-bearing mice	Inhibited tumor growth
Patrinia scabra Bunge	U14-bearing mice	Inhibited tumor growth, induced cell apoptosis and cell cycle arrest
Phaseolus vulgaris L.	HT-29 cells	Upregulated SIAH1, PRKCA, and MSH2 genes, downregulated CHEK1 and GADD45A genes
Prunella vulgaris L.	Lewis lung carcinoma mice model	Inhibited tumor growth, enhanced immune function
Pulsatilla chinensis	C6-bearing mice	Inhibited tumor growth, prolonged survival
Punica granatum	MCF-7 and K562 cells	Inhibited cell proliferation
Safflower	S180- and LA795-bearing mice	Inhibited tumor growth, enhanced the cytotoxicity of immunocytes
Schisandra chinensis leaf	L5178Y-bearing mice	Inhibited tumor growth, enhanced functions of immune system
Sweet potato	K562 and Hca-F cells	Inhibited tumor cells fissiparity
Taxus yunnanensis	HeLa, HT1080 cells	Inhibited cell growth
Tea leaf	H22-bearing rats	Inhibited tumor growth, decreased microvessel density in tumor, regulated immune function
Tea plant flower	S180-bearing mice	Inhibited tumor growth, prolonged the mice survival day, regulated immune function
Tea seeds	K562 cells	Inhibited cell proliferation
Thuja occidentalis	B16F-10 bearing mice	Stimulated cell-mediated immune system, decreased pro-inflammatory cytokines, inhibited metastasis of tumor
Ziziphus jujuba	Melanoma cells	Inhibited cell proliferation, induced cell apoptosis

source of PS, and their effect on tumor cells. Isolation and evaluation of anticancer PS from terrestrial and underground plant parts is becoming an active area of research.

6 Conclusion

From the above-described narrative, there appears a strong "proof of concept" in support of PS ability to augment conventional cancer therapy. However, their translational input into clinics as monotherapy is taken with skepticism in the field of Western medicine, except for their general use as immune-stimulating agents. This is because of a general lack of integration with modern biomedical advancements relating to proficiency testing. More rigorous nonrandomized clinical control trials are warranted with GMP standard single agent before PS becomes a realm in the field of cancer control strategy. Some initiative in this direction has

been initiated by the National Cancer Institute, USA. Nevertheless, the safety profile of PS, especially medicinal mushrooms, makes them attractive candidates for the development of chemopreventive agents. Unlike Basidiomycetes, PS from terrestrial herbs with potent promise need to be developed into GMP standard single agent and need to undergo rigorous evaluation for relevant comprehensive toxicity data and safety profile in preclinical and clinical models and identify potential adverse effect. Moreover, there exists severe deficiency regarding data relating to bioavailability, pharmacokinetics, and pharmacogenomics of PS. With recent developments in the availability of genetically modified murine models of diseases, it is imperative to evaluate the efficacy and safety profile of GMP standard PS in such models that closely mimic human situation recapitulating sequential stages of tumor initiation, promotion, and progression. Although some molecular targets of PS have been identified such as IL-6, IL-1β, TNFα, and others, in silico analysis and molecular modeling will further enhance the current state of knowledge and develop novel analogs with much greater potency. Together, this will strengthen our understanding towards an endeavor to convince oncologists and federal authorities in accepting this novel class of compound to complement along with modern medicine to prolong survival and improve QOL of cancer patients.

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Polysaccharides from the Fungus Scleroderma

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Marília da Silva Nascimento Santos and Edda Lisboa Leite

Contents

Abstract

Research with natural products is an alternative, which includes several bioproducts from bacteria, plant, animal, insect, and marine organisms. Such bioproducts include polysaccharides, proteins, dietary fibers, and many other biomolecules. Studies on fungus have attracted considerable interest for its application in the pharmaceutical and food industry. The anti-inflammatory potential of polysaccharides from the fungus *Scleroderma nitidum* Berk. and their possible action mechanism were studied. The effect of these polymers on the inflammatory process was tested using the carrageenan- and histamine-induced paw edema model and the sodium thioglycolate- and zymosan-induced models. The polysaccharides from *S. nitidum* were effective in reducing edema and cell infiltrate in both inflammation models tested. Nitric oxide, a mediator in the inflammatory process, also showed a low reduction. Analysis of pro- and anti-inflammatory cytokines showed that in the groups treated with polysaccharides from *S. nitidum,* there was an increase in cytokines such as IL-1ra, IL-10, and MIP-1β concomitant with the decrease in 17INF-γ and IL-2. The influence of polysaccharides on the modulation of the

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expression of nuclear factor κB showed that this compound reduced the expression of NF-κB by up to 64 %. This suggests that NF-κB modulation also explains the anti-inflammatory effect of polysaccharides from the fungus *S. nitidum.*

Keywords
Mushroom • *Scleroderma nitidum* • Anti-inflammatory properties • Immunomodulators • NF-KB • Polysaccharides • Glucans • Fungi

1 Introduction

The growing study of microorganisms such as fungi, algae, and bacteria is due to their essential importance in the different food chains and the possibility of commercial application in different areas such as nutrition and human and animal health. Mushrooms are used in the treatment of waste water, in energy production, and in the production of compounds of interest to food, chemical, and pharmaceutical industries, among others (Kirk et al. 2001).

The cell wall of fungi provides both protective and aggressive functions. It is protective, as it acts as an initial barrier that is in contact with hostile environments encountered by the fungus (Latgé 2007; Bowman and Free 2006). For a long time the cell wall was considered as an inert exoskeleton, but this wall is now seen as a dynamic structure that is continuously changing as a result of the modification of culture conditions and environmental stresses. Edible mushrooms are widely consumed due to their flavor, nutritional value, and texture (Cheung and Lee 2000; Giavasis 2014). They are also believed to have therapeutic properties in relation to cancer, heart disease, viral infection, cholesterol levels, and hypertension, which are attributed to dietary fiber, mainly chitin, the polysaccharide making up the cell walls (Wasser and Weiss 1999; Ofodile et al. 2005a). Mushrooms also contain considerable amounts of proteins. Amino acids occurring in the greatest quantities are alanine, asparagine, glycine, and glutamine (Akindahunsi and Oyetayo 2006; Manzi et al. 1999). Furthermore, the fungi are rich in polysaccharides.

The composition of nutrients in fungi differs from fungi to fungi. Breene (1990) analyzed the percentage composition of the fungus *Lentinus edodes* and observed that it is composed of 24.6 % proteins and 44 % carbohydrates. The fungi *Caripia montagnei* was found to be composed mainly of carbohydrates (63.3 %) and lipids (21.4 %). The lipid content in this fungus was considerably higher than that reported by Demiate and Shibata 2003, who found 0.89 % of lipids in the fungus *Agaricus blazei*. The chemical analyses carried out in this study show that the aqueous extract of *Caripia montagnei* is composed mainly of polysaccharides (98.7 %) and low protein content (1.3 %), in addition to being composed basically of glucose units (Queiroz et al. 2010). This result differs from those of other studies conducted in our laboratory. Dore et al. 2007 analyzed the fungus G*eastrum saccatum* and observed that it is composed of a large amount of carbohydrates (84 %) containing glucose and traces of galactose. The presence of protein in the extract may imply glucan-protein complexes (Wang et al. 1996; Gonzaga et al. 2009), which could be fundamental for

Table 1 Chemical components of the fruiting body of *Scleroderma nitidum* "in nature"

Fungi	Carbohydrates (%)	Proteins (%)	Lipids (%)	Moisture (%)	Fibers (%)
S. nitidum	35.31	3.59	1.86	33. 69	25.61

the action of glucans (Dore et al. 2007). The chemical composition is variable with the extraction, fungi species, and maturity of species. The chemical analyses conducted after obtaining polysaccharides from the extract of fungus *S. nitidum* fractionated with ethanol showed high carbohydrate content (94.71 %), low protein content (5.29 %), and no sulfate (Nascimento et al. 2012). The acid hydrolysis of polysaccharides by 2 M HCl showed the presence of glucose, galactose, mannose, and xylose, which were detected by HPLC analysis and found to be present in a molar ratio of 1: 0.4: 0.6: 0.2, respectively (Table 1).

The polysaccharides are natural biomolecules found in all living organisms and are a group of the most abundant and important compounds in the biosphere (Glazer and Nikaido 1995). These polymers are composed of monosaccharide units joined by glycosidic linkages that differ from each other in unity and degree of branching, the type of connections between them, and the length of their chains, with different compositions and functions, being classified as homopolymers or heteropolymers (Pazur 1994). These glucose polymers are widely distributed in nature and classified according to the type of glycosidic linkage [α,β] of the main chain. The β glucans are the predominant form found in fungi. In the cell walls of fungi, these polymers are normally linked to proteins, lipids, and other carbohydrates such as mannan (Williams 1997).

Fungal polysaccharides, belonging to the extracellular cell wall, have been investigated for a variety of biological defense responses, such as antitumor, anti-inflammatory, and immunomodulatory activities (Wasser 2002; Kiho et al. 1993). The exopolysaccharides are defined as extracellular polysaccharides produced by bacteria and some fungi (Kang et al. 1979). In yeast, exopolysaccharides constitute a major proportion of the biomass, accounting for over 75 % of the constituent polysaccharides of the hyphal wall. About these polysaccharides, the glucans are the most abundant in the cell walls of fungi which helps to maintain the optimum pH for ligninolytic enzymes, besides preventing dehydration hyphae and regulating the concentration of extracellular glucose (Gutiérrez et al. 1996).

Polysaccharides derived from mushrooms have been considered an important class of bioactive compounds, and their anti-inflammatory potential has been widely studied (Dore et al. 2007; Carbonero et al. 2008; Smiderle et al. 2008; Seviour et al. 1992). Glucans are widely distributed in nature and could be α, β, or totally β. The β-glucans are the predominant form found in fungi. In cell walls of fungi, these polymers are normally linked to proteins, lipids, and other carbohydrates such as mannans (Williams 1997). Regarding these polysaccharides, the glucans are the most abundant in the cell walls of fungi (Fig. 1), helping to maintain the optimum pH for ligninolytic enzymes, in addition to preventing dehydration hyphae and regulating the concentration of extracellular glucose (Gutiérrez et al. 1996).

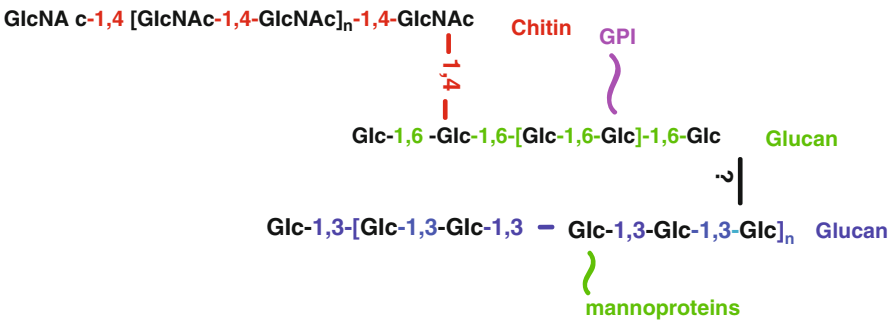

Fig. 1 Schematic representation of cell wall components and their linkages. GPI cell wall proteins, linkages in colors (Adapted from Guillaume and Bussey (2014))

β-glucans have both beneficial and deleterious effects. Zymosan is a crude glucan from yeast (*Saccharomyces cerevisiae*) and is used in the research on the acute inflammatory response. Several studies have demonstrated that zymosan induced arthritis, inflammation, and septic shock in rats (Chittaragi et al. 1998; Paiva et al. 2011). There are several glucans with variable structure. However, this glucan shows various biological activities related to inflammatory responses.

Several glucans are highly branched with linkages 1,3/1,6 being at the cell surface. However, form 1,3-linear of glucans is linked to chitin (Fig. 2). This contains a -1,4-acetyl-glucosamine homopolymer. However, their microfibrils are homopolymer similar to cellulose (β-1,4 glucose), enforcing the strength of the cell walls (Wessels 1990; Carbonero et al. 2008). Chitins are responsible for maintaining the shape, strength, and integrity of the cell structure. The production of chitosan from fungal mycelia has many advantages over crustacean chitosan such as the degree of acetylation, molecular weight, viscosity, and charge distribution of the fungal chitosan (Vetter 2007; Guo et al. 2007a, b). Chitosan from fungal mycelia has also been shown to be more stable than crustacean chitosan. Some investigators reported that chitin and chitosan induce analgesia (Okamoto et al. 2002).

Lentinan, a β-(1→)-glucan produced by *Lentinus edodes*, increases the body's resistance against infection by parasites and its sulfated form has been shown to exhibit potent anti-HIV action (Battle et al. 1998). The botryosphaeran exopolysaccharides produced by the ascomycete fungus *Botryosphaeria rhodina* is considered useful in immunotherapies (Barbosa et al. 2003). Among other examples of biologically active polysaccharides, the D-type β-glucan (1→3) and β-(1→3, 1→6) proved to be potent compounds, as well as being effective against syngeneic, allogeneic, and autologous tumors. Lentinan and schizophyllan, two fungal glucans β-(1→3, 1→6), have become clinically relevant as immunoadjuvants in cancer therapy in Japan. These glucans, non-cytotoxic, supposedly express their antitumor effect by stimulation of the immune host. It is believed that the action occurs mainly by T cell activation helper cells, such as natural killer and cytotoxic macrophages, as well as by an increase in cytotoxic T cells (Gomaa et al. 1992; Zhang and Cheung et al. 2002; Chen and Seviour 2007). Glucans β-(1→3) modulate the immune

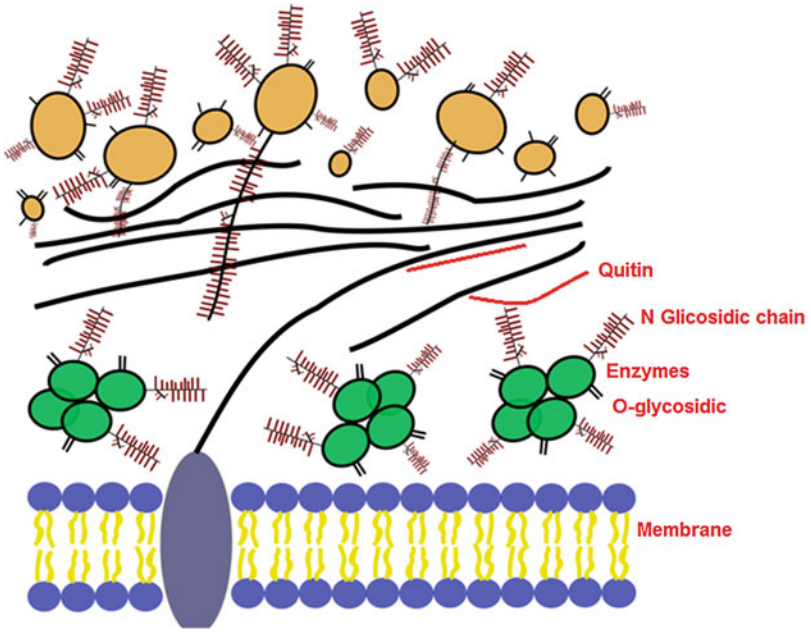

Fig. 2 Cell walls of fungi (Adapted from Seviour et al. (1992))

system by activating macrophages through binding of the polymer to the specific receptors of these cells (Williams 1997; Young et al. 2001). Subsequently was observed that insoluble glucan extracted from the inner cell wall of the fungus *Saccharomyces cerevisiae,* when administered intraperitoneally in mice in an abdominal sepsis model, improved survival by inducing protection against the formation of bacterial colonies in the peritoneal liquid with an increase in leukocyte migration (Freitas et al. 2006; Lesage and Bussey 2006).

In vivo studies have suggested that β-glucans of different molecular weights can directly activate leukocytes and stimulate phagocytic, cytotoxic, and antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates. These carbohydrates can also stimulate the production of proinflammatory mediators and cytokines such as IL-8, IL-1 β, IL- 6, and TNF-α (Rice et al. 2004; William et al. 1997).

2 Occurrence/Sources of Mushroom

The world's largest mushroom producers are the United States, France, Germany, Netherlands, China, and Japan and the main consumers may be seconded to Germany, Netherlands, Japan, and China (Moda et al. 2005). The fungi have a biodiversity found in Brazil that accounts for 15–20 % of the total biodiversity worldwide and is considered a source of biologically active compounds with

Fig. 3 *Scleroderma* genus Pers: Fr (Gasteromycetes, Sclerodermatales)

enormous potential as a source of new drugs (Barreiro and Fraga 1999). In contrast, consumption of mushrooms in Brazil is small compared to that of European and Asian peoples and is restricted to certain ethnic groups or larger cultural and economic statuses (Dias et al. 2004).

Mushrooms have been well studied because of their potential biotechnological importance. A number of mushroom-based compounds with pharmacological potential can be mentioned, such as triterpenes, lectins (Koyama et al. 2002), and polysaccharides (Carbonerro et al. 2008). Among the various polysaccharides isolated from Basidiomycetes are glucans, heterogalactans, and heteromanans (Schepetkin and Quinn 2006).

Scleroderma genus Pers.:Fr. (Gasteromycetes, Sclerodermatales), *Scleroderma nitidum,,* or *Veligaster nitidus* (Berk),name proposed by Guzman and Tapia, contains 25 ectomycorrhizal species (Fig. 3). *Scleroderma nitidum* was collected in Mata da Estrela, Rio Grande do Norte, Brazil. Guzmán and Ovrebo 2000 have included species commonly known as "earth balls," distributed worldwide (Kirk et al. 2001). At least 5,000 species of fungi are involved in ectomycorrhizal associations, usually with a high degree of specificity (Raven et al. 2001). The ectomycorrhizae are mostly formed by basidiomycetes, including many kinds of mushrooms. Although this genus, *Scleroderma,* contains poisonous species, the species have been investigated for their anti-inflammatory and hemostatic properties (Guzmán and Ovrebo 2000; Liu 1984; Nascimento et al. 2012).

Scleroderma nitidum (Fig. 1) has basidiomata yellowish brown subglobose, 1.5–3 cm diameter. Peridium, 4–5 mm thick when fresh, verrucous surface, dark brown, evenly spread over the surface is irregular dehiscence; hyphae hyaline, 10–18 × 6–12 μm. Gleba powdery, olive gray. Dark brown basidiospores, globose, equinulados, 11–12 mM diameter.

3 Biotechnological Approaches

Mushroom is highly nutritious containing all the classes of food. Using biotechnological approaches, they can not only be domesticated but also cultivated in large quantity and be available all season.

Several in vitro and in vivo mechanisms are related to the anti-inflammatory activity of polysaccharides and extracts rich in fungal polysaccharides. Among these mechanisms are cyclooxygenase inhibition (Hong et al. 2004), inhibition or decrease in cytokine levels (Kim et al. 2003), and inhibition of inflammatory cell recruitment (Dore et al. 2007). Clinical evidence of the antitumoral effect of mushroom was verified in commercial polysaccharides such as lentinan, krestin, and schizophyllan, but some other polysaccharides also showed good activities (Mizuno 1999; Zaidman et al. 2005). Their activity was also good when associated with chemotherapy. Research has demonstrated that mushrooms do not affect tumoral cells, as their action is to stimulate cells of immune response Akramiene et al. (2007).

Mushrooms are rich in vitamins, amino acids, and minerals, besides being significantly involved in human detoxification processes that have also an important role to play in reducing blood cholesterol (Oyetavo and Oyetavo 2009; Jeong et al. 2010). Edible mushrooms are of low calorie with very low fat and are highly nutritive as good quality proteins (Naumann et al. 2006; Oyetayo et al. 2007). Therefore, they are the delight of the diabetics (Kiho et al. 1993; Gray and Flatt 1998; Zhang et al. 2006; Lo et al. 2006).

Several mushrooms have antibacterial properties (Sutherland 1998). The antibacterial activity test of the *Caripia montagnei* extract showed that it did not have an effect on gram-positive or gram-negative bacteria at the three concentrations tested (Queiroz et al. 2010). Although the genus *Scleroderma* includes poisonous fungi, they have been studied for their anti-inflammatory and homeostatic properties (Guzman 1970). This result differs from that found by Smania et al. 2007, who studied the Brazilian fungus *Ganoderma australe* and observed the presence of activity against fungi, as well as against gram-positive and gram-negative bacteria. Research conducted with extracts of four species of *Ganoderma* (*Ganoderma colossum, G. resinaceum, G. lucidum,* and *G. boninense*) of Nigeria has demonstrated that these have microbicides activities and were active against *Pseudomonas syringae* and *Bacillus subtilis* (Ofodile et al. 2005b).

4 Phytochemical Screening

The phytochemical screening of the mushroom extract revealed the presence of alkaloids, saponins, steroids, phlobatannins, flavonoids, and anthraquinones. Extract of Scleroderma with different solvents was subjected to phytochemical analysis for secondary metabolites. In mushroom extracts, these metabolites were found to contain steroids, saponins, glycosides, fibers, and phenols (Cheung and Lee 2000). The qualitative phytochemical screening of *Scleroderma bermudense* has revealed the presence of various secondary metabolites of therapeutic importance, namely, steroids, saponins, phenols, glycosides, and flavonoids, but the absence of alkaloids, tannins, and terpenoids (Chittaragi et al. 2013).

Aqueous extraction is one of the most widely used techniques in scientific studies (Barbisan et al. 2002; Bellini et al. 2006; Giacomini and Eira 2003; Lavi et al. 2006). Its efficiency in obtaining polysaccharides and the low cost may be the main reasons for its wide use. Most complex carbohydrates are technically water insoluble but do form gels to varying degrees when mixed with water. *Scleroderma nitidum* showed polysaccharides partially soluble in water.

The chemical analyses conducted after obtaining polysaccharides from the fungus *S. nitidum* revealed very high carbohydrate content, low protein content, and no sulfate. The polysaccharide of this mushroom was submitted to acid hydrolysis by HCL and showed the presence of glucose, galactose, mannose, and xylose, which were detected by HPLC analysis and found to be present in a molar ratio of 1:0.4:0.6 :0.2, respectively. This polysaccharide is heterogenous like other polysaccharides of mushroom. Also found in *Ganoderma applanatum* mushroom is the monomer composition of polysaccharides containing glucose, galactose, mannose, and arabinose (Lee et al. 2007).

Many biological functions have been attributed to polysaccharides from fungi and much has been investigated with respect to their pharmacological potential (Novak and Vetvicka 2008). Many have Reid et al. (2009) been reported in the literature as having therapeutic properties, for example, members of the genus *Cordyceps*, which have antitumor, anti-metastatic, immunomodulatory, antioxidant, anti-inflammatory, microbicide, hypoglycemic, and hypolipidemic activities (Naumann et al. 2006; Zhang et al. 2006).

In addition, three cell receptor β-glucans have been identified: CR3, dectin-1, and lactosylceramide (Zimmerman et al. 1998; Ross et al. 1999; Brown and Gordon 2001, 2003). The CR3 are responsible for various cellular receptor activities in vitro and in vivo, stimulating the secretion of cytokines (TNF-α, IFN-α, IFN-γ, and IL-6) in NK cells, especially in the presence of pathogens (Ross et al. 1999). Dectin-1 is a receptor on cells of the monocytes and macrophages, has an exogenous ligand for binding polysaccharides and other co-stimulatory signals for T cell activation. Studies have shown that these cellular receptors and the lactosylceramide recognize β-glucans, particularly with the structure of type β-(1→3) and β-(1→6), and lymphocytes (Zimmerman et al. 1998; Brown and Gordon 2003; Stier et al. 2014).

The phytochemical screening of the mushroom extract revealed the presence of alkaloids, saponins, steroids, phlobatannins, flavonoids, and anthraquinones

(Adebayo et al. 2012). Extract of Scleroderma with solvents different was subjected to phytochemical analysis for secondary metabolites. In mushroom extracts this metabolites were found to contain steroids, saponins, glycosides, flavonoids, and phenols. The qualitative phytochemical screening of *Scleroderma bermudense* has revealed the presence of various secondary metabolites of therapeutic importance, namely, steroids, saponins, phenols, glycosides, and flavonoids, whereas absence of alkaloids, tannins, and terpenoids (Chittaragi et al. 2013).

Aqueous extraction is one of the most widely used techniques in scientific studies (Barbisan et al. 2002; Bellini et al. 2006; Giacomini and Eira 2003; Lavi et al. 2006). Its efficiency in obtaining polysaccharides and the low cost may be the main reasons for its wide use. Most complex carbohydrates are technically water insoluble; they do form gels to varying degrees when mixed with water. *Scleroderma nitidum* showed polysaccharides partially soluble in water.

Many activities have been reported in the literature as having therapeutic properties, for example, the genus *Cordyceps* which have antitumor effects, antimetastatic, immunomodulatory, antioxidants (Fu and Shieh 2002), anti-inflammatories, microbicide, hypoglycemic, and hypolipidemic (Kim et al. 2005; Badole et al. 2006; De et al. 2012).

The chemical analyses conducted after obtaining polysaccharides from the fungus *S. nitidum* showed very high carbohydrate content, low protein content, and no sulfate content. The polysaccharide of this mushroom submitted the acid hydrolysis by HCL and showed the presence of glucose, galactose, mannose, and xylose that were detected by HPLC analysis and found to be present in a molar ratio of 1:0.4:0.6:0.2, respectively. This polysaccharide is heterogenous as other polysaccharides of mushroom. *Ganoderma applanatum*, is a mushroom whose composition of polysaccharides contain sugars as glucose, galactose, mannose, and arabinose (Lee et al. 2007).

Many biological functions have been attributed to polysaccharide from fungi and much has been investigated with respect to their pharmacological potential (Lindequist et al. 2005). Three cells receptors β-glucans were proposed and were identified: CR3, dectin-1, and lactosylceramide (Zimmerman et al. 1998; Ross et al. 1999; Brown and Gordon 2003). The CR3 are responsible for various cellular receptor activities in vitro and in vivo, stimulating the secretion of cytokines (TNF-α, IFN- α, IFN- γ, and IL-6) in NK cells, especially in the presence of pathogens (Ross et al. 1999). Dectin-1 is a receptor on dectin-1 type on cells of the monocytes and macrophages has an exogenous ligand for binding polysaccharides and other co-stimulatory signals for T cell (Brown 2006). Fungal β-1,3-glucans can be recognized by a number of receptors apart from Dectin-1, such as CR3, lactosylceramide, and scavenger receptors. Studies have shown that these cellular receptors and the lactosylceramide and also lymphocytes recognize β-glucans, particularly with the structure types β-(1→) and β-(1→6) Dectin-1 and complement receptor 3 (CR3) pathways leading to NF-κB activation and cytokine production (Zimmerman et al. 1998; Brown and Gordon 2003). There are evidence that the antitumural and immunomodulatory effects of glucans fungal and in particulate β-glucans, despite their large size occurr pathway these receptors (Barbayar et al. 2012; Volman and Ramakers 2008).

The study of natural products derived of mushrooms and other bioactive compound of plants offers many perspectives for the development of new effective and safe immunomodulation agents. Polysaccharides derived from mushrooms have been considered an important class of bioactive compounds, and their anti-inflammatory potential has been widely studied (Dore et al. 2007; Carbonero et al. 2008; Smiderle et al. 2008).

5 *S. nitidum* and Their Effect on Inflammation

Inflammation is a complex event that consists of recognizing the lesion stimulus and the consequent attempt at restoring the damaged tissue (Nathan 2002; Medzhitov 2010). This involves a series of cellular and vascular events (Nussler and Billiar 1993; Rocha and Silva 1978). In inflammatory reactions, nitric oxide derived from cells stimulated by the action of cytokines promotes changes in the vascular permeability of the inflamed tissue (Barnes and Karin 1997; Winter et al. 1962). Adverse conditions, adverse conditions induce inflammation triggering the recruitment of leukocytes and plasma proteins to the affected tissue site (Medzhitov 2008). Macrophages and monocytes are proinflammatory cells that respond to invading pathogens by releasing proinflammatory mediators, as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-12 (IL-12), cyclooxygenase-2 (COX-2), and nitric oxide (NO) (Lawrence et al. 2002).

Acute inflammation is essentially mediated by exudate formation and leukocyte migration. Innumerable studies have proven the potential of polysaccharides extracted from fungi in reducing leukocyte migration to the inflammation sites. To confirm the anti-inflammatory potential of fungal polysaccharides, the aim of this study, we tested their effect in models of plantar edema induced by carrageenan and histamine and of peritonitis induced by sodium thioglycolate and zymosan (Cui et al.; Nascimento et al. 2012).

The inflammatory process of the extract of *S. nitidum*, rich in polysaccharides, was assessed in *Wistar* rats. Polysaccharides of this mushroom reduced carrageenan-induced paw edema at the inflammatory peak (second hour) with major reduction at the end of the fourth hour. In the histamine-induced paw edema, all the doses of polysaccharides (10–50 mg/kg) were efficient in reducing the edema. The greatest reduction observed occurred in the second hour, where the effect exceeded that obtained with L-NAME. At the end of the fourth hour, all the groups treated with the different doses of polysaccharides did not exhibit edema (Nascimento et al. 2012).

We observed that histological examinations demonstrated that the animals sensitized only with histamine, a positive control, exhibited intense cell infiltrate, characteristic of the inflammatory reaction. However, those that received only saline solution (negative control) showed an absence of inflammatory reaction. It was also observed that the animals treated with polysaccharides from the fungus *S. nitidum* (10, 30, and 50 mg/kg) showed a significant decrease in PMN cell recruitment at the inflammation site.

To check if the polysaccharides were capable of potentiating drug action, two drugs were used for this assay: L-NAME and diclofenac. Diclofenac is a COX 1 and 2 inhibitor, whereas L-NAME is a competitive inhibitor of arginine that promotes a decrease in the enzymatic activity of the iNOS (Bogle et al. 1992). Polysaccharides of *S. nitidum*, when tested at low doses and applied jointly with diclofenac, exceeded the action of this drug by around 10 %. However, the polysaccharides showed no significant differences when L-NAME was applied. The data demonstrate that these fungal polysaccharides may act synergically with other inflammatory drugs, potentiating their effect.

Nitric oxide (NO) is important in the physiological and biochemistry aspects including cell to cell communication in the nervous system and brain (Davies et al. 1995), as well as functioning in the immune system (Ignarro 2009). Its synthesis occurs by actions of enzyme called nitric oxide synthase (NOS). Three distinct isoforms of NOS are known to exist; two were originally described as constitutive and one inducible (Moncada et al. 1997; Nussler and Billiar 1993). Expression of enzymes (iNOS) is regulated, positively and negatively, by mediators present during infection and inflammation. The iNOS enzymes induction occur by lipopolysaccharide (LPS), interferon-y, interleukin (IL)-10, and tumor necrosis factor (TNF)-α. The expression of these enzymes may be increased by combinations of TNFα and interferon-γ, via the transcription factor NF-κB (Sato et al. 2003).

Nitric oxide in most body fluids is rapidly metabolized to stable products such as nitrite and nitrate. According to the results obtained in the sodium thioglycolate-induced model, the polysaccharides from the fungus *Scleroderma nitidum* showed a reduction of around 32.4 % in NO_2/NO_3 content. However, in the zymosan-induced peritonitis model, no significant reduction in NO_2/NO_3 levels was observed.

Polysaccharides of *S. nitidium*, rich in glucose, had no toxic effect at the doses tested. Such glucans showed an effect on cytokine expression. We observed a twofold and threefold increase in the release of anti-inflammatory cytokines IL-1ra and IL-10, respectively, in the groups treated with polysaccharides when compared with the positive control. A significant decrease (75 %) was also observed in IFN-γ levels as well as a slight reduction in IL-2 and MIP-1β levels of 22 % and 29 %, respectively, in the groups treated with the different polysaccharide doses. In addition, the effect of polysaccharides was not dose dependent.

The nuclear factor κB (NF-κB), an important transcription factor in the progression of inflammation, is related to gene activation in many of the mediators involved in the inflammatory response, such as TNF-α, IL-1β, and iNOS (Barnes and Karin 1997; Ben-Neriah and Schmidt-Supprian 2007). NF-κB exists in the cytoplasm in an inactive form associated with regulatory proteins (κB). NF-κB is clearly one of the most important regulators of proinflammatory gene expression (Fig. 4). This nuclear factor kappa B plays a principal role in the induction of eosinophilia in allergic airway inflammation, gastritis, arthritis, and other inflammatory diseases.

Nuclear factor kB activation is a critical event in inflammation. NF-kB activation is related to gene activation for many cytokines, chemokines, and adhesion molecules. This experiment was conducted to determine whether the polysaccharides from the fungus *Scleroderma nitidum* are capable of modulating

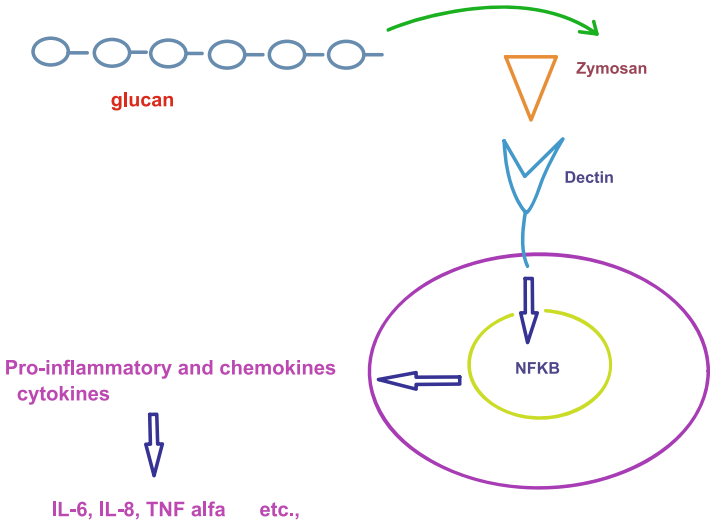

Fig. 4 Effect of zymosan in cell

NF-κB expression. According to the results obtained, the dose that had the best effect was 30 mg/kg, reducing NF-κB expression by 63 ± 8 %, whereas doses of 50 and 100 mg/kg reduced it by only 30 ± 1 %. Thus, it can be observed that all the fungal polysaccharide doses tested significantly reduced nuclear factor NF-kB expression (Fig. 4c).

During the inflammatory process, monocytes are recruited for parenchyma tissue, where they are activated to become cells with a phagocytic function, that is, macrophages. These cells can release inflammatory cytokines, free radicals, and nitric oxide, which can mediate the tissue lesion related to inflammatory response (Almeida et al. 1980; Gordon 2001; Hermann et al. 2001; Fu and Shieh 2002). Studies on mushrooms with medicinal potential have been conducted to discover compounds that can positively or negatively modulate the immunological system. The significant reduction of edema observed in the rats treated with polysaccharides from the fungus *Scleroderma nitidum* can be observed in two plantar edema models, suggesting their anti-inflammatory effect (Nascimento et al. 2012)

Macrophage activation via the classic pathway leads to the secretion of nitric oxide and proinflammatory cytokines, whereas activation via the alternative pathway leads to the release of anti-inflammatory cytokines (Gordon 2003). The polysaccharides from the fungus (glucans) *Scleroderma nitidum* did reduce INF-γ levels in the peritoneal wash of the mice. Furthermore, we observed a reduction in the levels of IL-2, a cytokine that stimulates T cell proliferation (Malek and Bayer 2004). The macrophage inflammatory protein 1 beta (MIP-1β) is a chemokine that induces chemotaxis and T cell adhesion (Tanaka et al. 1993). The MIP-1β levels were found to be reduced after treatment with polysaccharides from *Scleroderma nitidum*. IL-1ra can inhibit the proinflammatory effects of IL-1 (Dinarello 1992, 1994). Polysaccharides from the fungus *Scleroderma nitidum* caused a threefold

increase in IL-1ra levels in the peritoneal wash of mice with zymosan-induced peritonitis. In the present study a substantial increase in IL-10 levels was also found.

The increase in anti-inflammatory cytokine levels concomitant with the decrease in proinflammatory cytokine release is a plausible explanation for the anti-inflammatory activity of polysaccharides extracted from the fungus *Scleroderma nitidum.* Given that polysaccharides from the fungus *Scleroderma nitidum* were capable of decreasing nitric oxide levels and modulating cytokine expression, we decided to assess a possible action mechanism to explain their biological effects. Thus, an assay was conducted to determine the expression of transcription factor NF-kB. In the groups treated with polysaccharides from the fungus *Scleroderma nitidum,* a significant reduction in NF-κB expression was observed, showing that the polysaccharides could modulate nitric oxide and cytokine levels through the regulation of NF-κB expression. This fact may explain the anti-inflammatory effect of these polysaccharides.

6 Conclusion

The macrophage inflammatory protein 1 beta (MIP-1β) is a chemokine that induces chemotaxis and adhesion of T cells. Levels of MIP-1β were found to be reduced after treatment with polysaccharides of *Scleroderma nitidum*. The increased levels of inflammatory cytokines concomitant with decreased release of proinflammatory cytokines suggest an explanation for the anti-inflammatory activity of polysaccharides extracted from fungus *Scleroderma nitidum*. As the polysaccharides of the fungus *Scleroderma nitidum* were able to decrease the levels of nitric oxide and modulate cytokine expression, we decided to evaluate a possible mechanism of action to explain their biological effects. Thus, a test was performed to verify the expression of the transcriptional factor κB (NF-kB). In the groups treated with the polysaccharides of the fungus *Scleroderma nitidum,* a significant reduction of the expression of NF-kB was observed, indicating that the polysaccharides could act by modulating the levels of nitric oxide and cytokines by regulating the expression of NF-kB. This fact could explain the anti-inflammatory effect of these polysaccharides. Knowing the importance of evaluating the cytotoxicitys of a compound.

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