Nicola Volpi
Editor

Capillary Electrophoresis of Carbohydrates

From Monosaccharides to Complex Polysaccharides

Humana Press

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Preface

Capillary electrophoresis (CE) encompasses a range of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. Due to its high resolving power and sensitivity, CE has been applied in the analysis of simple and complex carbohydrates, such as intact oligosaccharides and glycosaminoglycans-derived oligosaccharides and disaccharides, providing concentration and structural characterization data essential for understanding their biological functions.

Simple carbohydrates and complex oligosaccharides and polysaccharides are a class of ubiquitous (macro)molecules exhibiting a wide range of biological functions. The recent advent of enhanced enzymatic, chemical, and analytical tools for the study of these sugars has triggered a genuine explosion in the field of glycomics. In particular, the study of complex oligosaccharides and heteropolysaccharides has led to deeper insight into how specific sugar structures modulate cellular phenotypes. An increased understanding of the structure-function relationship has led to the discovery of new pharmaceuticals for the treatment of serious diseases, such as cancer. This area of research is rapidly expanding and is expected to have a major impact on future therapeutic regimens.

This volume on the capillary electrophoresis of carbohydrates provides the reader with the latest breakthroughs and improvements in CE and CE techniques applied to monosaccharides up to complex oligo- and polysaccharides. Chapter 1 presents an overview on the application of CE and CE–mass spectrometric (MS) in the analysis of simple carbohydrates without any previous derivatization step. Various detection techniques such as spectrophotometric detection, electrochemical detection, and MS are discussed, as are less common techniques. A wide-ranging list of CE and CE-MS applications in the field of carbohydrate analysis published during the last decade is reported.

Chapter 2 covers all the currently used derivatization procedures, by means of chromophore or fluorophore incorporation, their mechanistic details, and the merits attributed to each approach, with the aim of enhancing sensitivity and also of improving analyte separation.

Chapter 3 focuses on CE, CE-MS, and tandem MS, on the separation and characterization of lipopolysaccharides along with some acidic polysaccharides and

derived oligosaccharides and disaccharides, which are important (macro)molecules belonging to bacteria.

Chapter 4 discusses microchip-based CE analysis of complex natural heteropolysaccharides, known as glycosaminoglycans, which affords rapid analysis on a time scale of seconds. This technology has great potential as a tool for routine assessment of pharmaceutical preparations and for clinical diagnosis.

Chapter 5 discusses the use of CE as an analytical approach for the detection of biofilm positivity in particular microorganisms, as well as for the separation of biofilm-positive and -negative strains, considering that the biofilm-positive surfaces are usually covered with specific extracellular polysaccharide substances that play a key role in biofilm formation and function.

Chapter 6 illustrates the capacity of CE in the structural characterization of polysaccharide mono- and oligomer constituents, surveying several applications on chemically and enzymatically degraded polysaccharides. Furthermore, CE was also demonstrated to be highly reliable for the determination of polysaccharides in biological samples, due to the possibility of analyzing rather complex matrices even without any pretreatment, a distinctive feature with respect to other separation strategies. In relation to this versatility, Chapter 7 features a survey on the more recent applications and developments of CE to study reactions involving saccharide-bearing molecules, such as strategies applied to monitoring the synthesis of carbohydrate-based molecules, research channeled toward the investigation of the action of enzymes on carbohydrates, and studies showing how CE has been recently applied to investigate naturally occurring processes, pursuing the ultimate goal of monitoring single cell reactions.

Chapter 8 investigates *biopharmaceuticals,* a term commonly employed to define therapeutic proteins produced by biotechnology and specifically via genetic engineering. Many of the biopharmaceuticals currently produced are glycoproteins, in which the oligosaccharide chains can impact markedly on bioactivity and several other properties. It is therefore mandatory to control glycoproteins by robust methods capable of providing adequate details regarding minor modifications. CE has proved its usefulness not only for the characterization of glycoprotein pharmaceuticals but also as a quality-control tool enabling accurate quantitation.

Chapter 9 highlights recent developments in the analysis of proteins glycosylated of the amino groups of asparagine residues by CE and CE-MS. The analysis of intact glycoproteins is reviewed followed by a detailed analysis of *N*-glycans released from glycoproteins through chemical or enzymatic reactions. Furthermore, to obtain detailed structural information, the advantages and limitations of various methodological approaches and techniques, as well as MS instrumentation, are discussed specifically in the context of glycan analysis.

Chapter 10 evaluates CE as a powerful analytical method to effectively and accurately determine the monosaccharide composition of glycoproteins and glycolipids in biological and biomedical samples and in relation to its potential application in the diagnosis or prognosis of diseases.

As the editor of this volume, I would like to thank all the contributors for their first-rate work, which will certainly impact the area of CE separation of carbohydrates by providing a better understanding of the underlying analytical phenomena and by widening the scope of their possible applications. Acknowledgement is due to the editors at Springer for their assistance in bringing this issue to publication.

Modena, Italy Prof. Nicola Volpi

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Chapter 1
Analysis of Simple Carbohydrates by Capillary Electrophoresis and Capillary Electrophoresis–Mass Spectrometry

Christian W. Klampfl, Markus Himmelsbach, and Wolfgang Buchberger

Abstract An overview of the application of capillary electrophoresis and capillary electrophoresis–mass spectrometry in the analysis of simple carbohydrates without any previous derivatization step is given. Besides electrolyte systems for carbohydrate separation, detection techniques employed in capillary electrophoresis, such as spectrophotometric detection, electrochemical detection, and mass spectrometric detection, are discussed, as are less common detection techniques. Thus, the chapter focuses on the assessment of the strong and weak points of these detection techniques for carbohydrate analysis. A table included in this chapter gives a comprehensive listing of capillary electrophoresis and capillary electrophoresis–mass spectrometry applications in the fields of carbohydrate analysis published within the last decade.

Keywords Capillary electrophoresis • Carbohydrates • Monosaccharides • Mass spectrometry • Spectrophotometric detection • Electrochemical detection

Abbreviations

AU	absorbence units
CAPS	3-cyclohexylamino-1-propane-sulfonic acid
CE	capillary electrophoresis
CTAB	cetyltrimethylammonium bromide
CTAH	cetyltrimethylammonium hydroxide
EOF	electroendoosmotic flow
FT-IR	Fourier transform infrared spectroscopy
GC	gas chromatography
HDB	1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene hexadimethrine bromide)

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HPLC	high-performance liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MTAB	myristylmethylammonium bromide
NAA	1-naphthylacetic acid
PAD	pulsed amperometric detection
PDC	2,6-pyridinedicarboxylic acid
PNP	*p*-nitrophenol
SIM	selected ion monitoring
TTAOH	tetradecyltrimethylammonium hydroxide
TIC	total ion current
TMP	thermomechanical pulp

1.1 Introduction

Carbohydrates play an important role in a variety of biological processes. Therefore, there is increasing interest in the analysis of these compounds holding essential bioinformation within a wide range of fields. Some of the most abundant fields of application (regarding carbohydrate analysis) are food analysis [1], analysis of biomedical samples [2, 3], and investigations on plants and plant-related materials [2, 3]. Focusing on the instrumental techniques commonly employed for carbohydrate analysis, important exponents are gas chromatography (GC) [4], high-performance liquid chromatography (HPLC) [3], and capillary electrophoresis (CE) [3]. Of these three techniques, HPLC, especially in combination with pulsed amperometric detection (PAD), is the most widely applied technique in the fields of carbohydrate analysis. Although GC plays only a minor role, as it is applicable only after derivatization of the sugars, CE has attracted substantial interest in the analysis of both derivatized and underivatized carbohydrates. This can be seen from the number of review articles and book chapters published in this field within the last decade [5–10]. This chapter gives a detailed overview of the analysis of simple carbohydrates by CE without a previous derivatization step. The analysis of derivatized carbohydrates is discussed in Chapter 2.

1.2 Determination of Simple Carbohydrates by CE

This section presents an overview of the reports on the analysis of simple carbohydrates using CE that have been published throughout the last decade (Table 1.1). These comprise a wide range of fields, from the separation and subsequent detection of simple standard mixtures to the analysis of relatively complex real samples. Thus, different approaches for separation systems and detection systems are presented. As detection can be regarded as the crucial issue in the determination of sugars by CE, applications included in Table 1.1 are classified based on the type of detector employed.

Table 1.1 Analysis of simple carbohydrates by CE and CE-MS

Sample	Carbohydrates determined	Carrier electrolyte	Detection	References
Standard solution	D-erythrono-1,4-lactone, D-ribono-1,4-lactone, D-galactono-1,4-lactone, D-glucono-1, 4-lactone, D-mannono-1,4-lactone, D-xylono-1, 4-lactone, quinic acid, isosaccharino-1, 4-lactone, D-erythronic acid, L-threonic acid, tartaric acid, D-xylonic acid, D-ribonic acid, isosaccharic acid, D-gluconic acid, L-mannonic acid, D-galactonic acid, glucaric acid, glucuronic acid, 2-keto-gluconic acid, 5-keto-gluconic acid	600 mM Sodium borate (pH 7.1)	UV 200 nm	[16]
Plant fiber	Galactose, glucose, rhamnose, mannose, arabinose, xylose	130 mM NaOH/36 mM disodium hydrogen phosphate (pH 12.6)	UV 270 nm	[14]
Fruit juices and cognac	Xylitol, mannitol, sucrose, fucose, cellobiose, galactose, glucose, rhamnose, mannose, arabinose, xylose, ribose	130 mM NaOH/36 mM disodium hydrogen phosphate (pH 12.6)	UV 270 nm	[15]
Juice	Glucose, fructose, maltose	200 mM Borate (pH 9.5)	UV 195 nm	[17]
Standard solution	Sucrose, ribose, glucose	6 mM $CuSO_4$, 500 mM NH_3 (pH 11.6)	UV 245 nm	[18]
Standard mixture	Glucose, rhamnose, mannose, fructose, lyxose, xylose, ribose, arabinose, galactose, fucose, lactose, lactulose, turanose, sucrose, raffinose, trehalose, maltotriose, gluconic acid	6 mM Sorbic acid with (a) 5% DEA (pH 12.6) (b) NaOH (pH 12.2) 6 mM Riboflavin/50 mM borate (pH 9.5)	Indirect 256 nm Indirect 267 nm (for riboflavin)	[27]
Low-calorie soft drink	Sucralose, sucrose, glucose, fructose	3 mM 3,5-Dinitrobenzoic acid (pH 12.1)	Indirect 238 nm	[39]
Heroin	Dextrose, lactose, sucrose, inositol, mannitol	Agilent basic anion buffer modified to pH 12.1	Indirect 275 nm	[20]

(continued)

Table 1.1 (continued)

Sample	Carbohydrates determined	Carrier electrolyte	Detection	References
Food and beverage	Talose, ribose, mannose, fructose, xylose, glucosamine, glucose, galactosamine, allose, galactose, fucose, sucrose, mannitol, xylitol, inositol	20 mM PDC and 0.5 mM CTAH (pH 12.1)	Indirect 350 nm	[21]
Standard solution	Glucose-6-phosphate, glucose-1-phosphate, 2-deoxyribose-5-phosphate, ribose-1-phosphate, ribose-5-phosphate, fructose-1,6-diphosphate	6 mM Potassium sorbate (pH 5.8)	Indirect 256 nm	[26]
Foliage	Ribose, mannose, fructose, rhamnose, glucose, galactose, sucrose, arginine, mannitol, sorbitol, pinitol, inositol	10 or 20 mM Sodium benzoate/0.5 mM MTAB (pH 12.0)	Indirect 225 nm	[32]
Juice samples	Glucose, fructose, xylose, rhamnose, ribose, arabinose, xylitol, mannitol, fucose, galactose, mannose, lactose, maltose, lyxose, sucrose	50 mM Glycyl–glycine (pH 12)	Indirect 207 nm	[40]
Cereals and dairy products	Fructose, glucose, galactose, lactose, maltose, maltotriose, sucrose	(a) 15 mM Sorbate/0.2 mM CTAB/35 mM NaOH (b) 15 mM Sorbate/0.3 mM CTAB/55 mM NaOH	Indirect 254 nm	[28]
Sports drinks, nutrient-added drinks, fruit juices, dairy products, serum samples	Glucose, galactose, mannose, fructose, arabinose, ribose, xylose, lyxose, melibiose, cellobiose, turanose, rhamnose, L-sorbose, lactulose, lactose, maltose, sucrose, raffinose, glucuronic acid, gluconic acid	2 mM NAA (pH 12.2)	Indirect 222 nm	[33]
Orange juice, yogurt, pickled ume apricot (prunus mume), sake mash	Mannuronic acid, glucuronic acid, galacturonic acid, *N*-glycolylneuraminic acid, *N*-acetyl-neuraminic acid, ribose, mannose, xylose, glucosamine, glucose, galactosamine, galactose, fucose, mannitol, sorbitol, xylitol, inositol, fructose, rhamnose, lactulose, lactose, sucrose, galactitol, *N*-acetylmannosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, raffinose	20 mM PDC/0.5 mM CTAH (pH 12.1)	Indirect 350 nm	[22]

Orange juice	Fructose, glucose, sucrose	70 mM NaOH/2 mM naphthol blue-black/80 mM sodium phosphate (pH 12.5)	Indirect 570 nm	[35]
Orange juice	Fructose, glucose, sucrose	40 mM Sodium phosphate/1 mg mL^{-1} naphthol blue-black (pH 12.5)	Indirect 570 nm	[36]
Soft drinks and fruit juice	Mannuronic acid, glucuronic acid, galacturonic acid, gluconic acid, *N*-acetylneuraminic acid, fructose, rhamnose, glucose, galactose, 2-deoxy-D-ribose, sucrose, xylose, cellobiose	6 mM Sorbate/0.001% w/v HDB/5% v/v acetone (pH 12.1)	Indirect 254 nm	[29]
Non-alcoholic beer, dietetic fruit juices, and chocolate	Sucrose, maltotriose, maltose, glucose, fructose	1 mM NAA (pH 12.5)	Indirect 222 nm	[34]
Urine	Lactulose, mannitol	6 mM Sorbic acid/1.25 mM CTAB/50 mM LiOH (pH 12.5)	Indirect 254 nm	[30]
Standard solution	2-, 3- and 4-Sulfated-L-fucose	12 mM Ethanolamine/2 mM trimesic acid in methanol–ethanol (1:1, v/v)	Indirect 200 nm	[37]
Standard solution	Sucrose, cellobiose, maltose, lactose, 4-*O*-galactosyl-mannopyranoside, isomaltose, fructose, mellibiose, glucose	175 mM Sodium borate (pH 10.0)/6 mM PNP	Indirect 400 nm	[38]
Soy sauce, nutrient tonic, pineapple	Galacturonic acid, *N*-acetylmannosamine, ribose, fructose, *N*-acetylglucosamine, mannose, xylose, *N*-acetylgalactosamine, ramnose, glucosamine, mannosamine, lactose, arabinose, glucose, maltose, galactosamine, lactulose, galactose, fucose, sucrose, raffinose, mannuronic acid, mannitol, trehalose, sorbitol, galactitol, glucuronic acid, xylitol, erythritol, inositol	20 mM PDC/0.5 mM CTAH (pH 12.1)	Indirect 350 nm	[23]

(continued)

Table 1.1 (continued)

Sample	Carbohydrates determined	Carrier electrolyte	Detection	References
Glycoproteins after hydrolysis	Monosaccharides liberated from fetuin	20 mM PDC/0.5 mM CTAB (pH 12.1)	Indirect 350 nm	[24]
Lycopersicon extracts	Fructose, glucose, sucrose	20 mM PDC/0.1% HDB (pH 12.1)	Indirect 200 nm	[25]
Standard solution	Glucuronic acid, galacturonic acid, gluconic acid, mannose, rhamnose, glucose, galactose, 2-deoxy-D-ribose, raffinose	6 mM Sorbate/0.001% HDB (pH 12.2)	Indirect 254 nm	[31]
Must and wine	Gluconic acid, galacturonic acid, glucuronic acid	5 mM β-Resorcylic acid/1 mM TTAOH (pH 3.0)	Indirect 214 nm	[41]
Soft drinks, isotonic beverages, fruit juice, sugarcane spirits	Fructose, glucose, sucrose	10 mM NaOH/4.5 mM disodium hydrogen phosphate/0.2 mM CTAB	Conductivity	[49]
Coconut water	Fructose, glucose, sucrose	10 mM NaOH/4.5 mM disodium hydrogen phosphate/0.2 mM CTAB	Conductivity	[50]
Standard solution	Sucrose, lactose, galactose, glucose, mannose, fructose, xylose, ribose	80 mM NaOH	Amperometric	[60]
Urine and beverage	Sucrose, lactose, galactose, glucose, mannose, fructose	100 mM NaOH	Amperometric	[51]
Honey samples	Glucose, sucrose, fructose	50 mM NaOH	Amperometric	[58]
Standard solution	Glycerol, raffinose, fucose, galactose, mannose, arabinose, xylose, glucose	100 mM NaOH	Amperometric	[59]
Standard solution	Glucose, sucrose	100 mM NaOH	Amperometric	[81]
Coke and diet coke	Sucrose, glucose, fructose	50 mM NaOH (pH 12.7)	Amperometric	[82]

Standard solution	Glucose, glucosamine	5 mM Borate, 2.4 mM SDS	Amperometric	[66]
Soft drinks	Glucose, raffinose, galactose, cellobiose, xylose, fructose, sucrose	80 mM NaOH	Amperometric	[56]
Standard solution	Glucose, sucrose, galactose, fructose, sodium gluconate, glucuronic acid	35 mM NaOH	Amperometric	[57]
Honey	Trehalose, sucrose, lactose, galactose, glucose, mannose, fructose, xylose	100 mM NaOH	Amperometric	[83]
Standard solution	Glucose, maltose, xylose	12 mM NaOH/8 mM potassium carbonate (pH 12.0)	Amperometric	[65]
Standard solution	Glucose, fructose, lactose, sucrose, maltose	10 mM Sodium borate (pH 12.0)	Amperometric	[67]
Moutan cortex	Paeoniflorin, sucrose, paeonoside, glucose, fructose	75 mM NaOH	Amperometric	[61]
Black tea beverage	Glucose, sucrose, fructose	30 mM NaOH	Amperometric	[84]
Standard solution	Maltose, glucose	60 mM Tetraborate/50 mM sodium sulfate (pH 9.3)	Electrochemical with a bienzyme electrode	[69]
Samples were obtained by heating glucose with amino acids	Fructosyl derivatives of amino acids	20 mM Ammonium acetate (pH 6.9)	ESI-MS and MS/MS	[72]
Urine	4-*O*-α-D-Galactopyranosyl-D-galactopyranose, 5-*N*-acetyl-neuraminic acid, 1-*O*-methyl-α-D-mannopyranoside, *O*-glycosylated peptides	50 mM Ammonium acetate/ammonia (pH 11 and 12)	ESI-MS	[73]
Wine samples	Deoxyribose, arabinose, ribose, xylose, xylitol, fucose, galactose, glucose, mannose, fructose, inositol, mannitol, sucrose, maltose, raffinose, glucosamine, galactosamine	300 mM DEA	ESI-MS	[74]

(continued)

Table 1.1 (continued)

Sample	Carbohydrates determined	Carrier electrolyte	Detection	References
E. coli lysates	α-D-Galactosamine-1-phosphate, α-D-talose-1-phosphate, 2-deoxy-α-D-galactose-1-phosphate, 3-deoxy-α-D-glucose-1-phosphate, 6-azido-6-deoxy-α-D-glucose-1-phosphate, 6-bromo-6-deoxy-α-D-galactose-1-phosphate, 6-deoxy-α-D-galactose-1-phosphate, 6-deoxy-6,6-difluoro-α-D-galactose-1-phosphate, 6-deoxy-6-fluoro-α-D-glucose-1-phosphate, 6-deoxy-6-thio-α-D-galactose-1-phosphate, β-L-altrose-1-phosphate, β-L-glucose-1-phosphate, α-D-galactose-1-phosphate, α-D-glucose-1-phosphate, 6-azido-6-deoxy-α-D-galactose-1-phosphate, 6-chloro-6-deoxy-α-D-galactose-1-phosphate, 2-deoxy-α-D-glucose-1-phosphate, 6-deoxy-α-D-glucose-1-phosphate, 4-deoxy-α-D-galactose-1-phosphate, α-D-allose-1-phosphate, α-D-glucosamine-1-phosphate, 6-amino-6-deoxy-α-D-galactose-1-phosphate	30 mM Morpholine/formate (pH 9.0)	ESI-MS	[75]
Orange fruit juice	Sucrose, galactose, glucose, fructose	50 mM Sodium carbonate (pH 12.3)	FT-IR	[76]
Standard solution	Glucose, fructose, saccharose	25 mM Sodium borate (pH 8.0)	Refractometric	[77]
Standard solution	Maltose, lactose, and ribose	33 mM Borate/13.3 mM CAPS	Refractometric	[78]
Standard solution	Sucrose, lactose, maltose, raffinose, galactose, xylose, glucose	50 mM NaOH	Aerosol chemiluminescent detector	[79]

1.2.1 Detection Systems in CE

As in any other separation technique, detection plays an important role in CE. In principle, all types of detectors suitable for liquid chromatography can also be used in CE. The most commonly employed detection techniques are spectrophotometric (either absorbance or fluorescence) detection, electrochemical detection (potentiometry, voltammetry, conductometry), and mass spectrometric detection [11]. In addition to these widely used techniques, other detection systems include infrared spectroscopy [12] and refractometry [13] in combination with CE.

1.2.1.1 Spectrophotometric Detection for the Determination of Underivatized Carbohydrates by CE

The majority of commercially available instruments for CE are equipped with a spectrophotometric detector, because of the simplicity of this approach (the capillary also serves as the detection cell) as well as its suitability for the detection of a wide range of (mostly organic) compounds. On the other hand, this very simple detector layout has a substantial drawback: the small inner diameter of the separation capillaries commonly employed in CE results in a short optical path length, leading to reduced sensitivity (according to Beer's law). For the analysis of simple carbohydrates, the use of spectrophotometric detectors is additionally compromised by their low ultraviolet (UV) absorbance (within the commonly used range of detection wavelengths). For this reason, spectrophotometry is not the perfect choice when it comes to the detection of carbohydrates, unless a UV-absorbing or (in the case of fluorescence detection) fluorescent tag is attached in a preceding derivatization step. Capillary electrophoresis analysis of such derivatized carbohydrates is discussed in detail in Chap. 2; the present chapter reviews the CE analysis of simple carbohydrates in their native form.

Starting with direct UV detection (i.e., the measurement based on the UV absorbance of the analytes), there are a few strategies for the use of this approach for carbohydrate analysis with CE. Highly alkaline electrolyte solutions (pH >12.5) enhance the detectability of carbohydrates in the UV range [14, 15]. This increase in UV absorbance is due to a reaction cascade of ionization, mutarotation, enolization, and isomerization, resulting in the formation of enediolate anions. The proposed mechanism for this reaction is depicted in Fig. 1.1. These enediolate anions show sufficient absorbance at a wavelength of 270 nm, with a molar absorptivity of about 40, leading to limits of detection (LODs) and limit of quantification (LOQ) values of 0.01–0.05 mM [15]. An example for the application of this strategy to the analysis of hydrolyzed cellulosic fiber of different plants is depicted in Fig. 1.2. Also complexation with borate [16, 17] or copper [18] can facilitate the detection of sugars by UV absorbance. However, the use of borate requires relatively low wavelengths (195–200 nm), whereas the copper (II) system allows detection at 245 nm, with detection limits in the range of 50–100 μM.

The most widely used approach for the detection of compounds showing very little or no UV absorbance using CE with a spectrophotometric detector is the indirect detection mode. This mode uses a carrier electrolyte and a strongly UV-absorbing probe.

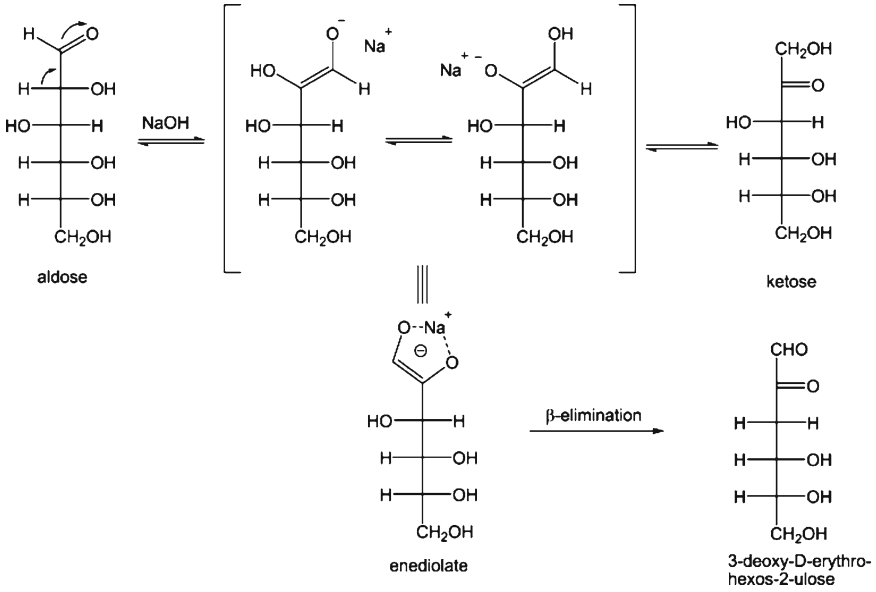

Fig. 1.1 Proposed reaction mechanism for enediolate formation and subsequent ß-elimination reaction (from Rovio et al. [15], with permission)

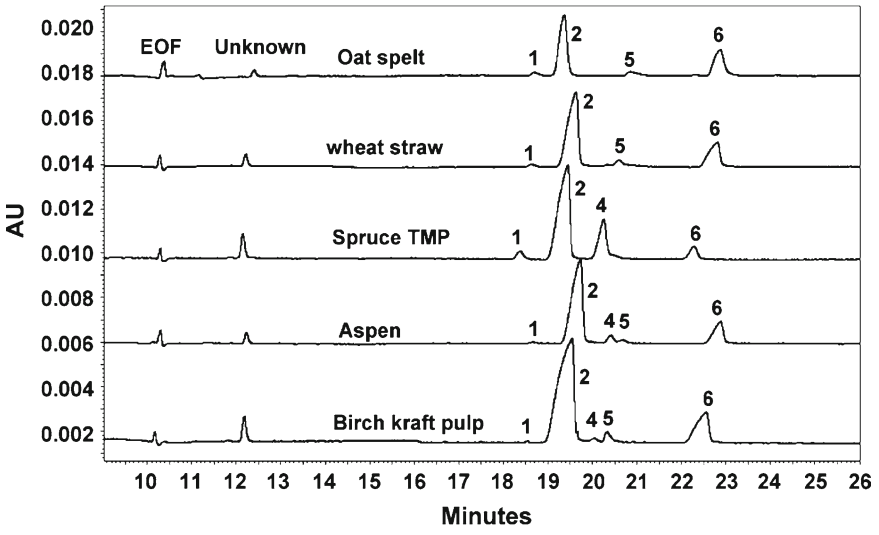

Fig. 1.2 Electropherogram of undiluted acid hydrolyzed samples. Experimental conditions: fused-silica capillary 50 μm × 60 cm (50 cm effective length); separation voltage of 17 kV; background electrolyte, 130 mM NaOH/36 mM NaH_2PO_4 (pH 12.6); UV detection at 270 nm. 1, galactose; 2, glucose; 3, rhamnose; 4, mannose; 5, arabinose; 6, xylose. (From Rovio et al. [14], with permission). *AU* Absorbance Units; *EOF* electroosmotic flow; *TMP* thermomechanical pulp

Analyte zones lead to a decrease in UV absorbance, due to exchange of the absorbing probe ions, generating negative peaks that can be flipped by 180° employing suitable software thereby leading to positive peaks and a more conventional appearance of the electropherogram [19]. Although this approach can be seen as a step toward an almost universal detection system, it has a number of drawbacks. Problems with sensitivity occur if the simultaneous detection of UV-absorbing and nonabsorbing solutes is desired. Indirect UV detection is often biased by the frequent occurrence of system peaks, which probably interfere with analyte signals. The use of strongly alkaline electrolytes (such as in the case of carbohydrate analysis) is problematic, as the accumulation of carbonate from the ambient air leads to additional signals in the electropherogram. Nevertheless, several researchers have reported the use of indirect UV detection in the CE separation of native carbohydrates. Probes employed for indirect UV (or visible)-detection of carbohydrates comprise pyridine-2,6-dicarboxylic acid [20–25], sorbate [26–31], benzoate [32], 1-naphthylacetic acid [33, 34], naphthol blue-black [35, 36], trimesic acid [37], 4-nitrophenol [38], 3,5-dinitrobenzoic acid [39], glycyl–glycine [40], and resorcylic acid [41]. Typical LODs for simple sugars achieved using these probes are in the range of 20–100 μM. An additional advantage of this approach is that it enables determining a variety of

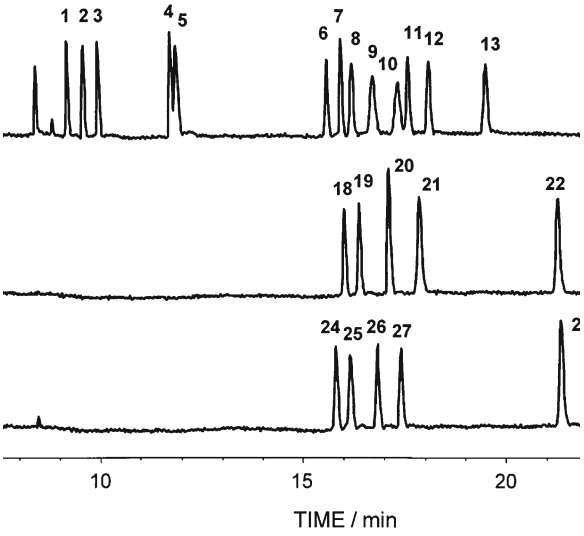

Fig. 1.3 Separation of 28 carbohydrates by CE. Experimental conditions: fused-silica capillary 50 mm × 112.5 cm (104 cm effective length); separation voltage, −25 kV; background electrolyte, 20 mM pyridinedicarboxylic acid (pH 12.1); injection, 50 mbar × 6 s; temperature 20°C; indirect detection at 275 nm. Peak assignment: 1, mannuronic acid; 2, glucuronic acid; 3, galacturonic acid; 4, *N*-gylcolneuraminic acid; 5, *N*-acetylneuraminic acid; 6, ribose; 7, mannose; 8, xylose; 9, glucosamine; 10, glucose; 11, galactosamine; 12, galactose; 13, fucose; 14, mannitol; 15, sorbitol; 16, xylitol; 17, inositol; 18, fructose; 19, rhamnose; 20, lactulose; 21, lactose; 22, sucrose; 23, galactitol; 24, *N*-acetylmannosamine; 25, *N*-acetylglucosamine; 26, *N*-acetylgalactosamine; 27, arabinose; 28, raffinose; 200 mg/L each. (From Soga and Serwe [22], with permission)

different compounds ranging from inorganic anions to carboxylic acids, amino acids, and sugars employing a single separation and detection system [21]. Figure 1.3 shows the separation of three standard mixtures with a total of 28 carbohydrates using the pyridine-2,6-dicarboxylic acid electrolyte system and indirect UV detection at 275 nm.

1.2.1.2 Electrochemical Detection for the Determination of Carbohydrates by CE

Electrochemical detection can be seen as a complement to the more widely employed spectrophotometric detection, providing substantial advantages for substances showing only little or no UV absorbance [42–44]. Therefore, in carbohydrate analysis the interest in conductometric or amperometric detection equals or even exceeds that of spectrophotometry, a situation that is rather uncommon for most other classes of analytes.

Conductivity detection is a perfect choice for CE, as detection is based on an intrinsic property of CE analytes, namely their charge [45]. There are two different basic designs for conductivity detection (in combination with CE): a contact mode and a contactless design, first reported in 1998 [46, 47]. Today only the latter plays a significant role, as the detectors based on galvanic contact between the electrodes and the electrolyte solution suffered from several shortcomings, such as tedious electrode alignment or electrode fouling [48]. For sugars, detection limits achieved with contactless conductivity detection are in the micromolar range [49, 50] and thus are comparable with those achieved in indirect UV detection.

Amperometric detection may be well suited for miniaturized separation systems like capillary electrophoresis, because the use of microelectrodes does not necessarily deteriorate the detection limits. Unfortunately, the high voltage applied for the separation may seriously interfere with the amperometric measurement. Various ways have been suggested to avoid these problems, such as the decoupling of the separation voltage from the detector, which can be done by combining the separation capillary with a short transfer capillary, at the end of which the detection electrode is positioned. These two capillaries are connected by a piece of porous materials, through which the electric current of the electrophoresis is grounded so that the detection electrode remains unaffected. Alternatively, the use of separation capillaries of somewhat smaller inner diameter makes the use of systems without a decoupler feasible, although the positioning of the detection electrode relative to the end of the separation capillary is quite critical.

Amperometric detection of carbohydrates after CE separation entails different approaches, such as detection at constant potential, PAD, and detection at enzyme-modified electrodes.

Detection of carbohydrates at constant potential uses metallic nickel electrodes [51, 52], metallic copper electrodes [53–56], copper composite electrodes [57], or electrodes modified with nickel oxide [58] or coppe oxide [59, 60]. In all cases,

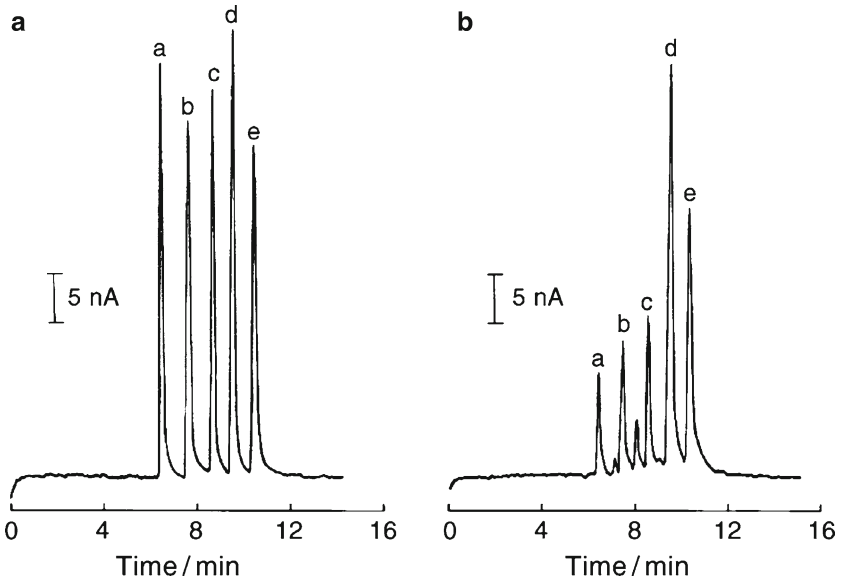

Fig. 1.4 CE separation and amperometric detection of gycosides and sugars in traditional Chinese medicine Moutan Cortex: (**a**) standard solution, (**b**) sample extract. Experimental conditions: fused-silica capillary 25 mm × 40 cm; separation voltage, 12 kV; carrier electrolyte, 75 mM NaOH; injection, electrokinetical, 12 kV × 6 s; temperature, 25°C; amperometric detection, working electrode: metallic copper at 0.6 V (vs. saturated calomel electrode). Peak assignment: a, paeniflorin; b, sucrose; c, paenoside; d, glucose; e, fructose. (From Chen et al. [61], with permission)

strongly alkaline conditions are required, and a constant oxidative potential is applied. Under these conditions, a layer of an oxide of higher valency is generated, such as NiOOH, which oxidizes the carbohydrate and is reduced to a lower valency oxide such as $Ni(OH)_2$. The latter is again oxidized by the applied voltage, thereby generating the analytical signal. Typical detection limits at about 1 μmol/L have been reported. An example of the separation and detection of glycosides and sugars in the traditional Chinese medicine Moutan Cortex is given in Fig. 1.4 [61].

The use of gold electrodes under strongly alkaline conditions also enables the oxidative detection of carbohydrates, but rapid electrode fouling due to accumulation of adsorbed oxidation products is the consequence if the electrode is operated at constant potential. This problem can be circumvented if PAD is employed. In this case, a moderate positive potential is applied to oxidize the analyte and to generate the analytical signal, followed by a high positive potential oxidizing the electrode to clean the surface, and a negative potential to reactivate the surface by reducing the oxide generated during the cleaning step. This cycle of three potentials is repeated at a frequency of 1–2 Hz. PAD has become a routine tool for detection of carbohydrates after liquid chromatographic separation, and has also been evaluated for its suitability in CE using a classic instrumental setup [62–64] as well as instrumentation in the chip format [65–67]. Detection limits are in the low μM range.

Enzyme-modified electrodes reported so far for the detection of carbohydrates in CE are based on glucose oxidase immobilized in carbon paste [68] or on platinum [55]. The enzyme catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, which can be detected in the oxidative amperometric mode. The disadvantage is the specific action of the enzyme for glucose, so that other carbohydrates cannot be detected. The immobilization of both amyloglucosidase, which converts maltose to glucose, and glucose oxidase on the surface of the electrode [69] enables the detection of both maltose and glucose. Similar approaches with additional enzymes might provide an option for detection of a wider range of analytes.

1.2.1.3 Other Detection Methods for the Determination of Carbohydrates by CE

Mass spectrometry detection in combination with CE separations is a highly powerful technique, as can be seen from the increasing number of publications demonstrating the use of CE-MS in a variety of fields [70, 71]. Mass spectrometry is especially suitable for the detection of compounds with very little or no UV absorbance. In addition to its often-superior sensitivity, MS (and even more MS-MS) detection provides additional spectroscopic information, thereby increasing the certainty in peak assignment. This development is also reflected in carbohydrate analysis, although up to now there have been only a few reports on the use of this technique for the analysis of simple carbohydrates [72–75]. Nevertheless, it should be noted that the majority of CE-MS and CE-MS-MS reports can be found in the fields of bioanalysis, focusing mostly on structurally more complex carbohydrates [8]. The type of MS instrumentation employed for CE-MS of simple carbohydrates ranges from basic quadrupole mass selective detectors operated in the selected-ion-monitoring mode [74] to instruments with MS-MS capabilities [72, 75], and finally high-resolution MS [73], providing excellent mass accuracy, thus leading to the proposal of molecular formulas for unknown compounds. Typical LODs using this type of instrumentation are in the low μM range. Figure 1.5 depicts the application of CE-MS determination of carbohydrates in wine samples.

In addition to the three common detection techniques (spectrophotometric, electrochemical, and MS), other types of detectors have been combined with CE [11]. Focusing on carbohydrate analysis, the use of mid-infrared detection [76], refractometric detection [77, 78], and an aerosol chemiluminescence detector [79] has been reported. As in the case of MS, mid-infrared spectra provide additional information on the structure of the detected molecules, although detection limits are substantially higher (low millimolar range). Figure 1.6 shows the application of this combination to the analysis of a carbohydrate standard. Although there is no commercially available instrumentation, refractometry might be a possible alternative to indirect photometric detection, providing the advantage of unbiased sensitivity for both UV absorbing and nonabsorbing analytes.

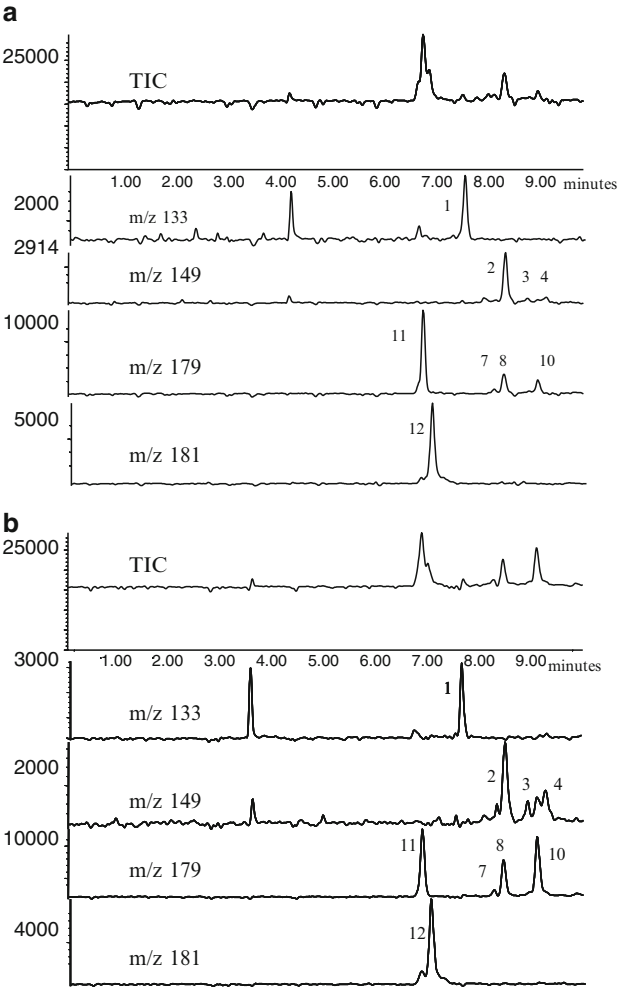

Fig. 1.5 CE-MS Total Ion Current (TIC)- and Single-Ion Monitoring (SIM)-electropherograms for a red wine (**a**) and a white wine (**b**) sample. Experimental conditions: capillary, fused silica 70 cm×50 μm; separation voltage, +20 kV; carrier electrolyte, 300 mM diethylamine; injection, 9 s at 50 mbar; sheath liquid, 4 μL/min of 0.25% diethylamine in 2-propanol/water 80:20% v/v. Peak assignment: 1, deoxyribose; 2, arabinose; 3, ribose; 4, xylose; 5, xylitol; 6, fucose; 7, galactose; 8, glucose; 9, mannose; 10, fructose; 11, inositol; 12, mannitol; 13, sucrose; 14, maltose; 15, raffinose; 16, glucosamine; 17, galactosamine. (From Klampfl and Buchberger [74], with permission)

1.2.2 Electrolyte Systems for the CE-Separation of Simple Carbohydrates

According to the basic principles of separation, only charged analytes can be resolved employing capillary zone electrophoresis (CZE). In the case of an

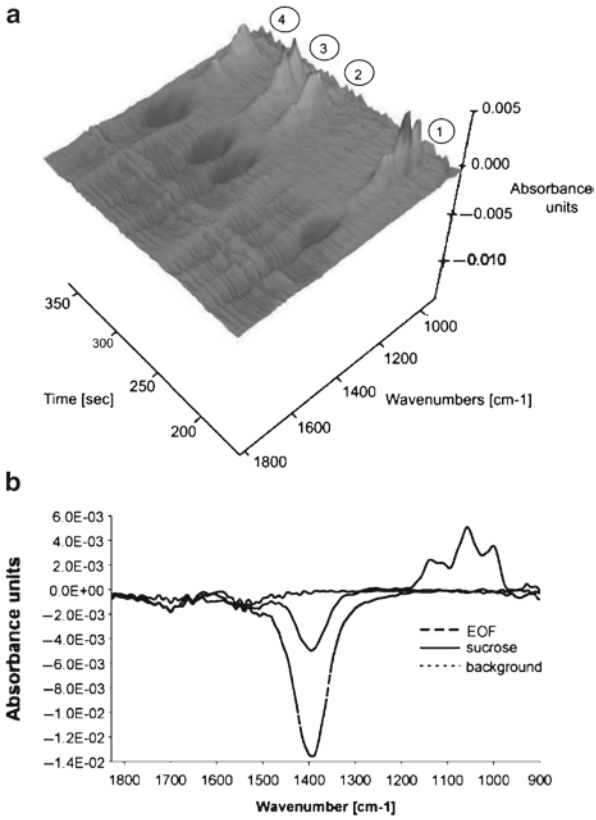

Fig. 1.6 On-line Fourier transform infrared spectroscopy (FTIR) detection of carbohydrate standards. Experimental conditions: fused-silica capillary 50 μm×118 cm (58 cm effective length); separation voltage, 21 kV; background electrolyte, 50 mM Na_2CO_3 (pH 12.3); injection, 9 nL by elevation of the injection vial; temperature, 20°C. (**a**) Detection FTIR 320 kHz scanning speed, co-addition of 64 spectra×8 cm^{-1}. Peak assignment: 1, sucrose; 2, galactose; 3, glucose; 4, fructose. (**b**) Extracted spectra from three-dimensional plot. (From Kölhed and Karlberg [76], with permission)

underivatized carbohydrate, ionization is primarily achieved by using an alkaline carrier electrolyte, resulting in the deprotonation of the sugar–OH groups. Commonly employed electrolytes are based either on alkali hydroxides or on carbonates, phosphates, alkali borates, and aromatic carboxylic acids adjusted to basic pH by alkali hydroxides. Due to the low acidity of sugar–OH groups (pK_a 11–13) [80], typical pH values for such electrolyte systems lie in the range of pH 9.5 [17, 27] to pH 13 [81], although there are a few reports on the use of less alkaline electrolytes (pH 8) [77]. Electrolytes with an even lower pH are suitable only if sugars showing additional ionizable functionalities such as acids [16, 41, 72] or phosphates [26] are separated. Of course, electrolyte ingredients have to be chosen based on their compatibility with the detection method employed. For electrochemical detection, most electrolytes are based on solutions of NaOH. Indirect UV detection requires the presence

of a UV-absorptive probe, which often serves also as the buffering agent, such as sorbic acid or pyridine-2,6-dicarboxylic acid. The combination of CE with MS detection implies the use of volatile electrolyte ingredients; thus, for CE-MS, mostly organic amines are used to achieve the basic pH value necessary for the separation of carbohydrates.

1.2.3 Analysis of Simple Carbohydrates in Real Samples by CE

In addition to its use in fundamental studies showing new approaches for the separation or detection of standard mixtures, CE is also an excellent tool for the determination of simple carbohydrates in a variety of matrices. Major fields of application are the analysis of foods and beverages [15, 17, 21–23, 28, 29, 33–36, 39–41, 49–51, 56, 58, 74, 76, 82–84], plant materials [16, 25, 32, 61], and biological samples and body fluids [30, 51, 73, 75]; other reports address the determination of sugars in uncommon matrices such as illicit drugs [20]. The latter paper is a good example of the applicability of CE in the field of forensic analysis; CE was compared with nuclear magnetic resonance (NMR) for the determination of common diluents such as carbohydrates in heroin samples. For analyzing foods and beverages, CE has proven its capabilities in a variety of different samples, including wines and juices, for which CE allows direct analysis after a simple dilution step, and products requiring substantial sample pretreatment [28]. Regardless of the complexity of the matrix of a real sample, CE has two advantages over techniques involving stationary phases like chromatography: unwanted sample components can be simply flushed out after analysis, and replacement of a deteriorated separation capillary is far less costly than acquiring a new HPLC column.

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Chapter 2
Fluorophores and Chromophores for the Separation of Carbohydrates by Capillary Electrophoresis

Michael Breadmore, Emily Hilder, and Artaches Kazarian

Abstract Carbohydrates are known to be the most abundant assembly of biopolymers with immense diversity, as a consequence of the complex glycosylation processes that occur within living organisms. This complexity inevitably complicates the ability to study these structures (Wells et al., Science 291:2376–2378, 2001). Viral infection and tumor malignancy have been pinpointed as a result of subtle changes in the structure of oligosaccharides, implying the significance of carbohydrates and ultimately the importance of the field of glycobiology (Chen et al., Nat Med 3:866–871, 1997; Isogai et al. Cancer Res 56:3902–3908, 1996). In most cases capillary electrophoresis (CE) offers greater advantages for studying carbohydrates when compared to other techniques, resulting in utilization of smaller samples, better separation efficiency, and shorter separation time. Typical analysis of carbohydrates in CE requires incorporation of a chromophore or a fluorophore through derivatization, which in due course enhances sensitivity and can also improve the separation of the analytes. This chapter discusses the currently used derivatization procedures, their mechanisms, and the advantages of each approach. The discussion mainly focuses on the publications from 2003 to 2009.

Keywords Capillary electrophoresis • Carbohydrates • Fluorophores • Chromophores • Reductive amination • *N*-methyl-glycamine derivatives • Pyrazolone compounds • On-column derivatization

Abbreviations

AcOH	acetic acid
ANDSA	7-aminonaphthalene-1,3-disulfonic acid
CBQCA	3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde
CE	capillary electrophoresis

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CL	chemiluminescence
DAD	diode array detection
DMSO	dimethylsulfoxide
HVAox	2,2′-hydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid
Kdo	2-keto-3-deoxyoctuolosonic acid
LED	light-emitting diode
LIF	laser-induced fluorescence
LODs	limits of detection (measured in nM unless stated otherwise)
MALDI	matrix-assisted laser desorption ionisation
MeOH	methanol
NBD-F	7-nitro-2,1,3-benzoxadiazole 4-fluoride
NMP	1-(2-naphthyl)-3-methyl-5-pyrazolone
NPBA	3-nitrophenylboronic acid
NS	not stated
PEG	polyethylene glycol
PITC	phenylisothiocyanate
PMP	1-phenyl-3-methyl-2-pyrazolin-5-one
QY	quantum yield
SA	sulfanilic acid
SAP	(S)-3-amino-1,2-propanediol
SDS	sodium dodecyl sulfate
THF	tetrahydrofuran
TRSE	4-carboxytetramethylrhodamine succinimidyl ester
UV	Ultra violet
Vis (or vis)	visible

2.1 Introduction

The application of capillary electrophoresis (CE) for carbohydrate separation can be difficult, as most carbohydrates are uncharged and do not contain any chromophore or fluorophore. In some cases detection by ultraviolet (UV) and refractive index of free carbohydrates is possible, although the sensitivity and selectivity are poor, which presents a major problem for such approaches [4–6]. Incorporation of a fluorophore or a chromophore is the most commonly used strategy to facilitate detection of the carbohydrates and to provide the low limits of detection (LODs) needed for many applications. In many instances, reagents used for derivatization also possess additional charged functionalities, which can aid the separation by improving the selectivity, efficiency, and resolution.

Chromophores, absorb light at a specific wavelength and are used for absorbance detection, with the detector response proportional to the concentration of the chromophore according to Beer's law, $A = \varepsilon bc$, where A is the absorbance, ε is the molar absorptivity, b is the path length, and c is the concentration [7]. It is important to note that the detector response is directly proportional to ε, and more sensitive detection is obtained by reagents with a higher molar absorptivity. What is less obvious is that the wavelength of maximum absorbance should also correspond to

a strong output signal from the light source, and thus traditionally UV tags have been used predominantly as chromophores because of their compatibility with the high-intensity light output from deuterium light sources used in most CE units.

For more sensitive detection, a reagent containing a fluorophore can be used. Light of a suitable wavelength is focused onto the capillary, where a species of interest is excited by a photon of light and allowed to de-excite, with the result being the emission of a photon at a slightly higher wavelength. The emitted light is collected at a 90° angle from the excitation source to minimize the amount of stray light, and then this light is subjected to spectral and spatial filtering to isolate the required wavelength for detection [8]. Detector response is proportional to the quantum yield (QY) of the reagent, which is the ratio of photons absorbed to photons emitted [9]. Similar to absorbance detection, the wavelength and intensity of the light source also play a significant role, and a more intense light source usually provides more intense emission, thus producing a higher detector response. For this reason, lasers are the most commonly used light source for fluorescence detection in CE.

The way in which the chromophore or fluorophore is attached to the carbohydrate is very important, and much research has been performed to develop efficient and fast derivatization procedures. These are summarized in Fig. 2.1. These methods are

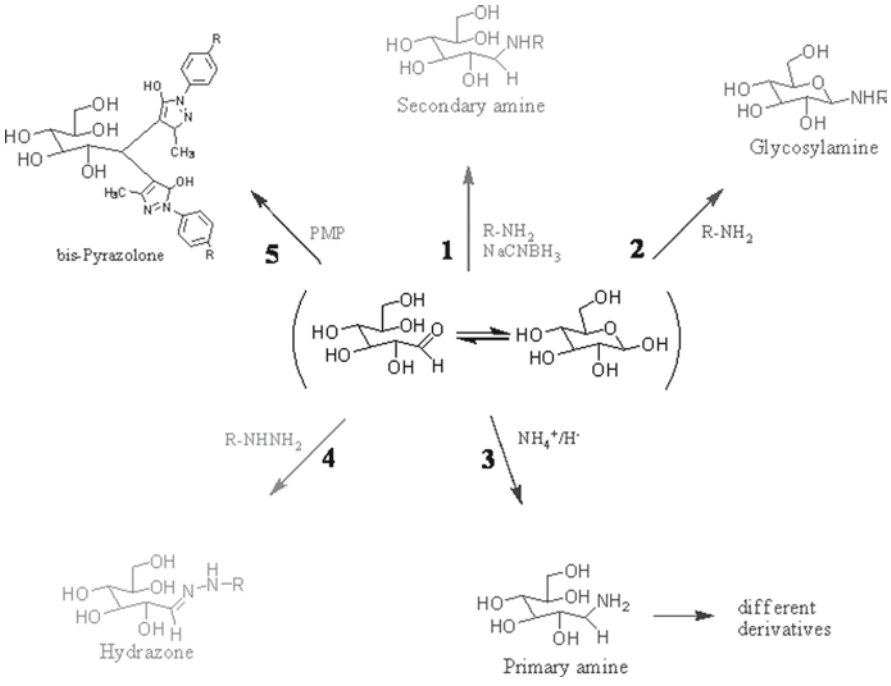


Fig. 2.1 A diagram showing different strategies, assigned as numbers 1–5, for pre-column labeling of carbohydrates: 1, reductive amination; 2, amination via formation of glycosylamine; 3, derivatization with *N*-methylglycamine derivatives; 4, formation of hydrazones; 5, pyrazolone derivatives of carbohydrates. The above derivatization strategies highlight their representative reagents; however, these are not the exclusively used reagents for each derivatization approach

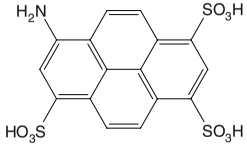
now well established and referred to as reductive amination, amination via formation of glycosylamine, derivatization with *N*-methylglycamine derivatives, formation of hydrazones, pyrazolone derivatives of carbohydrates, and derivatization at carboxylic acid functionalities. Each one of these derivatization strategies is discussed in detail in this chapter, including the mechanism of reaction as well as the advantages and disadvantages of each approach and the range of reagents that has been used, which are summarized in Table 2.1. The table provides information regarding the reagents that have been used, including a short description of structures, properties, and separation and derivatization conditions, as well as commercial availability, LODs, and the sample analytes that have been separated. The applications of each derivatization approach for the separation of carbohydrates that have been published from 2003 to 2009 are discussed. For publications prior to 2003, readers are directed to the large number of comprehensive reviews published by Lamari et al. [10], Gao et al. [11], Suzuki and Honda [12], Paulus and Klockow [5], Shilova and Bovin [13], and Oefner and Chiesa [14] on this topic.

2.2 Reductive Amination

Reductive amination is the most commonly used method for the derivatization of carbohydrates. The reaction facilitates the covalent attachment between the carbonyl group, known as the reducing end of the sugar, and an aromatic complex with a primary amino group, resulting in the formation of a secondary amine. The mechanism of reductive amination is depicted in more detail in Fig. 2.2 [15]. In solution, carbohydrates exist in a number of conformations in equilibrium [1, 2]. Importantly for reductive amination, it is the open chain form 1 that is essential, as the first step in the mechanism is the nucleophilic attack of the electrophilic carbon on the carbonyl function by the lone pair on the amine nitrogen 3. The reaction then proceeds via acid-catalyzed elimination of water to give an imine 4. Imine formation exists in equilibrium with an aldehyde; thus for quantitative derivatization it is necessary to convert the imine into a secondary amine 5 via nonreversible reduction with a hydride source such as sodium cyanoborohydride.

As already stated, reductive amination is the most popular way in which to introduce a chromophore or fluorophore, as is evidenced by Table 2.1. This stems from the fact that there is a vast choice of fluorophores and chromophores that can be used and also from the ability to quantitatively study carbohydrates, provided that each chosen analyte contains only one reducing end. Examination of the most recent publications in this area reveals that a large variety of reaction conditions have been trialed over the years. General conditions for derivatization via reductive amination are as follows: reaction time of 1 h to overnight; temperature of 37–90°C; reducing agent is $NaCNBH_3$ typically dissolved in tetrahydrofuran (THF) for higher derivatization efficiency; acid used is acetic, citric, or formic; derivatization solvent is methanol, dimethylsulfoxide (DMSO), or sometimes water depending on the solubility of the derivatization agent.

Table 2.1 Names, structures, spectral properties, commercial availability, separation and derivatization conditions, limits of detection, analytes being studied, and references of various derivatization reagents

Chromophore fluorophore	Structure	Excitation (nm) Emission (nm)	Commercial availability	CE conditions	Derivatization conditions	LODs (nM)	Analyte	Ref
1-Aminopyrene-3,6,8-trisulfonic acid (APTS)		424 505	Yes	BGE: pH 5.0, 25 mM acetate Sample: in H_2O Injection: 5 kV, 10 s Separation: 15 kV Detection: *LIF 488 nm	2 μL, 0.2 M APTS, 15% AcOH, 2 μl, 1 M $NaCNBH_3$ in THF 75°C, 1 h.	NS	Maltooligosaccharides, xylose, arabinose, glucose, galactose	[24, 27]
				BGE: CARB separation Solution: (eGene) Sample: in H_2O Injection: 2 kV, 20 s Separation: 2–8 kV Detection: LED 460–470 nm	2 μL, 0.2 M APTS, 15% AcOH, 2 μl, 1 M $NaCNBH_3$ in THF 75°C, 1 h.	NS	Maltooligo saccharides	[30]
				BGE: pH 3.7, 1% AcOH, 15 mM, triethylamine, pH 9.15, 20 mM borate Sample: in H_2O Injection: 34.5 mbar, 5 s Separation: −20 kV, +20 kV Detection: LIF 488 nm	0.3 μL, 0.2 M APTS, 15% AcOH, 0.3 μl, 1 M $NaCNBH_3$ in THF, 55°C, 90 min	NS	Oligosaccharides (dp 1–10)	[21]

(continued)

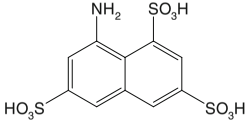
Table 2.1 (continued)

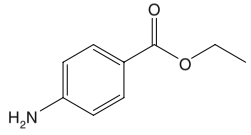
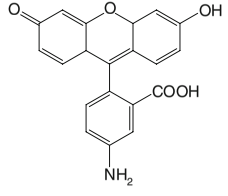
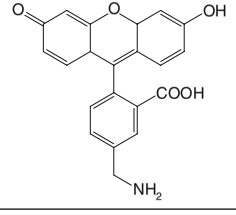
Chromophore fluorophore	Structure	Excitation (nm) Emission (nm)	Commercial availability	CE conditions	Derivatization conditions	LODs (nM)	Analyte	Ref
				BGE: pH 9.2, 25 mM sodium tetraborate (normal), pH 2.8, 40 mM phosphoric acid 28 mM triethylamine (reverse) Sample: in H_2O Injection: 100 mbar, 10 s Separation: −30 kV, +30 kV Detection: LIF 488 nm	2 μL, 0.2 M APTS, 15% AcOH, 2 μl, 1 M $NaCNBH_3$ in THF 55°C, 90 min	NS	Polysaccharides, oligosaccharides, rhamnose, mannose, glucose, fucose, halactose, Kdo	[20]
				BGE: pH 5.0, 25 mM acetate Sample: in H_2O Injection: 34.5 mbar, 5–10 s Separation: 8.5 kV, 25°C Detection: LIF 488 nm	2 μL, 0.2 M APTS, 15% AcOH + 2 μL, 1 M $NaCNBH_3$ in THF 75°C, 1 h.	NS	Oligosaccharides, xylose, arabinose, glucose, galactose	[25]
				BGE: pH 8.4, 80 mM borate and pH 8.5, 50 mM phosphate Sample: in BGE or H_2O Injection: 34 mbar, 10 s Separation: 25 kV, 25°C Detection: LIF 488 nm	10 μL oligomer stock + 2 μL, 0.2 M APTS, 15% AcOH + 10 μL, 1 M, $NaCNBH_3$, 75°C, up to 5 h.	NS	Oligosaccharides (dp 2–6)	[17]
				BGE: pH 2.5, 100 mM phosphate Sample: in H_2O Injection: 50 mbar, 5 s Separation: 20 kV, 25°C Detection: LIF 488 nm	0.4 mM oligoguluronates, 12 mM APTS, 200 mM $NaCNBH_3$, 300 mM AcOH 60°C, 3.5 h.	0.4–0.6	Oligoguluronates	[29]

	BGE: pH 10.2, 135 mM borate Sample: in H_2O Injection: 34.5 mbar, 5 s Separation: 25 kV Detection: LIF 488 nm	10 μL, 0.16 μmol of each carbohydrates + 2 μL 0.1 M APTS in 4.2 M AcOH, 4 μL, 1.0 M, $NaCNBH_3$ in THF, 75°C, 1 h	100	Maltose, lactose, D-mannose, D-glucose, D-ribose, D-xylose, L-arabinose, D-galactose [19]
	BGE: pH 5.0, 25 mM lithium acetate Sample: in H_2O Injection: 34.5 mbar, 5–10 s Separation: 10 kV, 20 or 15°C Detection: LIF 488 nm	5–50 nmol oligosaccharide, 2 μL, 0.2 M APTS, 15% AcOH + 2 μL 1 M $NaCNBH_3$ in THF, 75°C, 1 h	NS	Oligosaccharide, structural isomers [26]
	BGE: pH 4.75, 25 mM acetate, 0.4% polyethylene oxide Sample: in H_2O Injection: 34.5 mbar, 4 s Separation: 30 kV, 25°C Detection: LIF 488 nm	50 nmol monosaccharide or 0.1 mg carbohydrate + 2 μL, 0.2 M APTS, 15%, AcOH + 2 μL, 1 M $NaCNBH_3$ in THF, 60°C, 90 min	NS	Plant polysaccharides (mono-oligosaccharides) [22]
	BGE: pH 7.0, 50 mM acetate, PEG7000 Sample: in H_2O Injection: 34.5 mbar, 5 s Separation: 18 kV, 25°C Detection: LIF 488 nm	Oligosaccharides + 2 μL, 0.1 M APTS, 15% AcOH, 2 μL, 1 M, $NaCNBH_3$ in THF, 55°C, 90 min	NS	Oligosaccharides [23]

(continued)

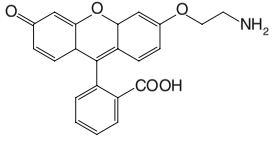
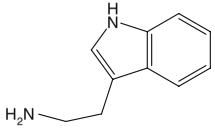
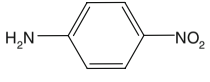
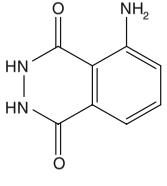
Table 2.1 (continued)

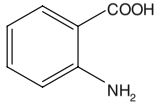
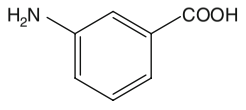
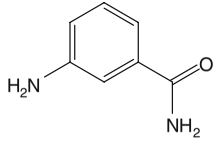
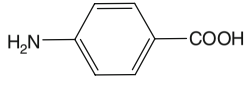
Chromophore fluorophore	Structure	Excitation (nm) Emission (nm)	Commercial availability	CE conditions	Derivatization conditions	LODs (nM)	Analyte	Ref
				BGE: pH 5.0, 25 mM acetate Sample: in H_2O Injection: 34.5–138 mbar, 10 s Separation: 25°C Detection: LIF 488 nm	Oligosaccharides + 2 μL, 0.2 M, APTS, 15% AcOH, 2 μL, 1 M $NaCNBH_3$ in THF, 75°C, 1 h	NS	Maltooligosaccharide	[28]
				BGE: pH 10.2, 120 mM, borate Sample: in H_2O Injection: 20 mbar, 14 s Separation: 30 kV Detection: LIF 488 nm Separation: 15 kV Detection: LIF 488 nm	Carbohydrate + 0.1 M APTS in 0.6 M citric acid + 1.0 M $NaCNBH_3$, 75°C, 1 h	NS	Mono-oligosaccharides	[18]
8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS)		353 535	Yes	BGE: pH 9.2, SAP-200 mM, 200 mM borate buffer, 10% acetonitrile Sample: in H_2O Injection: 50 mbar, 2 s Separation: +30 kV, 30°C Detection: 245 nm	10 μL, 40 mg/ml dextran 15 in H_2O + 2 ml 0.1 M ANTS in 25% AcOH + 10 μL, 1 M $NaCNBH_3$ in THF/MeOH 10:1, 37 or 75°C overnight	NS	Mannose, galactose, fucose, glucose, xylose, arabinose	[32]
				BGE: pH 8.8, 1,500 mM borate and pH 8.8, 1,500 mM HCl Sample: in 40% v/v glycerol Injection: – Separation: 400 V, 4°C Detection: –	10 μL + 30 mM ANTS + 15%, AcOH, 25°C, 15 min. Add 10 μL, 1 M $NaCNBH_3$ in DMSO, incubate 45°C, 4 h.	NS	Heparins (polysaccharides)	[31]

4-Aminobenzoic acid ethyl ester (ABEE)		290 354	Yes	BGE: pH 10.2, 50 mM tetraborate, SDS Sample: in BGE Injection: 34.5 mbar, 3 s Separation: 30 kV, 16°C Detection: DAD 300 nm	10 mg/ml ABEE + AcOH 100 mg/ml in + 10 mg $NaCNBH_3$ in 1 ml MeOH to give stock soln. 500 µL stock soln + 500 µL acidic hydrolysate, standard-calibration-soln, 80°C, 1 h	51,400 91,700	Rhamnose, xylose, ribose, glucose, mannose, arabinose, fucose, galactose	[33]
5-Aminofluorescein		495 520	Yes	BGE: pH 9.2, 100 mM borate Sample: 95 mM formic acid in H_2O Injection: 50 mbar, 10–500 s Separation: −20 kV, 30°C Detection: LED 488 nm	50 µL 0.1 mM tag in MeOH + sugar 50 µL 0.1 mM + 1 µL formic acid + 1 µL 1 M, $NaCNBH_3$, 70°C, 1 h	85	Glucose, lactose, maltotoriose	[35]
5-Aminomethyl fluorescein		492 516	Yes	BGE: pH 8.65, 100 mM borate Sample: pH 2.4, AcOH in H_2O Injection: 50 mbar, 10 s Separation: −30 kV, 25°C Detection: LIF 488 nm	Bulk soln: 120 µL + 33.6 µL, 6.4 µL. Take 7 µL of bulk soln + 2 µL, 1 M $NaCNBH_3$ + 1 µL, 99.7% AcOH + 50 nmol sugar, 70°C, 1 h	≈1	Corn syrup, oligosaccharides (dp 4–10), heptaose	Unpublished

(continued)

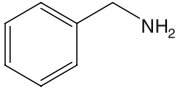
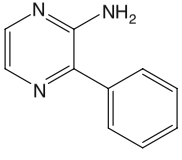
Table 2.1 (continued)

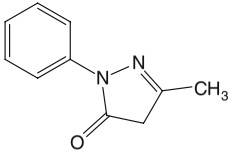
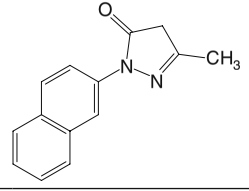
Chromophore fluorophore	Structure	Excitation (nm) Emission (nm)	Commercial availability	CE conditions	Derivatization conditions	LODs (nM)	Analyte	Ref
O-[2-Aminoethyl] fluorescein		504 526	No	BGE: pH 8.55, 100 mM borate Sample: in H_2O Injection: 50 mbar, 10–100 s Separation: −30 kV, 25°C Detection: LIF 488 nm	Bulk soln: 120 μL + 33.6 μL +6.4 μL. Take 7 μL of bulk soln + 2 μL, 1 M $NaCNBH_3$ + 1 μL 99.7% AcOH + 50 nmol sugar, 70°C, 1 h	≈1	corn syrup oligosaccharides (dp 4–10), heptaose, monosaccharides	Unpublished
Tryptamine		304 452	Yes	BGE, pH 9.7, 35 mM cholate, 100 mM borate, 2% 1-propanol Sample: in H_2O Injection:, 50 mbar, 1 s Separation: 30 kV, 30°C Detection: UV 220 nm	100 μL sugar + 12.5 μL, 0.15 M, tryptamine in 10% propanol, 90°C, 10 min. Add 0.3 g/ml, 4.5 μL $NaCNBH_3$, 90°C, 1 h	NS	Monosaccharides, disaccharides, oligosaccharides	[36]
p-Nitroaniline		380 nm	Yes	BGE: pH 9.7, 170 mM borate Sample: in H_2O Injection: 170 mbar, 2 s Separation: −20 kV, 30°C Detection: UV 380 nm, LED 406 nm	5 mM, 50 μL sugar mixture +50 μL glacial AcOH + 100 μL 0.036 M PNA in MeOH + 75 μL, 0.08 M $NaCNBH_3$, in MeOH, 90°C, 60 min	<1,100	Mono-, disaccharides	[37]
3-Aminophthal hydrazide (luminol)			Yes	BGE: pH 10.0, 200 mM borate, 100 mM H_2O_2, 3 M NaOH, 25mM hexacyanoferrater Sample: in H_2O Injection: 20 s, 10 cm height Separation: 15 kV Detection: chemiluminescence	0.08 mmol luminol + 0.5 mmol $NaCNBH_3$, 0.8 ml DMSO + 0.2 ml, AcOH to give reagent soln, 20 μL reagent soln + 5 μL, 0.002–5 mM sugar soln, 30°C	100	Rhamnose, glucose, arabinose, fucose, galactose, glucuronic acid	[39]

2-Aminobenzoic acid (2-AA)		330 420	Yes	BGE: pH 5.5, 50 mM phosphate Sample: in BGE Injection: 10 cm × 15 s Separation: 20 kV, 25°C Detection: UV 214 nm	20 μL, 30 nmol saccharide + 0.2 M 2-AA + 1.0 M, $NaCNBH_3$, 65°C, 2 h	NS	Mono-, di-saccharides	[34]
3-Aminobenzoic acid (3-AA)		≈280 415	Yes	BGE: pH 8.3 100 mM borate, 10% PEG70000 Sample: in H_2O Injection: 69 mbar, 10 s Separation: +25 kV at 25°C Detection: LIF 325 nm	30 μL, 0.7 M 3-AA in DMSO-AcOH (7;3 v/v) + 30 μL, 2 M $NaCNBH_3$, 50°C, 1 h	NS	Oligosaccharides	[23]
3-Aminobenzamide (3-ABA)		306 452	Yes	BGE: pH 8.3, 100 mM borate, 10% PEG70000 Sample: in H_2O Injection: 69 mbar, 10 s Separation: +25 kV at 25°C Detection: LIF 325 nm	1 mg enzyme reaction mixture + 30 μL 0.7 M 3-AA in 70% DMSO, 30% AcOH + 30 μL, 2 M $NaCNBH_3$, 50°C, 1 h		Oligosaccharides	[38]
p-Aminobenzoic acid (PABA)		305 425	Yes	BGE: pH 10.2, 20 mM borate Sample: in H_2O Injection: 8 s Separation: +20 kV, 25°C Detection: 280 nm	20 mg L^{-1} carbohydrate + 250 mM PABA, 20% CAN in 1 ml MeOH + 20 mg $NaCNBH_3$, 40°C, 1 h	7,600–22,000	Glucose, maltose, maltotriose, maltopentaose, maltohexaose, maltoheptaose, maltotetraose	[40]

(continued)

Table 2.1 (continued)

Chromophore fluorophore	Structure	Excitation (nm) Emission (nm)	Commercial availability	CE conditions	Derivatization conditions	LODs (nM)	Analyte	Ref
Benzylamine		Detection carried out at 230 and 248 nm	Yes	BGE: pH 9.2, 50–100 mM borate. pH 4.5, acetate Sample: NS Injection: 50 mbar, 4 s Separation: +20 kV, 25°C Detection: 230 nm, 248 nm	1 nmol, 50 μL oligosaccharide + 10 μL benzylamine in 100 μL, MeOH + 0.05 mmol $NaCNBH_3$ + AcOH 5 μL, 80°C, 2 h (unquarternised). 1 nmol benzy-lamine-oligosaccharide in 100 μL. MeOH + 3 mg $NaHCO_3$ + 20 μL CH_3I, 30–40°C, 3 h (quaternised)	30 fmol	Oligosaccharides	[44]
2-Amino-3-phenylpyrazine (3-APP)		340 440	No	BGE: 100 mM borate Sample: in H_2O Injection: 5 nl (volume) Separation: +25 kV Detection: LIF 325 nm	100 nmol monosaccharide +10 μL, 0.5 M 3-APP in AcOH, 90°C, 30 min. Then 10 μL, 0.7 M, dimethy-lamine-borane in AcOH was added, mixture heated, 90°C, 15 min	5–10 fmol	Monosaccharides	[43]

1-Phenyl-3-methyl-5-pyrazolone (PMP)		245	Yes	BGE: pH 6.0, 25 mM phosphate, 70 mM SDSSample: in H_2O Injection: 1 kV, 1 s Separation: +20 kV, 26°C Detection: 254 nm	8.25 nmol monosaccharide in 0.3 M NaOH + 50 µL 0.5 M PMP in MeOH, 30 min, dried under vacuum at 100°C. Extraction with chloroform	<0.9 ng	D-mannose, rhamnose, D-galactose, glucose, D-xylose	[62]
				BGE: pH 9.2, 200 mM SAP, 200 mM borateSample: chloroform/MeOH, H_2O Injection: 50 mbar, 2 s Separation: +20 kV, 10–50°C Detection: 245 nm	10–500 pmol oligosaccharide + 50 µL 0.5 M PMP in MeOH + 50 µL, 0.3 M $NaCNBH_3$, 70°C, 30 min. Extraction with chloroform/H_2O	NS	D-mannose, galactose, fucose, glucose, xylose, arabinose	[61]
1-(2-Naphthyl)-3-methyl-5-pyrazolone (NMP)		243	No	BGE: pH 9.46, 15–65 mM borateSample: in acetonitrile Injection: 50 mbar, 10 s Separation: 22 kV, 20°C Detection: DAD 251 nm	20–30 µL carbohydrate + 200 µL, 0.05 M NMP in MeOH + 20 µL ammonia, 70°C, 30 min. Excess NMP removed in Sep-Pak C18	<85	Mannose, rhamnose, glucuronic acid, glucose, galactose, xylose, fucose, galacturonic acid	[60]

The first report on the use of reductive amination for the derivatization of carbohydrates was in 1978, when 2-aminopyridine was used to study oligosaccharides using paper electrophoresis [16]. These labeled sugars showed good stability and produced a near-quantitative reaction, which made 2-aminopyridine an attractive tool to study carbohydrates. Later research introduced a copious assortment of derivatization reagents, which are covered in the reviews by Lamari et al. [10], Gao et al. [11], Suzuki and Honda [12], Shilova and Bovin [13], and Oefner and Chiesa [14], with more recent publications shown in [1].

From the table, a number of fluorescent and absorbing tags have been reported, including APTS [17–30], ANTS [31, 32], ABEE [33], 2-AA [34], 5-aminofluorescein and its analogues [35], tryptamine [36], *p*-nitroaniline [37], 3-AA [23, 38], 3-ABA [38], luminal [39], and PABA [40]. Of these reagents, APTS is currently the most widely utilized derivatization reagent for the separation of carbohydrates. This reagent contains three sulfonate functionalities, which introduce multiple charges throughout a wide pH range and even allows complex long-chained carbohydrates to acquire significant electrophoretic mobilities, enabling very high resolution separations to be obtained. This is clearly illustrated in Fig. 2.3, which shows an early report by Stefansson and Novotny [41] on the use of APTS and other reagents to separate complex oligosaccharides. The figure shows a very impressive separation of oligosaccharides of degree of polymerization up to 80 (dp) made possible because of the highly charged nature of the reagent.

Studies show that APTS in its free form is less fluorescent than the conjugated derivatives, which typically exhibit 40 times the enhancement in fluorescence intensity. Importantly there is also a Stokes shift to a higher wavelength of the derivatized carbohydrates, with the excitation maxima approaching 488 nm, the output of the argon-ion laser. When used with this type of laser, excellent sensitivity can be obtained, with LODs in the range of 10^{-12} M being reported [29, 42]. When combined, the high charge state of the reagent and the excellent fluorescence properties make this an outstanding reagent for the separation of carbohydrates, and its importance to the field can be seen by the high number of publications using this reagent, with 14 research articles since 2003 highlighting the use of APTS to analyze oligosaccharides, carbohydrates, and even more simple monosaccharides.

In general, the following conditions are used for derivatization with APTS: 0.2 M APTS is reacted in 15% AcOH and reduced with 1 M $NaCNBH_3$ at 55–90°C for 1–5 h. Separation is performed in acetate or borate buffers at high pH. Phosphate can be used, although it has not been used very frequently. Yamamoto and colleagues [43] introduced 2-amino-3-phenylpyrazine (3-APP) as a sensitive alternative to APTS. This was labeled to monosaccharides using dimethyl amine-borane as the reducing agent in this case. The reaction was performed in two steps: first, acetic acid was added to 3-APP to generate the imine complex; second, dimethyl amine-borane was added to induce reduction of the derivatized monosaccharides. Both steps were completed within 35 min, producing highly fluorescent complexes that resulted in LODs at 5–10 fmol. These impressive results were attributed to the flexible nature of 3-APP, showing no significant fluorescence intensity change throughout the pH range as well as being closely matched with the 325-nm helium–cadmium laser, where 3-APP demonstrated maximum excitation wavelength at 331 nm.

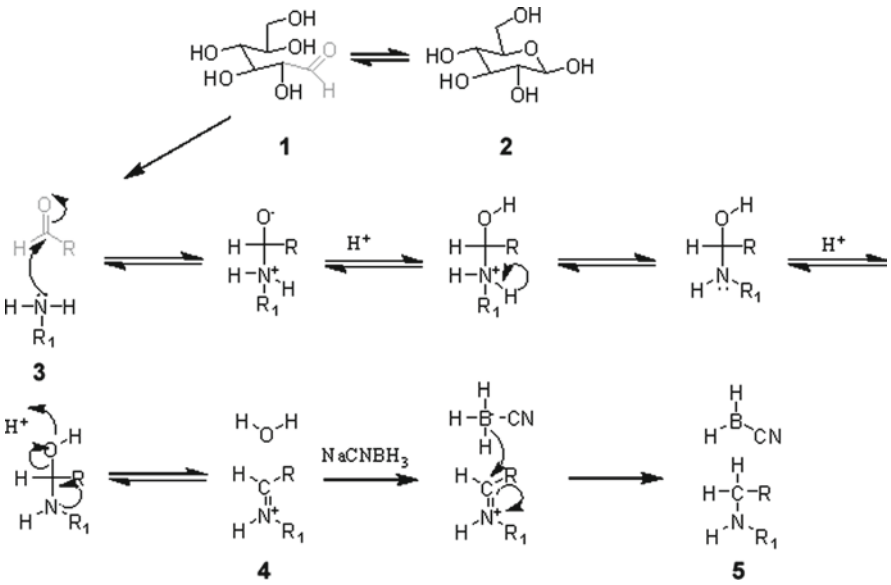


Fig. 2.2 Mechanistic details of reductive amination [15]

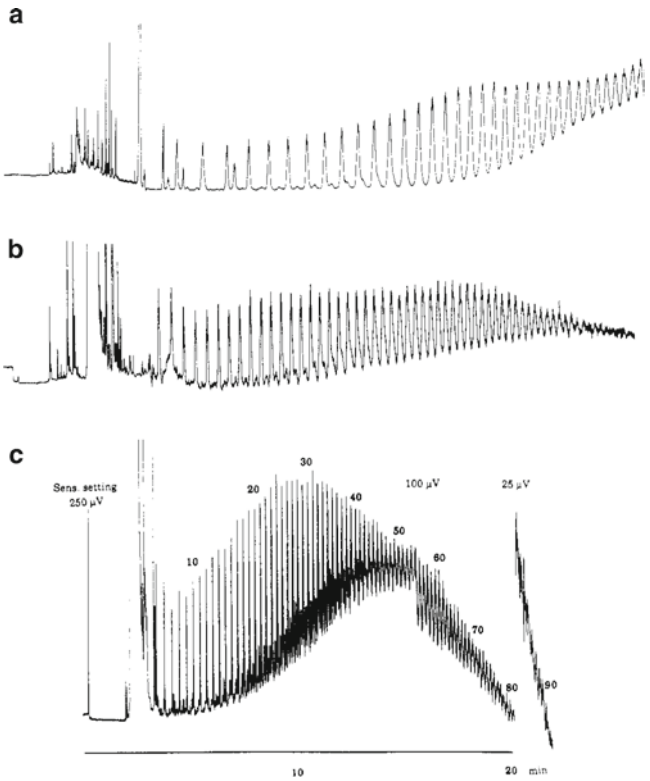


Fig. 2.3 Electropherograms demonstrating separations of dextran standard using 2-aminopyridine, ANTS, and APTS labeling reagents. Electropherogram C, describing APTS, is of particular interest and is discussed in this chapter [41]

While APTS is clearly the dominant fluorescent reagent, a wider range of absorbing reagents have been used to derivatize carbohydrates, such as ABEE [33], 2-AA [34], tryptamine [36], *p*-nitroaniline [37], benzyl amine [44], and luminal [39]. Most of these reagents absorb strongly in the UV region and are thus compatible with the high output intensity of lamps commonly used for CE.

The use of *p*-nitroaniline warrants discussion, as this reagent was selected because of its compatibility with light-emitting diodes (LEDs) that output in the near UV region (360–400 nm). LEDs are an attractive light source for separations not only because of their low cost, high stability, and exceptionally long lifetimes, but also because of their high light output and low noise levels, which translates into improved LODs [45]. When applied to simple mono- and disaccharides, LODs at <1.1 μM were obtained. While acceptable separations were obtained, the reagent is not charged, and separation was achieved via borate complexation. This same rationale was used to select 5-aminofluorescein as a derivatization reagent. This reagent has a high molar absorptivity of approximately 80,000 $L\,mol^{-1}\,cm^{-1}$ at 480–500 nm, which makes it perfectly compatible with high-intensity blue LEDs [46]. In addition, because the reagent has ionizable functional groups (phenolic and carboxylate), this allows on-line concentration via the use of a pH discontinuity between the samples, allowing very large injections to be performed without compromising resolution. Using this system, nanomolar LODs for mono-, di-, and trisaccharides were described by Kazarian et al. [35], and while these LODs are still higher than can be achieved with laser-induced fluorescence (LIF), this system is both instrumentally and chemically cheaper and will no doubt find a number of applications that do not require ultralow detection limits. The same authors have extended the idea of using on-line concentration via dynamic pH junction using 5-aminomethyl fluorescein and a novel fluorescent compound known as *O*-(2-aminoethyl) fluorescein, with improvements in sensitivity of 50-fold over the use of a normal injection. Surprisingly, there are very few reports on the use of on-line concentration methods to improve the sensitivity of carbohydrates.

The separation electrolytes used for these UV/Vis reagents remains essentially the same when compared to the fluorescent analogues with mostly borate and phosphate buffers used. The selection of analytes for these UV/Vis tags is predominantly based on mono- and disaccharides, with an exception of oligosaccharides derivatized with benzyl amine and later subjected to matrix-assisted laser desorption Ionisation (MALDI) mass spectrometry (MS) analysis [44].

In 2003 Wang et al. [39] studied 3-aminophthalhydrazide for labeling of seven carbohydrates via reductive amination, although detection was not accomplished using light absorption but rather chemiluminescence detection. The merits of the work were highlighted by the use of a brain microdialysis sample with its complex matrix where spiked glucose was identified. The outcome suggested that the sample matrix did not interfere with glucose analysis, making the method well suited for studying complex biological samples.

In summary, reductive amination is a very attractive strategy for labeling simple and more complex carbohydrates, particularly due to the large diversity of fluorophores and chromophores available commercially at relatively modest cost and in high purity.

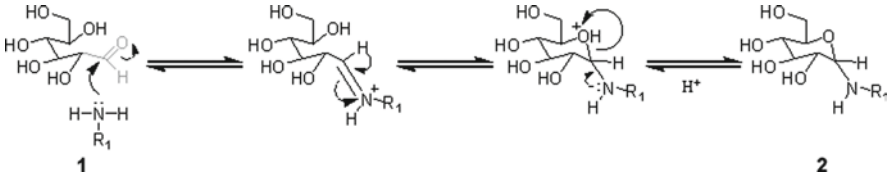


Fig. 2.4 Amination reaction via formation of glycosylamine [47]

2.3 Amination Via Formation of Glycosylamine

This labeling strategy is a variation of the reductive amination, where an amino-based compound 1 reacts with the reducing end of the carbohydrate to yield a glycosylamine 2 (Fig. 2.4) [47]. The reaction proceeds without the involvement of reducing agents, generating a closed ring amine with an *N*-glycosidic bond, resulting in glycosylamine formation as opposed to an open ring conformation of secondary amines observed in reductive amination. This strategy has not been extensively utilized over the years; nevertheless, Li and colleagues [48, 49] performed a number of studies related to amination via glycosylamine formation. The main goal of the research was to gain better insight about the structure of disaccharides by employing *p*-aminobenzoic acid and *p*-aminobenzoic acid ethyl ester as labeling reagents. The merits of this approach are highlighted by the ability to study interglycosidic linkages as well as to provide more insight into an anomeric configuration of saccharides. This derivatization scheme is also appealing because the derivatization process can be reversed, producing carbohydrates in their free form. The disadvantages of the glycosylamine approach for disaccharide derivatization were also apparent, with formation of two anomers for each disaccharide ultimately complicating the separation step. Overall, glycosylamine formation through derivatization with chromophores or fluorophores in CE has generated very little interest in carbohydrate analysis by CE; thus, additional research and detailed discussion is required to recognize the underlying strengths or limitations of this strategy.

2.4 Derivatization with *N*-Methyl-glycamine Derivatives

Formation of glycamines is achieved by introducing methylamine and dimethylborane, and converting the reducing sugars to acyclic *N*-methylglycamines. This strategy is another form of reductive amination recognized as *N*-methylamination, where the first step is initiated by the methylamine 1 attack of the electrophilic site on the reducing end of the carbohydrate. The reaction proceeds to form an imine ion 2, which is in due course reduced by dimethylamine borane 3 to give a glycamine 4 (Fig. 2.5). This is followed by labeling with a fluorophore or a chromophore of choice, such as 7-nitro-2,1,3-benzoxadiazole 4-fluoride (NBD-F). The advantage of using NBD-F derivatization when compared to other fluorophores such as

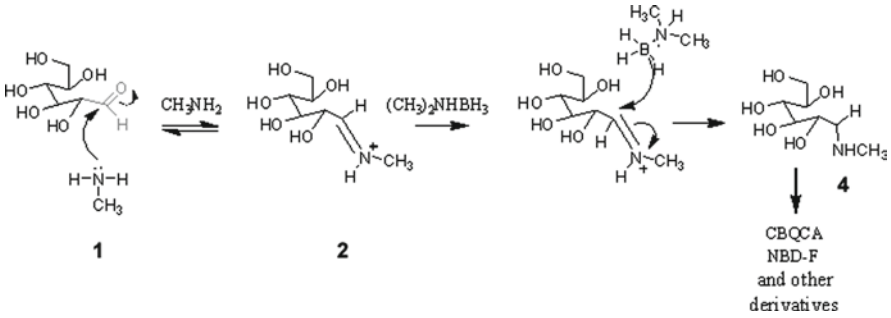


Fig. 2.5 Formation of glycamine followed by labeling with various derivatization reagents such as NBD-F, CBQCA, and other derivatives. The reagents shown above are not exclusively used to label various derivatives, but rather used for derivatization with NBD-F

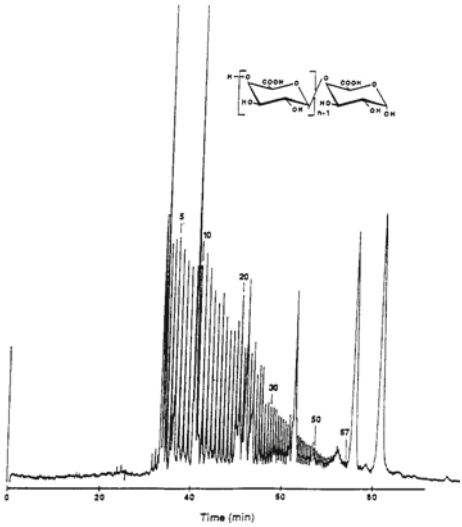


Fig. 2.6 An electropherogram describing a separation of oligomers [52]

3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) is highlighted by the use of mild conditions, while CBQCA employs toxic cyanide salt in its derivatization pathway [50]. The literature concurs that the main benefit of NBD-F derivatization via glycamine formation is the mild conditions that keep the sialic acids in place, allowing trace analysis of sialylated oligosaccharides [51]. On the other hand, CBQCA with its harsh derivatization conditions is still utilized in labeling of carbohydrates due the astonishing sensitivity that can be accomplished with this method. In 1992 Liu and colleagues [52] achieved a separation of oligomers obtained from hydrolysis of poly (galacturonic acid) and derivatized with CBQCA (Fig. 2.6) [52]. This was carried out using a gel-filled capillary with practically no electro-osmotic flow. The authors clearly emphasize the importance of using high

concentrations of gels to enhance separations of the larger oligosaccharide units, and using a charge to induce electrophoretic mobility. As well as reporting an impressive resolution of the oligomers, the authors stated that the minimum detectable quantities of the analytes were recorded at impressive low attomole levels.

There are a few other fluorophores included that fit into this derivatization category, such as 4-carboxytetramethylrhodamine succinimidyl ester (TRSE) [53] and phenylisothiocyanate (PITC) [54], and these have been previously mentioned in a review by Suzuki and Honda [12]. Overall formation of glycamine followed by labeling with various derivatization reagents has not been used extensively in recent years, even though the results indicate impressive potential.

2.5 Formation of Hydrazones

Hydrazone formation is another form of derivatization in which the reducing end of the sugar is reacted with the amine group of a hydrazine-based derivative 1 [55]. Hydrazine-based reagents are advantageous because they produce stable products, allow high detection sensitivity, and have high derivatization yield. The reaction is pH dependent and proceeds via formation of a hemiaminal 4 followed by dehydration to give a hydrazone complex 7. The mechanism of the reaction is shown in Fig. 2.7. The more nucleophilic nitrogen of the hydrazine attacks the electrophilic site on the protonated carbonyl function to eventually enable hemiaminal formation followed by a proton removal, which is assisted by base ($^{-}$OAc). The reaction then proceeds by acid-catalyzed removal of water to give a hydrazone complex. This particular reaction is catalyzed by acids, and unlike reductive amination does not require a reducing agent to form the final derivatized complex. The end product is a hydrazone with various side products, presenting a clear drawback of the reaction. Nevertheless, this approach provides practically constant reaction conditions, such as the temperature, time, and pH of the reaction mixture when using

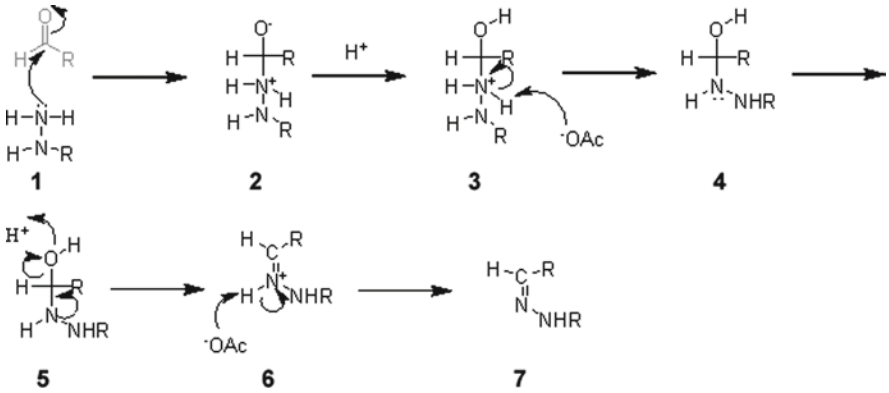


Fig. 2.7 Mechanisms of hydrazone formation [55]

dansylhydrazine as a labeling reagent, and generally shows good separations between the carbohydrates and the starting derivatization agent [13].

Some of the early research by Perez and Colon [56] introduced dansylhydrazine as a label for monosaccharides where optimized conditions showed impressively quick reaction times of 15 min at 68°C and good detection compatibility with a helium–cadmium laser at 325 nm, while separation was performed using borate buffer. Further outcomes of the study produced impressive LODs at 100 amol, and showed the ability to detect glucose in tear samples. The drawbacks of the method are apparent when closely examining electropherograms, as resolution is hampered and various other unidentified peaks are observed. More hydrazine reagents have been introduced over the years such as *N*,*N*-diphenylhydrazine [57] and 2,4-dinitrophenylhydrazine [58], with details of the research described in reviews by Lamari et al. [10]. The use of hydrazines over the last 5–6 years has been quite limited. This is perhaps due to the unsatisfactory resolution and the additional unknown peaks that in some cases interfere with separation of the analytes. Nevertheless, this group of fluorophores provides a great base for obtaining sensitive detection with exceptionally quick derivatization times while using mild labeling conditions, creating a good foundation and potential for successful carbohydrate analysis.

2.6 Derivatization with Pyrazolone Compounds

Pyrazolone compounds such as 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP) [61, 62], its methoxy derivative, and a novel 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) [60] analogue have been used to label reducing carbohydrates through base-catalyzed condensation between the carbonyl functionality and the hydrogens of PMP, as shown in Fig. 2.8. The reaction of PMP with carbohydrates is initiated by sodium hydroxide, which acts as a base, removing the α-hydrogen of the PMP. This in turn produces a nucleophile 1, which attacks the electrophilic site on the carbohydrate, allowing formation of the conjugated complex 2. Following this, another deprotonated PMP molecule attacks the electrophilic site of the conjugated complex through Michael 1,4-addition, resulting in formation of an enolate 3. Acid/base reaction then takes place in which the enolate deprotonates a water molecule, inducing formation of the final bis-PMP derivative 4.

Various advantages of using PMP-based derivatives are observed when labeling and detecting carbohydrates. The backbone of PMP contains the phenyl and methyl functionalities, which increase the hydrophobicity, allowing separations in micellar electrokinetic chromatography, therefore introducing additional flexibility to the analysis of carbohydrates. Additional benefits are apparent when considering the keto group of the pyrazolone ring, which can tautomerize based on pH, therefore introducing a negative charge that enables other modes of CE to be performed. Condensation using PMP-based derivatives also excludes the use of strong reducing agents such as sodium cyanoborohydride, making it an attractive option to derivatize carbohydrates. Despite these advantages, there are also drawbacks, particularly the extraction step

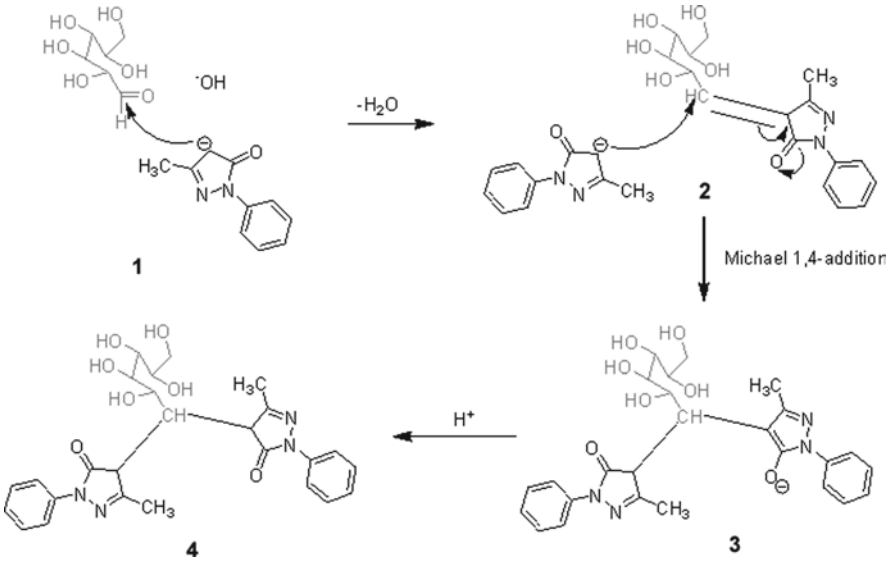


Fig. 2.8 Mechanism of derivatization of PMP-based compounds [59, 60]

required to remove the large excess of reagent that would otherwise potentially hamper the analysis. Further drawbacks of using PMP-based compounds are attributed to specific storage conditions required as a result of the labile nature of the bis derivatives under basic conditions, which can decompose to form mono-PMP analogues with different mobility. Nonetheless, decomposition can be minimized by storing these compounds in dry conditions at low temperature or by storing them in acidic buffer.

Typical reaction conditions for PMP-based derivatives entail using methanol as a solvent in the presence of sodium hydroxide where the reaction temperature is around 70°C and the time is in the range of 30 min–1 h. The PMP-based compounds undergo derivatization to form mono-PMP intermediates, followed by bis-PMP analogues that are strongly UV absorbing chromophores displaying high molar absorptivities of 30,000 $M^{-1}cm^{-1}$, with maximum absorption wavelength close to 245 nm [59]. A recent publication by You et al. [60] indicated that a novel labeling reagent NMP possesses even higher molar absorptivity of 55,800 $M^{-1}cm^{-1}$, enabling better sensitivity to be achieved.

A review of the literature of the past 7 years shows that PMP chromophores have not been very popular in CE, with a small number of publications reporting that the main analytes of interest were mono- and disaccharides. Kodama and colleagues [61] studied monosaccharides; mannose, galactose, and fucose labeled to PMP were enantioseparated by ligand-exchange CE. Further insight into the enantioseparation behavior was gained by introducing computer-based predictions, which enabled elucidation of the structure of the diastereomers aiding chiral resolution and ultimately separation, highlighting the novelty of the research. A very recent study by You et al. [60] introduced a newly synthesized NMP analogue to separate a range of nine monosaccharides using diode array detection (DAD). The merits of this work are

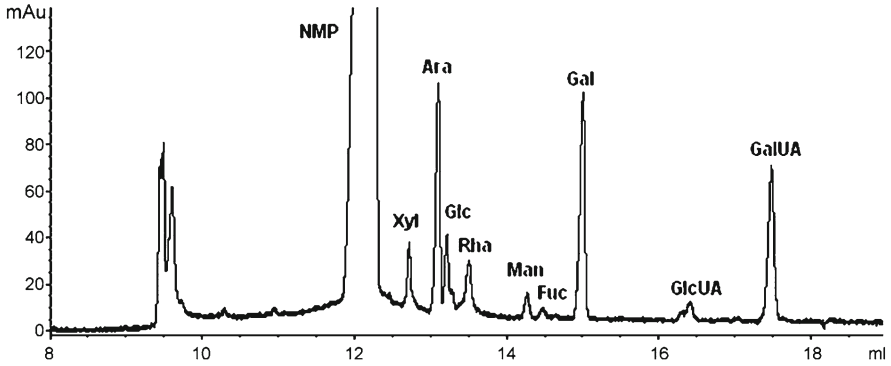


Fig. 2.9 A separation of carbohydrates found in the hydrolyzed rape bee pollen [60]

emphasized through the use of a highly absorbing chromophore that subsequently led to impressive LODs at nanomolar concentration levels. The sensitivity of this system is very similar to the previously discussed research by Kazarian et al. [35], which studied mono-, di-, and trisaccharides using LEDs for detection and dynamic pH junction as a means of preconcentration. Even though DAD detection typically lacks sensitivity, the applicability of NMP as a sensitive chromophore considerably improved the sensitivity of the system, allowing nanomolar-level LODs.

The suitability of the newly developed method was confirmed by analyzing carbohydrates from a rape bee pollen, confirming the presence of all nine monosaccharides, as described in Fig. 2.9.

Comparison of the separation conditions among PMP-based publications indicates that borate buffer at a pH above 9.0 is a general trend, while borate concentration has to be carefully adjusted to obtain the best resolution. Application of phosphate buffer was also demonstrated by Zhang and colleagues to analyze PMP-labeled carbohydrates; however, sodium dodecyl sulfate (SDS) was added to the buffer and micellar electrokinetic chromatography (MEKC) was used as the separation mode of choice. Sample composition remains relatively similar throughout the research publications described in Table 2.1; however, NMP-based samples do not need the extraction step to remove the excess reagent, while PMP-derivatized carbohydrates require chloroform for the extraction step. Overall, the use of PMP-based derivatives is an attractive option, offering impressive LODs in the nanomolar range as well as cheap means of detection such as DAD.

2.7 Derivatization at Carboxylic Acid Functionalities

Derivatization of acidic carbohydrates described in Fig. 2.10 was implemented by Mechref et al. [64] to derivatize acidic carbohydrates using amino functions of sulfanilic acid (SA) or 7-aminonaphthalene-1,3-disulfonic acid (ANDSA). This is achieved by adding carbodiimide, which activates carboxylic acid functionalities

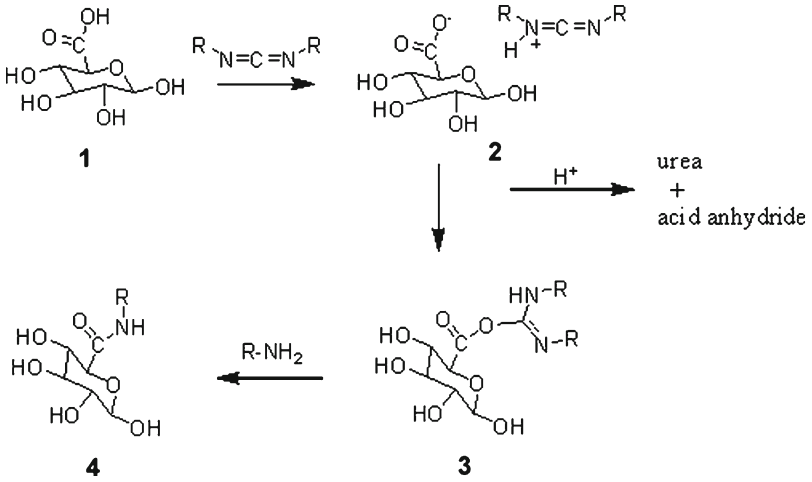


Fig. 2.10 Derivatization of acidic carbohydrates [63]

on the carbohydrate 2 to give a carboxylic ester intermediate, O-acylisourea 3, which consequently reacts with the amine of the derivatization reagent to form an amide bond, therefore producing a labeled carbohydrate with a peptide bond 4 (Fig. 2.10). The advantage of this condensation reaction is emphasized by the use of mild conditions that prevent formation of desialylated and desulfated products. However, this labeling approach can produce various side reactions, although they can be avoided by carefully adding stoichiometric amounts of the carbodiimide to the carbohydrate. This is still an important issue when an unknown sample is to be analyzed, as it is impossible to know the stoichiometric amount before analysis [63].

2.8 On-Column Derivatization

All of the work discussed above has been performed using pre-column derivatization. Undoubtedly pre-column labeling is time-consuming and in some cases requires purification or dilution of the sample to secure good resolution and efficiency, eventually yielding a desired separation. On-column derivatization offers a number of advantages, such as the ease of automation, reduced sample size, minimization of sample loss, and potentially quicker reaction and analysis times. Despite these advantages, only a few reagents have been reported for on-line labeling of carbohydrates, such as PMP [65] and *p*-hydrazinebenzenesulfonic acid [66]. In these reports, carbohydrate derivatization was accomplished using a sandwich or a tandem configuration at the tip of the capillary. In this case, the sample is injected between two reagent plugs and left to react for the appropriate time at the appropriate temperature. Afterward, the voltage is applied to perform the separation.

A different approach was undertaken by Jin and colleagues [67], in which glucose was determined by the use of two enzymes, namely glucose oxidase and horseradish peroxidase, and homovanillic acid was the fluorophore. The concept is based on the oxidation of glucose by glucose oxidase to produce gluconic acid and hydrogen peroxide. A nonfluorescent HVA (4-hydroxy-3-methoxyphenylacetic acid) is then oxidized by hydrogen peroxide and the action of horseradish peroxidase to produce a fluorescent 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (HVA_{ox}) derivative suitable for LIF detection. This on-column approach was very quick, with a mixing time of 1 min followed by an additional incubation of 2 min, allowing the very rapid analysis of glucose down to 800 nM.

More recently, Kaiser and colleagues [68] have used a dynamic boronate to derivatize and separate carbohydrates. Boronates have been known for over 100 years to complex with 1,2- and 1,3-diols [69], producing ring structures as shown in Fig. 2.11, and have been used extensively to separate sugars using borate buffers, but have very rarely been used to enhance detection. A few reports can be found on their use in high-performance liquid chromatography (HPLC) with post-column reaction, but until this recent work, the only similar report was by Robins and Wright, who used boronate complexation to facilitate separation and detection of organophosphonic acids. As shown in Fig. 2.11, boronic acids are capable of reactions with water, changing their geometry from the trigonal neutral state 1 to a negatively charged tetrahedral conformation 2, which in due course enables interactions with vicinal diol functionalities 3 found in carbohydrates to give a negatively charged complex 4. This leads to further transformations to give a trigonal neutral geometry 5 [69].

Kaiser and colleagues [68] eloquently demonstrated the concept of dynamic labeling of sugars using the UV absorbing reagent 3-nitrophenylboronic acid (NPBA) for the separation of polyol stereoisomers, namely mannitol, galactitol, sorbitol, catechol, galactonic acid, and gluconic acid. Detection was facilitated because of the slight change in absorbance maximum of NPBA when conjugated to the carbohydrates. While this approach is simple and rapid, only moderate

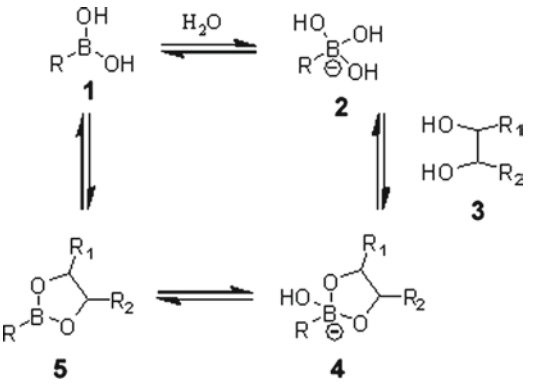


Fig. 2.11 Schematic showing boronic acid complexation with diols [69]

detection limits of approximately 20 μM were reported, primarily due to the high background absorbance of the NPBA added to the electrolyte. Future work to enhance the sensitivity of this strategy is clearly needed to make this an approach that has widespread application, but the potential benefits of this approach are significant.

2.9 Conclusion and Future Directions

While there is a range of derivatization strategies that can be used to facilitate the detection of carbohydrates, there is no current strategy that is perfect. Until there is a reagent for the fast, sensitive, and selective analysis of carbohydrates with high reaction yields in mild conditions without the use of toxic constituents and that is applicable for real carbohydrate samples providing highly efficient and well-resolved separations with minimal degradation and side products, there will still be much work to be done in this area. A number of reagents currently meet some, but not all, of these needs, with the two major issues that need to be addressed being the sensitivity of the system and the time for analysis. The first and the foremost issue of sensitivity can be addressed by the use and development of new hardware through the use of sensitive detectors such as LIF and chemiluminescence (CL), while the innate chemistry of the system can also be used to enhance the system via the use of on-line sample preconcentration methods. The design of new reagents would also be of value, and through a fundamental understanding of fluorescence on/off quenching mechanisms could dramatically ameliorate the synthetic design of these new reagents.

The second issue concerning fast analysis time of carbohydrates can be advanced by the use of on-line derivatization. Dynamic boronates show much promise in this area, but there is still much work in this field to be done before separations equivalent to those achievable with APTS are obtained. The alternative strategy of miniaturization through the implementation on microchips has significant potential to take current approaches and significantly improve reaction times and yields while also allowing seamless integration with electrophoretic separation. Movement is already headed in this direction, but as with the first issue, there still remains much that needs to be done before reliable systems can be developed.

Nobody knows what the future holds; however, it is quite clear that the technology is advancing toward faster, better, smaller, and cheaper ways to conduct analytical separations, and this will become evident in the years to come.

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Chapter 3
Capillary Electrophoresis of Bacterial (Lipo)Polysaccharides

Nicola Volpi and Francesca Maccari

Abstract Lipopolysaccharides (LPSs) are major components of the outer membrane of gram-negative bacteria, and, along with some acidic polysaccharides, are important macromolecules belonging to bacteria. The recent emergence of modern analytical tools for their study has produced a virtual explosion in the field of glycomics. Capillary electrophoresis (CE), due to its high resolving power and sensitivity, has been useful in the analysis of intact bacterial acidic polymers and derived oligo- and disaccharides such as LPS, affording concentration and structural characterization data essential for understanding their biological functions. Furthermore, the coupling of CE with mass spectrometry (MS) provides a powerful approach far rapid identification of target analytes, such as bacterial LPSs, present in biological matrices, and for their structural characterization. This methodology facilitates the determination of closely related LPS glycoform and isoform families by exploiting differences in their unique molecular conformations and ionic charge distributions by electrophoretic separation. On-line CE–MS also provides an additional tool to improve detection limits successfully applied to directly probe oligosaccharide LPS glycoform populations of bacteria. This chapter discusses the state of the art of CE, CE–MS, and tandem MS methods for screening LPSs and bacterial polysaccharides and derived oligosaccharides and disaccharides.

Keywords Capillary electrophoresis • Polysaccharides • Lipopolysaccharides • Bacteria • Meningococcal polysaccharides • Alginic acid • *Aeromonas salmonicida* • Glycotyping • *Pseudomonas aeruginosa* • Cystic fibrosis • K4 polysaccharide • K5 polysaccharide • Heparosan

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Abbreviations

2-AMAC	2-aminoacridone
2-AP	2-aminopyridine
4-ABN	4-aminobenzonitrile
6-AQ	6-aminoquinoline
ANDSA	7-aminonaphatalene-1,3-disulfonic acid
ANTS	8-amino-naphatalene-1,3,6-trisulfonic acid
APTS	8-aminoyrene-1,3,6-trisulfonate
BAP	biotin aminopyridine
CBQCA	3-(4-carbobenzoyl)-2-quinolinecarboxaldeyde
CE	capillary electrophoresis
CS	chondroitin sulfate
CZE	capillary zone electrophoresis
DOC	deoxycholic acid
DS	dermatan sulfate
ESI	electrospray ionization
ESI-MS	electrospray ion MS
FAIMS	high-field asymmetric ion mobility spectrometry
GAG	glycosaminoglycan
GalNac	*N*-acetyl-glucosamine
HA	hyaluronic acid
Hb	hemoglobin
Hep	heparin
HPLC	high-performance liquid chromatography
HS	heparan sulfate
IdoA	iduronic acid
IS-CID	in-source collision induced dissociation
KDO	keto-deoxyoctulosonate
KS	keratan sulfate
LIF	laser-induced fluorescence
LOS	lipooligosaccharides
LPSs	lypopolysaccharides
LRPS	liporhamnopolysaccharide
MALDI	matrix-assisted laser desorption ionization
MECC	micellar electrokinetic capillary chromatography
MECCKC	microemulsion electrokinetic capillary chromatography
MEKC-UV	micellar electrokinetic capillary chromatography-UV
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NBD-F	4-fluoro-7-nitrobenzofurazane
*O*ac	*O*-acetyl
O-PS	*O*-link polysaccharides
PE	phosphoethanolamine
PG	proteoglycan
PMP	1-phenyl-3-methyl-pyrazolone

PMPMP 1(4-methoxy)phenyl-3-methyl-5-pyrazolone
TOF time-of-flight

3.1 Introduction

Lipopolysaccharides (LPSs) are major components of the outer membrane of gram-negative bacteria [1]. They are amphipathic molecules consisting of a hydrophilic carbohydrate portion, core and *O*-link polysaccharides (*O*-PS), and a hydrophobic lipid A portion (Fig. 3.1). The *O*-PS is either absent or truncated in some bacteria as a result of genetic mutation or related to a specific characteristic of the bacterial strains. This type of LPS may be referred to as rough-type (R-type) LPS, to differentiate it from smooth-type (S-type) LPS. Some bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*, express R-type LPS as a heterogeneous mixture of core oligosaccharides both within and between strains, and it is often referred to as short-chain LPS or lipo-oligosaccharide (LOS). In contrast, certain bacteria, such as *Escherichia coli* and *Salmonella* spp., elaborate S-type LPS containing *O*-PS comprised of repeating oligosaccharide units providing the basis for serological classification [2]. The nature and structure of the lipid A portion are generally conserved within a particular bacterial species mostly composed of a disaccharide formed of β-glucosamine residues, such as *H. influenzae* and *N. meningitidis* [3], or comprising amino sugar analogs, *Campylobacter jejuni*, to which *O*- and *N*-linked fatty acids are attached [4, 5].

Glycosaminoglycans (GAGs) are linear, polydisperse, and complex polysaccharides formed from disaccharide repeating units of glucuronic acid or iduronic acid

Fig. 3.1 Structure of a generic lipopolysaccharide (LPS)

residue (in keratan sulfate the uronic acid residue is replaced by galactose) linked to a glucosamine or galactosamine residue [6–8]. With the exception of hyaluronic acid, the GAGs, chondroitin sulfate/dermatan sulfate, and heparan sulfate/heparin are sulfated macromolecules with different degrees of charge density due to varying numbers sulfo groups linked to the polysaccharide backbone at different positions [6–8].

The GAGs are ubiquitous components of all tissue-organized organisms, with a very peculiar distribution in mammalian and other vertebrate tissues as well as invertebrates and a large structural variation, depending on their origin [9]. Furthermore, some bacteria synthesize capsular polysaccharides having carbohydrate backbones identical to nonsulfated chondroitin sulfate or heparan sulfate [8], with very interesting applications in several fields.

Determining the structural properties of *O*-PS or LOS and GAGs/GAG-derived oligosaccharides represents a major methodological challenge because of sample complexity. In the case of polysaccharides or oligosaccharides isolated from biological samples, structural analysis often must be performed with limited amounts of heterogeneous analyte in the presence of a background of complex biological contaminants. As a consequence, many efforts have been made to develop improved methods for (lipo)polysaccharide analysis. Capillary electrophoresis (CE) is a high-resolution technique for the separation of complex biological mixtures and has been widely applied to biological analyses [10]. Furthermore, CE has proved to be a very attractive alternative separation technique for GAGs and GAG-derived oligosaccharides, as their high negative charge assures excellent resolving power even in the presence of other contaminating biological molecules. Also, CE offers many advantages over a variety of other analytical methods, including an extremely high separation efficiency, simple operation, short analysis time, automated and reproducible analysis, very low consumption of samples, and buffers. Finally, over the last 10 years, mass spectrometry (MS) has proved to be a remarkably effective tool in studies of protein, carbohydrate, and glycolipid structure and function such as applied for specific structural evaluation when online direct coupled with electrospray ion MS (ESI-MS) [8]. This chapter provides an overview of various separation approaches and detection methods, relying on CE to evaluate several aspects of research in (lipo)polysaccharides and derived oligosaccharides, due to their importance in biological and pharmaceutical samples, disease diagnosis, glycobiology and carbohydrate biochemistry, and chemical synthesis.

3.2 Capillary Electrophoresis of Lipopolysaccharides

As above mentioned, LPS is the major component of the outer membrane of gram-negative bacteria, contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. LPS also increases the negative charge of the cell membrane and helps stabilize the overall membrane structure. It is of crucial importance to gram-negative bacteria, whose death results

if it is mutated or removed. LPS is an endotoxin, and it induces a strong response from normal animal immune systems. It acts as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of proinflammatory cytokines in many cell types, but especially in macrophages [11]. In immunology, the term *LPS challenge* refers to the process of exposing a subject to an LPS that may act as a toxin. Lipopolysaccharides are of crucial importance to gram-negative bacteria, and are therefore candidate targets for new antimicrobial agents.

Lipid A is normally a phosphorylated glucosamine disaccharide decorated with multiple fatty acids (Fig. 3.2). 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 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).

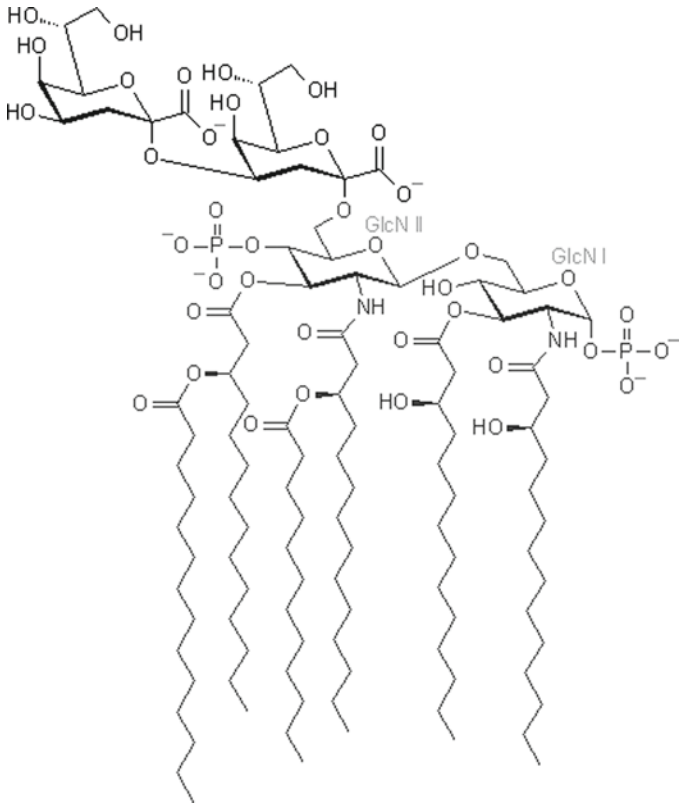

Fig. 3.2 The basis lipopolysaccharide of *E. coli*. GlcN, glucosamine

The core domain always contains an oligosaccharide component that attaches directly to lipid A and commonly contains sugars such as heptose and 3-deoxy-D-manno-octulosonic acid [also known as keto-deoxyoctulosonate (KDO)] [12]. The LPS cores of many bacteria also contain noncarbohydrate components, such as phosphate, amino acids, and ethanolamine substituents (Fig. 3.2).

The *O*-PS or LOS is attached to the core oligosaccharide, and comprises the outermost domain of the LPS molecule (Fig. 3.2). The composition of the *O*-chain varies from strain to strain; for example, there are over 160 different *O*-antigen structures produced by different *E. coli* strains [11]. *O*-antigen is exposed on the very outer surface of the bacterial cell, and as a consequence is a target for recognition by host antibodies.

Due to the high heterogeneity of the *O*-PS and LOS structures, various approaches and applications of CE in relation to the characterization of different properties of these complex carbohydrates will be discussed.

3.2.1 On-Gel Hydrolysis and CE-LIF Monosaccharide Composition of Excised Lipopolysaccharides

Many derived CE applications have been reported in mono- and polysaccharide analysis as such as using 9-aminopyrene-1,4,6-trisulfonate as labeling reagent associated with laser-induced fluorescence (LIF) for separation and detection [13, 14]. Reductive amination by 1-aminopyrene-3,6,8-trisulfonic acid (APTS) was also used to analyze, for example, chitin oligosaccharides [15] and big polysaccharides such as carrageenan [16], and to study the sequence of the polysaccharide parts of glycoproteins [17]. As a consequence, reductive amination is a common technique for the derivatization of reducing carbohydrates, providing appropriate chromophores or fluorophores to overcome native detection deficiencies [18]. Other dyes to label (oligo)saccharides were also used. A large variety of charged and uncharged UV-active or fluorophore derivatization reagents have been suggested in the literature for this purpose, such as 1-phenyl-3-methyl-5-pyrazolone [19], 4-aminobenzoic acid [20], and 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) [21]. 9-aminoacridone (AMAC) [22] works nicely and allows detection at nm concentrations of oligosaccharides. However, it is not charged, making it difficult to separate the uncharged species. In the same way, Imai's group [23] also proposed using the neutral dye 4-fluoro-7-nitrobenzofurazane (NBD-F). The fluorescence of the obtained compound is very sensitive to the solvent, and the obtained components are uncharged at detection limits of 100 nM. 3-(4-carbobenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [24] can also be used. A reductive amination using ammonia followed by condensation with CBQCA in the presence of the cyanide ion leads to a fluorescent isoindole derivative. Among these different strategies of labeling, APTS dye was the most developed and optimized [24], and it is charged and usable with the 488-nm argon-ion laser.

Sinorhizobium synthesizes polysaccharides in which large amounts of glucose can be found, as well as small quantities of galactose and mannose [18]. Nevertheless, only when grown under symbiotic-like conditions did substantial

amounts of rhamnose appear in S-surface polysaccharides. In association with this *de novo* synthesized sugar, and in addition to the normally present low-molecular-weight compounds, a new band appeared on the slab-gel [deoxycholic acid (DOC)–polyacrylamide gel electrophoresis (PAGE)] after silver staining (LPS-specific coloration). This compound displayed the electrophoretic behavior of a slow-migrating high-molecular-weight LPS (bearing the *O*-antigen polysaccharide) [18]. After a long step of purification, the sugar composition of this flavonoid-inducible LPS revealed that nearly half of its total sugar abundance is accounted for by rhamnose, making this compound a liporhamnopolysaccharide (LRPS).

To simplify the analysis protocol and to manage with less biological material (100 mL cultures instead of 5 L), a highly sensitive method was developed consistent with this complex sample [18]. After having demonstrated the high sensitivity of normal polarity CE-LIF analysis on standard monosaccharides (APTS-labeled sugar), on-gel analysis was performed [18]. A new DOC-PAGE was done on the crude extract using two lanes. The first lane was stained with silver nitrate. From the other lane the PAGE region corresponding to the slow-migrating LRPS was blind-excised and transferred to a glass vial. Reproducibility of gel migration from lane to lane was previously proved. To increase the probability of excising the totality of the band, only the central lanes, where no border effect can occur, were used. The hydrolysis protocol was performed directly on this gel fragment. The sugars released in the aqueous solution were APTS-labeled after removal of the gel. Blank analysis of excised gel demonstrated the absence of interfering compounds. CE-LIF analysis showed a very similar carbohydrate composition to the one obtained by GC-MS of the LRPS. Some fast-moving molecules probably due to incomplete hydrolysis of the targeted compound could be observed. Finally, the signal-to-noise ratio was very high, considering the small quantities loaded on the gel. The on-gel analysis was found to be a simple, direct, and unambiguous way to prove that the rhamnose-rich polysaccharide is effectively the compound revealed as a band on the PAGE and not another compound present in the high-molecular-weight fraction of size-exclusion chromatography.

The method described presents two enormous advantages for all types of polysaccharide analysis: (1) the very high sensitivity of CE-LIF for APTS-labeled mono- and oligosaccharides requires little material, which is easy to produce by microextraction of reduced quantities of cultured bacteria; and (2) the monosaccharide composition of polysaccharides can be monitored directly on the basis of PAGE analysis, avoiding long purification steps.

3.2.2 CE Chips for Screening of Endotoxin Chemotypes from Whole-Cell Lysates

Biochemical differences among LPSs from different bacterial serotypes and their mutants include variations within the lipid A part, the core oligosaccharides, and the composition of the *O*-polysaccharide chains. The R-type LPS can be extracted

and purified from bacteria by the method of Galanos et al. [25] (about 5 days), while the S-type LPSs can be purified by the long-lasting procedure of Westphal and Luderitz [26] (about 10 days). A short extraction process (about 40 h) leading to partially purified LPS from whole-cell lysates can be done by the procedure of Hitchcock and Brown [27], which needs a smaller culture volume. At the moment, the analysis of intact LPS preparations and the classification of the chemotypes are routinely checked by sodium dodecyl sulfate (SDS)-PAGE with silver staining [28]. The chemical composition is reflected by the complexity of the electrophoretic patterns, that is, by the presence of bands corresponding only to the simpler R-type structures or bands showing S-type structures having *O*-polysaccharide chains with increasing numbers of carbohydrate constituents (a distinctive ladder-like pattern).

Recently, a microchip electrophoretic method to fingerprint pure bacterial LPSs, based on LPS complexation with dodecyl sulfate chains and then with a fluorescent dye, has been introduced [29, 30]. This method is able to replace the SDS-PAGE and silver staining, with the advantage of high speed and quantitation. Endotoxin samples and LPS, either prepared from bacterial strains or prepared by suspending pure lyophilizates, were analyzed and compared by electrophoresis in microchips [30]. Although the CE profiles were found to be similar, the actual migration properties of the LPS components in the molecular sieving environment were different. Both the molecular masses and the relative amounts show a characteristic pattern to the respective bacterial origin.

When large numbers of bacterial mutants and their LPS content are to be compared, for example in the preparation of vaccines or for qualitative evaluation of the heterogeneity of LPSs and the *O*-polysaccharide components, LPSs are prepared directly from bacterial cultures, generally by the relatively short method of Hitchcock and Brown [27]. This method involves proteolytic digestion of whole-cell lysates producing protein-free total cellular LPS, but the procedure does not involve further purification steps and freeze drying. An important specialty of this method is that a culture volume of only 1 mL is necessary for the characterization of the LPSs. The microchip CE method for the qualitative and quantitative characterization of pure endotoxin preparations [29, 30] show that with the careful preparation of the LPSs from whole-cell lysates the electrophoretic profiles of the endotoxins can be used for the proper assignment of the LPS chemotypes. With the help of this microchip analysis, ten endotoxin assignments can be performed within about 1 h compared to the SDS-PAGE methodology, which needs a laborious gel-preparation and silver staining for visualization of 18–20 samples in one run. The R-types were observed to contain high-mobility/low-molecular-mass components presumably consisting of lipid A and core oligosaccharides. The S-type LPSs possessed components with lower mobility/higher molecular mass, and sometimes appeared as “waves” of peaks based on the number of repeating units in the *O*-side chains. The sensitivity of the method was high, since satisfactory patterns from 1 mL bacterial cell cultures and an LPS content less than 1 ng were obtained.

3.2.3 Capillary Zone Electrophoresis (CZE) Separation of *O*-Deacylated LPS and Polysaccharide Derivatives

Diversity of nonstoichiometric substituents on the inner R-core of *E. coli* C LPS, an Ra strain of the R1-type core of *E. coli*, using deacylated derivatives, has been observed [31]. The LPS was found to have diverse substitution with up to eight phosphate, three KDO, and two ethanolamine residues on the polysaccharide backbone (Fig. 3.3). The separation of the deacylated derivatives of the LPS based on the variation in the charged substituents was investigated by CZE [31]. The LPS of *E. coli* C was treated with anhydrous hydrazine at 37°C for 30 min to form *O*-deacylated LPS and then further treated with 4 M KOH at 125°C for 18 h to form *O*,*N*-deacylated LPS. The polysaccharide part of LPS was obtained by hydrolyzing the LPS in 1% acetic acid at 100°C for 2 h. This treatment degraded LPS to form derivatives further purified by gel filtration. CZE was investigated for the separation of these derivatives by operating with a buffer of 10 mM ammonium acetate pH 9.0 at 30 kV. These conditions afforded good separation efficiencies for the various derivatives [31]. However, it did not separate the more challenging de*O*-derivative, which consisted of more than 20 components. The concentration of the running electrolyte was thus changed to 50 mM to improve its separation, and the electrophoresis was carried out at 10 kV to keep the current below 15 mA. By on-line coupling and CZE separation with ESI-MS (see below for a more complete list of CE-MS applications), analysis of the separated species in the de*O*,*N*- and polysaccharide derivatives was achieved and clearly characterized for composition. The electropherogram accomplished the determination of the composition of the diverse species among de*O*-derivatives by monitoring the specific m/z numbers corresponding to the species having different numbers of phosphate residues. Due to this strong possibility, the present method was found to be valuable for understanding the diversity of LPS from gram-negative bacteria.

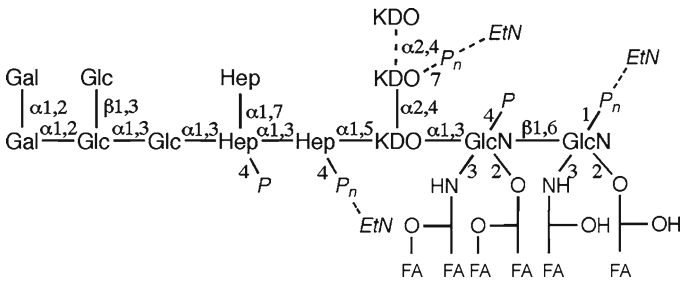

Fig. 3.3 Chemical structure of LPS of *E. coli* C strain. *Gal* d-galactose, *Glc* d-glucose, *Hep*, l-*glycero*-d-*manno*-heptose, *KDO* 3-deoxy-d-*manno*-octulosonic acid, *GlcN* 2-amino-2-deoxy-d-glucose, *EtN* ethanolamine, *P* phosphate, *FA* fatty acid

3.2.4 CE for Quantitative Analysis of Extracted Lipopolysaccharides

The rapid detection of microbial contaminants in food is critical for ensuring the safety of consumers. Traditional methods for detection of bacteria in food rely on time-consuming growth in culture media, followed by isolation, biochemical identification, and in some instances serology. Structural information regarding various unique molecules including lipopolysaccharide is continuously applied in the development of faster and more convenient biochemical assays. With LPS being an amphiphilic molecule, one would be led to think that a rapid electrophoretic technique not employing immunology would be the answer in LPS identification and substructure evaluation. As a consequence, CZE could be applied in the detection of the complete LPS molecule, with the only disadvantage being low sensitivity. A CZE method for the evaluation of LPS obtained with extraction methods has been developed [32].

Capillary zone electrophoresis was found to be suitable when applied in the evaluation of LPS derived with different extraction methods. This technique proved to be rapid and the results were reproducible. In situations where the immunological evaluation of LPS is hampered due to residual SDS and proteinase K, CZE could be applied for this purpose. Furthermore, CZE was able to distinguish between LPS derived from two different extraction procedures. Finally, the limited as well as the elaborate breakdown patterns of LPS extracted with various methods was found reproducible, producing a specific "fingerprint" that could in the future be applied in the rapid identification of the gram-negative bacteria present as contaminant in foods.

3.2.5 CZE for the Determination of Meningococcal Polysaccharides

The global disease burden of meningococcal meningitis remains high despite the widespread availability of effective antibiotics and medical care. Variability in the initial presentation of the disease and the possibility of progression from early symptoms to death in as little as 24 h contribute to a mortality rate of 7–19% [33]. Infants under 1 year of age have the greatest incidence of disease, but adults can also suffer serious illness and may actually have a higher fatality rate [34]. The serious and unpredictable nature of the disease emphasizes the importance of vaccination of at-risk populations as a way to contain and prevent *N. meningitidis*-induced illness. The meningococcal vaccine currently licensed in the United States consists of a mixture of native capsular polysaccharides from four serogroups (A, C, W135, and Y) and is effective in preventing disease caused by these serogroups in adults [33]. Purified polysaccharides are large, unbranched structures composed of homopolymers of mannosamine phosphate (serogroup A) or sialic acid (serogroup C) or heteropolymers of sialic acid alternating with either glucose (serogroup Y) or galactose (serogroup W135) [35] (Fig. 3.4).

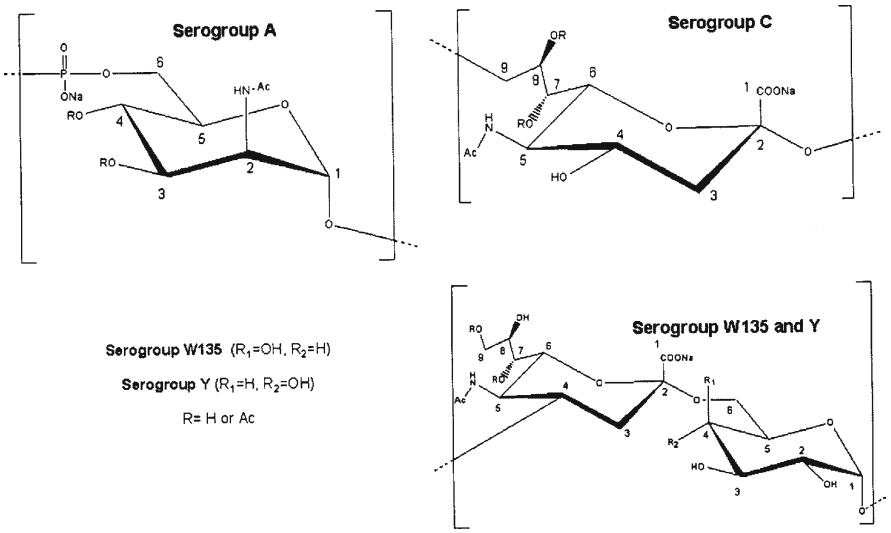

Fig. 3.4 Structure of meningococcal polysaccharides

Several methods for quantification of meningococcal polysaccharides have been used [35], providing accurate and sensitive measurement of meningococcal polysaccharides but requiring prior conversion to monosaccharides by acid digestion. The use of strong acids, often at elevated temperature, represents a potential safety concern for analysts performing the tests, and has a cost associated with material disposal. Furthermore, it is difficult to extend these approaches to detection of polysaccharides in mixtures, since serogroup C does not contain a unique saccharide. On this basis, CZE was applied [35] to quantify intact native meningococcal polysaccharides from the four serogroups currently used in human vaccines, and separation of the four serogroups was achieved in less than 30 min using a simple alkaline phosphate/borate buffer.

Effective and clear separation of a mixture of four native meningococcal polysaccharides (serogroups A, C, W135, and Y) using a phosphate/borate separation buffer was obtained (Fig. 3.5). Identity of the peaks was confirmed by testing a sample of each serogroup separately. Serogroup W135 and Y polysaccharides have very similar chemical structures, differing only in the relative orientation of adjacent hydroxyl groups on the alternating galactose and glucose residues, respectively. Good separation was observed in 40 mM of sodium phosphate (dibasic) in the absence of borate, and differential binding of borate was therefore not responsible for the separation observed. Consequently, the basis for separation of these two serogroups is unclear, although conformational differences between the polysaccharides or differential binding of other ions during their preparation may serve a similar role.

Increased ionic strength of the separation buffer resulted in increased separation of serogroups W135 and Y. These serogroups were also separated in a phosphate/borate

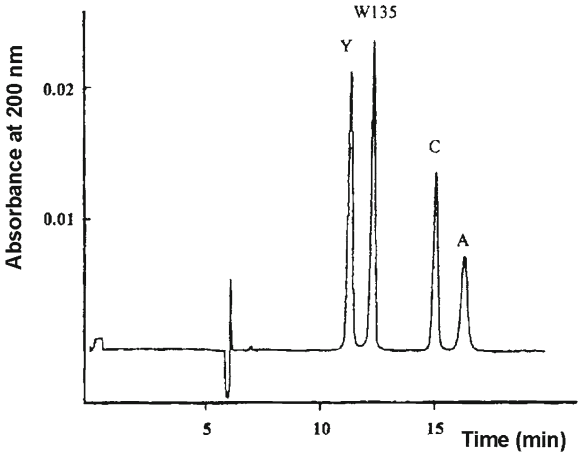

Fig. 3.5 Separation of a mixture of four meningococcal native polysaccharides using a bare silica capillary, 37 cm (30 cm effective length), 50 μm (inner diameter), with 40 mM phosphate/40 mM borate (natural pH) at 10 kV (normal polarity). Serogroup identity of peaks as marked (modified from [35])

solution adjusted to pH 10.3, but some deformation of peak shape was noted at the higher pH. Furthermore, temperatures between 20 and 40°C appeared to be suitable, but some deterioration of peak shape was noted at 45°C. The above reported results demonstrate that separation of native meningococcal polysaccharides by CZE is robust with respect to variations in the conditions used. The presence of SDS in the separation buffer resulted in the appearance of split peaks for all four serogroups. The cause of the peak splitting is not clear, but it has been shown that native meningococcal polysaccharides contain C16 and C18 fatty acids at the reducing end. Peak splitting therefore may be due to partial separation of differentially lipidated molecules.

Polysaccharides of varying sizes were also tested to examine the effect of this physical parameter on separation. Serogroup Y polysaccharide sized to 75 kDa by mild acid hydrolysis demonstrated the same migration time as did native material. Serogroup A polysaccharide sized to approximately 20 kDa and labeled on the reducing end by the addition of adipic dihydrazide also demonstrated the same migration time as did native polysaccharide of the corresponding serogroup. It may be that the charge density of linear, regularly repeating polysaccharides is constant over a range of lengths, and that size is not an important parameter when separation is based on this parameter.

Quantification of polysaccharides by CZE was undertaken, and the results were compared to values obtained by wet chemical determination. Polysaccharide levels determined by CZE were within 5% points of the expected value as measured by the wet chemical method, and, overall, good agreement was observed between the two analytical approaches. Furthermore, no apparent bias was observed for tests performed at two different concentrations. Based on these data, CZE appears to be a useful alternative to existing tests for determination of meningococcal polysaccharides.

3.2.6 CE to Monitor Lipopolysaccharides (Endotoxins) by Protein Complexation

As is well known, the lack of strong ultraviolet (UV)-absorbing groups in the LPS molecule excludes their detection with standard optical detectors (UV/Visible). It is also known that several proteins form complexes with LPSs, for instance, LPS-binding protein, and lipoproteins in plasma, albumin, transferrin, melittin, lysozyme, and complement proteins. Some of these endotoxin-binding proteins have been identified by affinity chromatography, autoradiography, and isoelectrofocusing [36]. Kaca et al. [37] showed that native human hemoglobin (Hb) forms stable complexes with bacterial endotoxins ($K_d = 3.1 \times 10^{-8}$ M).

Studies on the stoichiometry of forming Hb/endotoxin complexes have been performed [38], but the structure of the complexes and the interaction mechanism have not been elucidated. The analysis of absorption and circular dichroic spectra [39] revealed that the binding of LPS to Hb might result in oxidation of Hb to methemoglobin. Hb with its strong UV absorption at 205 nm and absorption maximum in the visible part of spectrum (415 nm) also served as a marker of rough and smooth LPSs. As a consequence, CZE of different kinds of endotoxins from *Salmonella minnesota* R595, *Shigella sonnei* R562H, and *S. minnesota* (wild-type), of free human Hb, as well as of the mixtures of the protein with the LPSs was conducted in Tris–HCl buffer at pH 8.5, following incubation [40]. The electrophoretic migration of the protein-containing zones was monitored in the UV and visible region. As expected, no UV signal was obtained with the various LPSs. The typical separation profile of pure Hb at 205 and 415 nm contained one dominating and two minor peaks, with the profile obtained at 415 nm showing reduced sensitivity. However, the incubation of Hb with LPS provided peaks in two time intervals in both the UV and the visible region (Fig. 3.6). Peaks appeared at the same migration times as did the free Hb, but an additional slightly deformed peak (with a small shoulder) appeared with a higher mobility. This component had similar absorption properties as free Hb, absorbing the light in both wavelength regions. Consequently, the complex of the LPS and Hb appeared in the electropherogram as a separate peak. The resolution between the complex and the pure Hb was very high (Fig. 3.6), and the short migration time indicated that the negative R-type LPS molecules (with high charge-to-mass ratio) attached to the protein molecules caused a significant increase in the mobility of the Hb. Furthermore, upon mixing the R-endotoxin extracted from *S. sonnei* R562H with Hb, a time-dependence in the complex formation was observed, since after a longer incubation (2 h) the free Hb peak disappeared and the complex peak increased (Fig. 3.6).

According to the above illustrated methodology, a new CE method based on the interaction of endotoxins with Hb to form soluble Hb/LPS complexes has been developed to overcome both the detectability and the solubility problems of LPS and thus to be used as a marker for LPSs. Differences in the structures of rough and smooth LPSs provided significantly different electrophoretic profiles when the migration properties of LPS-Hb complexes were monitored. It has been reported that

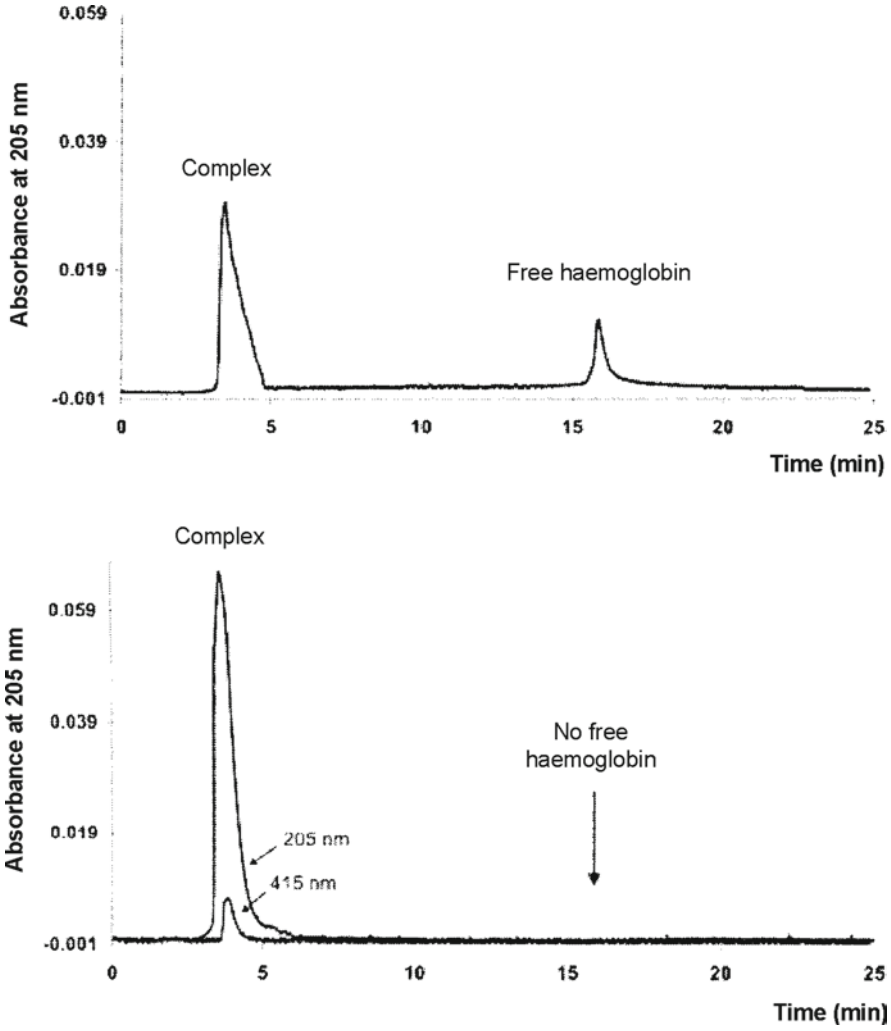

Fig. 3.6 Capillary zone electrophoresis (CZE) of a mixture of LPS from *S. sonnei* 562H and hemoglobin. The mixture was incubated at 37°C for 1 h (*upper panel*) and 2 h (*lower panel*) prior to electrophoresis (modified from [40])

each chain of the Hb tetramer might bind a single smooth LPS, or three to five rough LPSs [38]. Although electrophoresis cannot easily be used for such determinations, it is likely that only part of the smooth endotoxin molecules from *S. minnesota* (wild-type) was bound to Hb, since the small, overlapping peak of the complex did not change upon longer incubation. However, from the molar ratio of the smooth LPS to Hb, one can assume that probably not more than one or two LPS molecules bind to each protein molecule. In the cases of the R-form endotoxins, where the molar ratio is much higher, a significant amount of the LPSs should be bound to Hb.

The disappearance of the free Hb peak upon prolonged incubation with LPS from *S. sonnei* 562H indicates that the protein molecules bind several LPS molecules (up to 10–18) at the same time. As a consequence, the complex formation of the major and minor Hb components with endotoxins was different, since the ratios of the minor and the major components in the complexed forms and in the free forms vary.

3.2.7 CE of Alginic Acid

Alginic acid (AA) is a collective term to define linear binary block copolymers of (1→4) α-L-guluronic acid (G residues) and (1→4) β-D-mannuronic acid residues (M residues) synthesized by brown algae and some bacteria [41]. Owing to its gelling, stiffening, and stabilizing properties, AA is used as an ingredient in food and in a number of pharmaceutical applications such as in antacid formulations for the treatment of gastroesophageal reflux. The composition and the sequential arrangements vary widely from homopolymeric mannuronan to polymers with >70% guluronic acid. Apart from mannuronan, most naturally occurring alginate does not exhibit a repeating unit, and its sequence cannot be described by bernoullian statistics. This nonrandom structure has been attributed to the postpolymerization modification catalyzed by mannuronan C-5 epimerases. CE has been applied to analyze the direction, the extent of the process, and the subsite specificity of C-5 epimerase when acting on a homopolymeric mannuronan [41].

Hexamannuronic acid (DP6), heptamannuronic acid (DP7), and octamannuronic acid (DP8) were isolated by size-exclusion chromatography from a mannuronan hydrolysis mixture, and were used as well-defined substrates for biochemical subsite studies of C-5 epimerase. Micellar electrokinetic capillary chromatography-UV (MEKC-UV) was able to separate these AA species derivatized with 4-aminobenzonitrile (4-ABN) before enzymatic treatment [41]. The MEKC-UV profile of the hexamer showed a very similar appearance relative to the unreacted sample, also in terms of ratio between the molar concentrations of the components. No significant new compounds could be detected by MEKC-UV, confirming that the enzyme acts poorly on hexa- (and hepta-) mannuronic acid. In the case of DP7, treatment with epimerase induced the presence of some new compounds and a relatively large decrease in the amount of octamer with respect to heptamer. These results indicated that the efficiency of the epimerase action was greater for the octamer than for the heptamer, and the increase of enzyme efficiency with the number of monomeric units was confirmed by the CE analysis of the epimerized octamer.

Due to its molecular complexity and high molecular mass and to the lack of readily employed physicochemical properties, the analysis of AA has not been well defined. As a consequence, no simple, sensitive, convenient, and specific analytical procedure has been established for quality assurance in quality control laboratories. A titrimetric AA assay in a relatively pure state, which is clearly inapplicable to finished products due to interference with metal ions or other excipients, has been proposed [42]. Colorimetric methods, with pretreatment, have

also been widely used in the determination of AA content, but these methods were found to be inadequate due to interference by other polysaccharides in the sample, and also to obtain versatile calibration curves for the determination of AA with any ratio or distribution of uronic acids along the polymeric chain. High-performance liquid chromatography (HPLC) and gas chromatography have been applied after chemical or enzymatic AA treatment for structural studies, but no chromatographic method has been reported to date for its determination in pharmaceutical formulations or preparations [41, 42].

Alginate lyases [43], detected in several microorganisms, marine molluscs, and marine brown algae, catalyze a β-elimination reaction that cleaves the AA chain, producing an unsaturated uronic acid residue at the new nonreducing end, having a strong UV absorption at 230 nm. A new highly specific and sensitive CE method (electrokinetic chromatography with SDS) for the determination of the total AA content in pharmaceutical formulations has been developed by means of CE at 230 nm after treatment with alginate lyase [4.2.2.3] and separation of unsaturated products, D-oligomers (DHexA–[HexA]*n*), in particular DP3 (DHexA–HexA–HexA) and DP4 (DHexA–HexA–HexA–HexA) [42]. Using a buffer constituted with 10 mM of sodium borate and 50 mM of SDS at pH 9.0, micellar electrokinetic capillary chromatography was able to determine with very high resolution the AA D-oligomers produced by the action of the lyase (mainly DP3 and DP4) as one single species (Fig. 3.7). The intra- and interday variations (CV%) were between 6.3 and 9.1 for migration time and between 2.5 and 5.7 for peak area, respectively. The calibration curve showed good linearity for

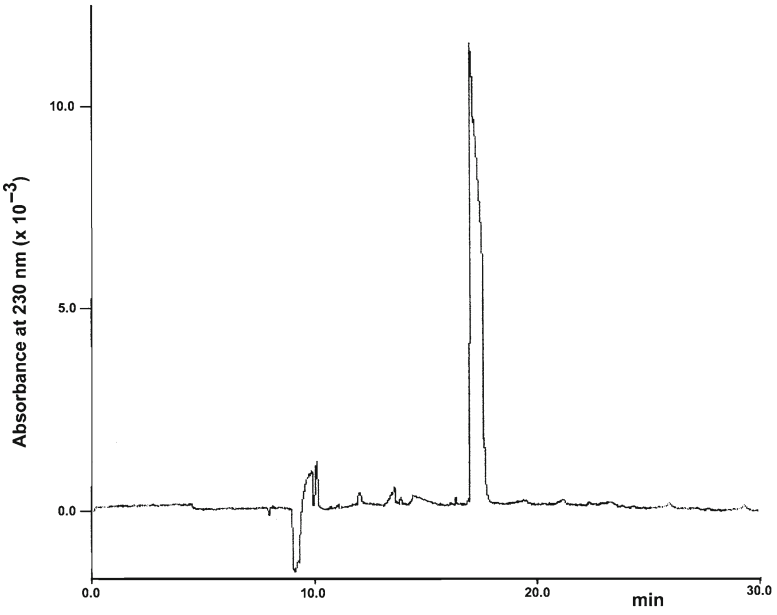

Fig. 3.7 Electrophoregram of alginic acid treated with alginate lyase (modified from [42])

the examined concentration range (60–360 ng), with an average correlation coefficient greater than 0.980. The lowest detection limit and the lowest quantitation limit of the method were 15 and 40 ng, respectively. The intra- and interday variations in terms of CV% were 5.5 and 8.6%, respectively, and the intra- and interday accuracy was estimated to range from 4.1 to 8.9%, while the percent recoveries of AA were calculated to be 102, 97, and 93% for different AA amounts. Variations in temperatures, voltage, and buffer composition in comparison with adopted conditions within a 10% limit do not modify the electrophoresis results.

The evaluation of AA was performed in both solid and liquid pharmaceutical formulations also in the presence of other ingredients, in particular, aluminium, sodium, and potassium bicarbonate, and emulsifying and flavoring agents. The quantitative results obtained were 101.2% of AA content in tablets and 98.4% in liquid formulation, in total conformity with the label claims.

3.2.8 Characterization of Lipopolysaccharides by CE-MS

The coupling of CE with MS provides a powerful approach for rapid identification of target analytes present at trace levels in biological matrices, and for structural characterization of complex biomolecules. This technique has been applied to the characterization of bacterial glycolipids and glycoconjugates from various bacteria, including *H. influenzae*, *N. meningitidis*, *Pasteurella multocida*, *Helicobacter pylori*, *C. jejuni*, and *Aeromonas salmonicida* (see [2] for a related bibliography). The application of CE-MS technique to the characterization of carbohydrates provides unparalleled resolution for identifying differences in LPS glycoform and phospho-form populations. In the rapidly developing field of glycobiology, such structural tools are playing an increasingly important role in the study of the molecules involved in host–pathogen interactions (cell adhesion, epitope recognition) as well as helping in delineating the genetic basis of oligosaccharide assembly and expression. CE-MS methodology facilitates the determination of closely related LPS glycoform and isoform families by exploiting differences in their unique molecular conformations and ionic charge distributions by electrophoretic separation. Furthermore, the location of functional groups, such as glycine, acetate, phosphoethanolamine, phosphocholine, and sialic acid, within the glycolipid structure is derived through tandem mass spectrometry.

The following subsections discuss various CE-MS applications to LPS characterization and determination.

3.2.8.1 Characterization of Intact LPS from the *H. influenzae* by Electrophoresis-Assisted Open-Tubular Liquid Chromatography-MS

The electrophoresis-assisted open-tubular liquid chromatography (LC)-MS method has been applied for analyzing intact LPSs from *H. influenzae* [44].

Structural informations on both core oligosaccharides and the lipid A moiety including the sialylation, glycylation, and distribution of fatty acid residues on the disaccharide backbone of lipid A were obtained. The fragmentation patterns of LPS molecules were investigated for determining the location of sialic acid. It was found that the tandem mass spectra of sodiated ions provided unambiguous evidence of both sialylated lactose and sialylated lacto-*N*-neotetraose. In contrast, the fragment ions of protonated ions only offered evidence for the existence of sialylated lacto-*N*-neotetraose. Intact LPS was directly introduced to a tandem mass spectrometer. An in-source dissociation strategy was employed, followed by multiple-stage MS/MS on the ions originating from the lipid part to obtain structural information. In the same way informations on the core oligosaccharides was obtained by MS/MS by focusing on ions originating from oligomers.

3.2.8.2 Carbohydrate Analysis of Typical and Atypical Isolates of *A. salmonicida* for the LPS-Based Classification

A total of 39 typical and atypical isolates of *A. salmonicida* have been screened using an in-source fragmentation technique [45]; their *O*-chain polysaccharide structure, composition, and linkage were analyzed, and comparison was made to the previously determined polysaccharide structure established by CE-MS. All the *A. salmonicida* strains examined could be divided into three structural patterns, types A, B, and C, based on the structure of their corresponding *O*-chain polysaccharides. The majority of typical *A. salmonicida* isolates belonged to structural type A and displayed the complete *O*-chain polysaccharide structure previously determined for *A. salmonicida* strains A449, 80204, and 80204-1, composed of the trisaccharide repeating unit containing L-rhamnose, 2-acetamido-2-deoxy-D-mannose, and D-glucose, and the noncarbohydrate *O*-acetyl substituent. Identical fragmentation patterns in the CE-MS spectra were obtained for 18 *A. salmonicida* strains examined belonging to this structural group. Characteristic fragment ions corresponding to one *O*-acetylated repeating unit were observed at *m/z* 554.5. Fragment ions at *m/z* 392.4 were consistent with the loss of D-glucose residues. Other characteristic fragment ions included ions at *m/z* 757.5 and *m/z* 945.7, which were consistent with consecutive additions of 2-acetamido-2-deoxy-D-mannose and L-rhamnose-*O*-acetyl residues, respectively.

The second group, structural type B, was represented by the *O*-chain polysaccharide of *A. salmonicida* strain 33659, consisting of disaccharide repeating units containing L-rhamnose and 2-acetamido-2-deoxy-D-mannose only. Identical fragmentation patterns in CE-MS spectra were obtained for 15 *A. salmonicida* strains examined belonging to this structural type. Characteristic fragment ions corresponding to one repeating unit were observed at *m/z* 350.4. Additional fragment ions corresponding to consecutive additions of 2-acetamido-2-deoxy-D-mannose (*m/z* 553.6) and L-rhamnose residues (*m/z* 699.7) were also observed.

Finally, six atypical *A. salmonicida* strains belonging to the third structural group, type C, were represented by the *O*-chain polysaccharide of atypical strain N4705 consisting of disaccharide repeating units containing L-rhamnose and 2-acetamido-2-deoxy-D-mannose and a noncarbohydrate *O*-acetyl component. The compositional and linkage analysis was performed on the purified *O*-chain polysaccharide of *A. salmonicida* strain N4705, confirming its structure. The CE-MS fragmentation pattern of *A. salmonicida* strains belonging to structural type C contained two characteristic fragment ions at *m/z* 392.4, corresponding to the *O*-acetylated repeating unit, and at *m/z* 350.4, corresponding to the non-*O*-acetylated repeating unit. Other characteristic fragment ions included ions at *m/z* 553.5 and at *m/z* 699.7 consistent with the consecutive additions of 2-acetamido-2-deoxy-D-mannose and L-rhamnose residues, respectively, and corresponding to a longer non-*O*-acetylated polysaccharide structure. Additional fragment ions corresponding to the *O*-acetylated polysaccharide were observed at *m/z* 595.5 and *m/z* 783.6, confirming the presence of L-rhamnose-*O*-acetyl in this structure. Based on relative intensities of fragment ions at *m/z* 392.4 (*O*-acetylated molecular species) and at *m/z* 350.4 (non-*O*-acetylated molecular species), the degree of *O*-acetylation of L-rhamnose residues was found to be approximately 30%.

3.2.8.3 CE and High-Field Asymmetric Waveform Ion Mobility Spectrometry MS for the Analysis of LPS

High-field asymmetric waveform ion mobility spectrometry (FAIMS) is a new technology for atmospheric pressure and room temperature separation of gas-phase ions. The FAIMS system acts as an ion filter that can continuously transmit one type of ion, independent of mass-to-charge ratio (*m/z*). The coupling of FAIMS to CE-MS provides a sensitive technique for the characterization of complex glycolipids, permitting the separation of trace-level LPS oligosaccharide glycoforms for subsequent structural characterization using tandem mass spectrometry. This was demonstrated for LPS from nontypeable *H. influenzae* strain 375 following *O*-deacylation with anhydrous hydrazine [46]. This strain of *H. influenzae* can express a triheptosyl-containing glycoform to which four hexose residues are linked forming the outer-core region of the molecule. This has been referred to as the Hex4 glycoform. Glycoforms have been identified that differ in the number of phosphoethanolamine (PE) substituents in the inner core. With the use of CE-FAIMS, isomeric Hex4 glycoforms containing two PE groups were separated and characterized by MS/MS. FAIMS provided a significant reduction in mass spectral noise, leading to improved detection limits (about 70 amol of the major glycoform) [46]. The extracted mass spectrum showed that the apparent noise was virtually eliminated. In addition to the reduction of chemical background, the ion current was increased by as much as 7.5 times as a result of the atmospheric pressure ion-focusing effect provided by the FAIMS system. The linearity of response of the CE-FAIMS-MS system was also studied, and the calibration curve was linear for approximately three orders of magnitude.

3.2.8.4 Glycotyping by CE-MS of *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis

P. aeruginosa is a gram-negative bacteria that causes chronic endobronchial infection in 60–70% of adult patients with cystic fibrosis [47]. The differences between the composition and structure of the *O*-chain polysaccharides of LPS provide the basis for the serological classification of *P. aeruginosa* isolates [48]. Reports of epidemic strains of *P. aeruginosa* have originated from cystic fibrosis clinics in the United Kingdom and Australia [49], and a Canadian *P. aeruginosa* epidemic strain has not been described previously [50].

To assess the application of the CE-MS-based approach for the rapid detection and differentiation of *P. aeruginosa* LPS phenotypes, 30 randomly selected clinical isolates were studied, along with a control strain [51]. Using a developed in-source fragmentation technique, the selected *P. aeruginosa* isolates were subjected to CE-MS analysis in the positive and negative ion modes carried out directly on bacterial cell samples. Chemical structures of the core and the *O*-chain polysaccharide repeating units of LPS belonging to serogroup O6a showed characteristic fragment ions corresponding to one *O*-acetylated repeating unit observed at *m/z* 796.0. Fragment ions at *m/z* 1649.9 were consistent with the loss of rhamnose residues, while fragment ions at *m/z* 1391.7 corresponded to the consecutive loss of rhamnose and GalNAcAN(OAc) residues. Based on the relative intensity of fragment ions at *m/z* 259.5 and *m/z* 649.9 (*O*-acetylated molecular species) and *m/z* 233.5 and *m/z* 607.9 (non-*O*-acetylated molecular species), the degree of *O*-acetylation of rhamnose residues was approximately 60–80%. Fragmentation patterns of the *O*-chain polysaccharides of *P. aeruginosa* LPS from clinical cystic fibrosis isolates confirmed that they belonged to a different, although structurally related, Lányi–Bergan subgroup O6a,6c, the main difference arising from the absence of the *O*-acetyl group on GalNAcAN residue. Characteristic fragment ions corresponding to one repeating unit were observed at *m/z* 753.6, while fragment ions at *m/z* 607.5 and *m/z* 391.5 were consistent with the consecutive loss of rhamnose and GalNAcAN residues. Nine of ten *P. aeruginosa* isolates belonging to clonal type A displayed a smooth LPS glycotype belonging to Lányi–Bergan subgroup O6a,6c and were serologically distinct from the control ATCC strain.

The second structural group represented *P. aeruginosa* strains producing a rough LPS containing a complete or occasionally incomplete or modified core oligosaccharide and frequently carrying *O*-acetyl (OAc) and phosphate or phosphoethanolamine substituents. Characteristic fragment ions corresponding to a complete core oligosaccharide were observed at *m/z* 875.3. Their fragmentation pattern was consistent with the previously published structure of the lipopolysaccharide core of the *P. aeruginosa* immunotype. While it is thought that phosphate groups play an important role in outer membrane interactions with antibiotics, this study [51] suggested that the presence of multiple *O*-acetylation sites also contributes to enhanced antibiotic resistance and multidrug resistance of *P. aeruginosa*. Finally, CE-MS-based screening of LPS glycotypes was proved to be a rapid and reliable method for differentiating clonality of *P. aeruginosa* isolates with LPS glycotypes also associated with antibiotic resistance.

3.2.8.5 Characterization of Short-Chain LPS Glycoforms by CE-MS

Lipopolysaccharide of *H. influenzae* is composed of an oligosaccharide moiety comprising a heterogeneous mixture of glycoforms, and a membrane-anchoring lipid A component.

The structural model shows the conserved L-glycero-D-manno-heptopyranosyl trisaccharide inner-core region observed in every stain studied to date. KDO carries phosphate or pyrophosphoethanolamine substituents at the O-4 position. LPS glycoforms containing *p*-*o*-glucopyranose, lactose, globotriose, globotetraose, sialyllactose, and sialyllacto-*N*-neotetraose oligosaccharide chains have been identified at one or more positions [2]. The relative proportions of these glycoforms in the LPS from a particular strain depends on the availability of the substrate sugar nucleotides and expression of phased variable genes. In addition, LPSs from several strains have been found to carry ester linked acetate and glycine substituents.

Direct infusion of LPS samples into the mass spectrometer without any front-end separation generally gives very complex mass spectra, since LPS glycolipids often consist of mixtures of closely related glycoforms varying by the length and the site of attachment of the oligosaccharide chains. When coupling a CE system to the mass spectrometer, one can obtain a contour profile of *m/z* versus time providing an additional dimension for the understanding of structural complexity [2]. CE-MS profiling of LPS-OH has been utilized for comparing the LPS glycoform populations of *H. influenzae* and *N. meningitidis* in different strains. In the spectrum of the *H. influenzae* LPS-OH, a series of doubly, triply, quadruply, and pentuply deprotonated molecules was observed and can be used to calculate the molecular masses of the different glycoforms. Due to the additional separation by CE, minor glycoforms containing oligosaccharide chains capped by a sialic acid residue were also readily detected [2]. In addition, the contour profile provided a useful method to identify closely related families of LPS glycoforms. The extension of one hexose residue to the LPS core unit results in an increase in molecular mass and a decrease in electrophoretic mobility (the reverse of the electro-osmotic flow), resulting in the observation of a shorter migration time. Similarly, the presence of LPS phosphoforms that differ in the presence and location of functional groups such as phospholipids is reflected by the changes of electrophoretic mobilities and masses.

3.2.8.6 Isomer Separation and Characterization by CE-MS

Capillary electrophoresis–MS is not only a technique for profiling bacterial LPS oligosaccharide glycoforms, but also a powerful technique for separation and characterization of isomeric glycoforms. For example, variation of the phosphatydilethanolamine location resulted in different glycoforms with the same composition in both *H. influenzae* and *N. meningitidis* [2]. The CE-MS data were obtained in both positive and negative ion mode on LPS-OH from *H. influenzae,* and triply and doubly charged ions corresponding to major glycolipids were identified at different

m/z values. Based on their molecular masses, the three glycolipids were assigned to correspondent composition. The observation that the triply deprotonated and doubly deprotonated species appeared in both of the CE-separated peaks indicated the existence of isomeric glycoforms [2]. Finally, to obtain structural information, tandem mass spectrometry (MS/MS) experiments were conducted on selected ions in both negative and positive ion detection modes.

3.2.8.7 In-Source Fragmentation Strategy for Polysaccharide Analysis

The electrospray ion is able to generate multiply charged ions, and the recorded spectrum can then be deconvoluted for the molecular mass of intact biopolymers [2]. However, no multiply protonated (positive ion mode) or deprotonated (negative ion mode) ions were detected for intact *O*-polysaccharide, when conventional orifice voltage is used. However, in-source collision-induced dissociation (IS-CID) can be applied to promote the formation of structurally relevant repeating units of heterogeneous *O*-polysaccharide and capsular polysaccharide that would remain undetected using conventional electrospray ionization conditions [52–54]. In fact, the fragmentation pattern of CE-IS-CID-MS was consistent with the reported structure of *O*-PS from *A. salmonicida* composed of a trisaccharide repeating unit containing Glc (162 Da), ManNAc (203 Da), and RhaOAc (188 Da) [2, 52]. This approach has proven particularly useful for probing the subtle structural differences in monosaccharide composition and functionalities arising across bacterial serotypes.

3.3 CE Analysis of Bacterial Glycosaminoglycan-Like Polysaccharides

3.3.1 General Considerations

Isolation and purification of GAGs from biological samples can be achieved by many different approaches. These include solubilizing the tissue with proteolytic enzymes, or fractionating the mixture by ion-exchange chromatography, gel-filtration columns, or sequential precipitation by organic solvents [55–57]. Currently, GAGs are isolated from a number of biological sources – tissues, cells, and fluids – either from proteoglycans (PGs) extracted with solutions containing chaotropic reagents and detergents or from direct protease digests of sample. These isolated macromolecules are generally quantified by using dye-binding assays or colorimetric reactions, and further characterized by chemical analysis and analytical and semi-preparative approaches.

Based on the information desired, analyses are performed by using either intact polymers or GAGs that are chemically or enzymatically degraded into oligosaccharides. Analysis of intact polysaccharides may give information on the amount,

charge density, molecular mass, and polydispersity of GAG chains. However, GAG structure is traditionally studied by controlled degradation of the chains using specific enzymes or chemical treatments and analysis of the produced disaccharides or oligosaccharides [58]. After treatment with specific enzymes, the products are subsequently either directly separated or derivatized and separated and then subjected to detection and quantification. The enzymes that are mainly used for the degradation of the GAGs belong to the family of polysaccharide lyases acting on the β(1→4) glycosidic linkage between hexosamine and uronic acid residues producing disaccharides and oligosaccharides possessing a double bond between C-4 and C-5 of the uronic acid [58]. This unsaturated uronic acid strongly absorbs ultraviolet light at 232 nm, and this wavelength is used for the quantification of the products. A combination of polysaccharide lyases having different specificities can also be used for the characterization of GAGs from biological samples. If correctly chosen, such a combination of polysaccharide lyases can ensure the nearly complete breakdown of GAGs to disaccharide products for subsequent disaccharide compositional analysis.

3.3.2 CE Analysis of E. coli K4 Polysaccharide

The uropathogenic *E. coli* K4 bacteria synthesizes a capsular polysaccharide with a carbohydrate backbone identical to chondroitin, consisting of [GlcA (β1→3) GalNAc (β1→4)]$_n$ backbone, to which β-fructofuranose units are linked to C-3 of GlcA residues. A rapid, highly sensitive and reproducible CE method (MECC with sodium dodecyl sulfate) has been developed for the determination of disaccharides present in this polysaccharide and its defructosylated product [59]. Following chondroitin ABC lyase digestion of K4 polysaccharide and its derivative, the two products ΔHexA(Frc)-GalNAc and ΔHexA-GalNAc were separated and determined within 20 min using normal polarity and detection at 230 nm (Fig. 3.8). Comparison was made by separation of these two disaccharides in isocratic strong-anion exchange HPLC. A linear relationship was found for the two unsaturated disaccharides over a wide range of concentrations, from approximately 0.5 to 5 μg for HPLC and from approximately 0.06 to 0.3 μg for CE. The CE separation produced a greater detection sensitivity (about ten times greater) than LC. The described methods were used to evaluate the defructosylation process of K4 under drastic acid conditions. Good correspondence was found for the amount of unsaturated disaccharides for the two techniques.

However, this analytical approach first requires treatment of the bacterial polysaccharide with polysaccharide lyases to produce unsaturated oligosaccharide products. A direct CE separation using normal polarity of native K4 and K4d polysaccharides has also been developed [60]. The two polyanions, K4 and defructosylated K4, were separated and readily determined within 30 min on an uncoated fused-silica capillary using normal polarity at 20 kV and detection at 200 nm (Fig. 3.9). A linear relationship was found for the two

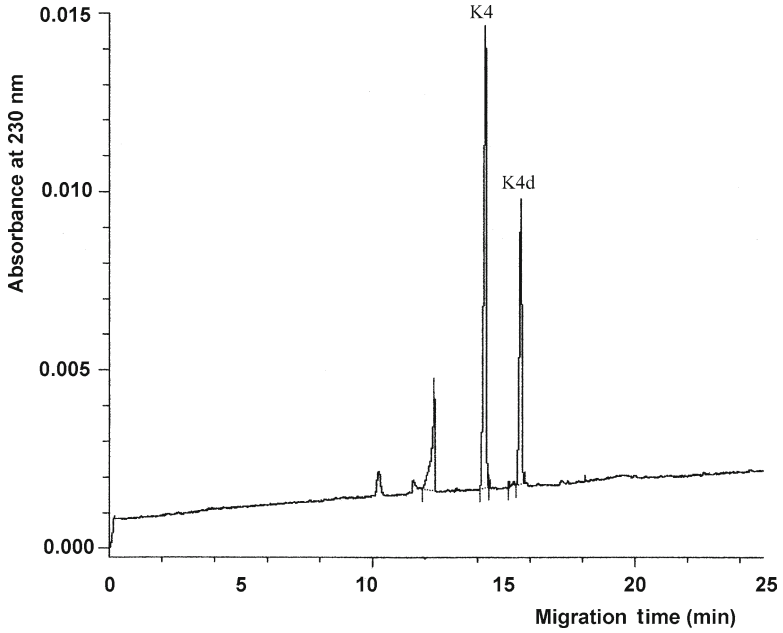

Fig. 3.8 Electropherogram of unsaturated disaccharides ΔHexAFrc-GalNAc from the polysaccharide K4 (K4) and ΔHexA-GalNAc of defructosylated K4 product (K4d) (modified from [59])

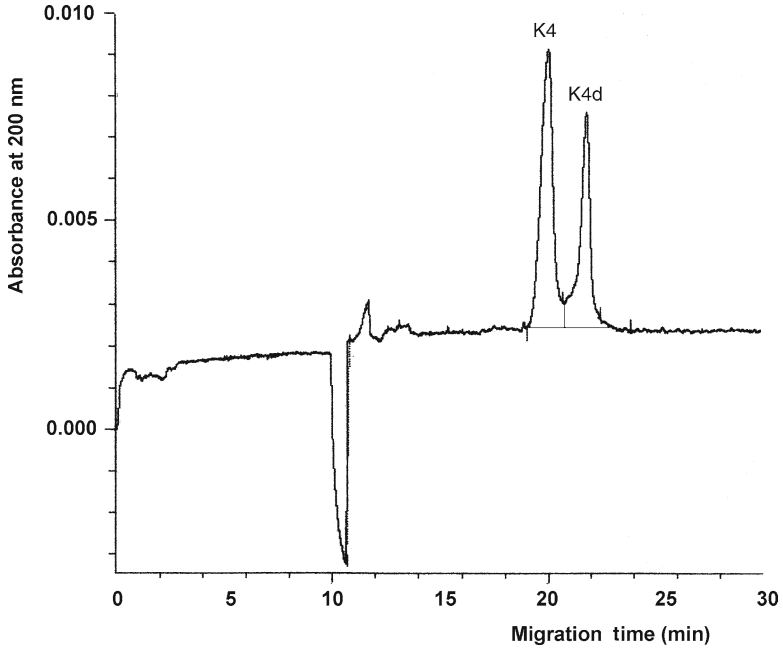

Fig. 3.9 Electropherogram of the intact polysaccharide K4 (K4) and of the defructosylated K4 polysaccharide (K4d) (modified from [60])

polysaccharides over a wide range of concentrations, from approximately 30 ng (0.5 μg/μL) to 210 ng (3.5 μg/μL). The described method was used to evaluate the defructosylation process of K4 under drastic acid conditions.

CE (electrokinetic chromatography with sodium dodecyl sulfate technique) was also applied to the extraction and purification process of the K4 polysaccharide from cultured bacteria in several stages [61]. It proved to be a technique with high resolution and sensitivity in analyzing K4 polysaccharide during its purification, in particular by using a strong anion-exchange resin. This is of paramount importance to monitor the product during the extraction and purification process or to test the purity of the final product. Furthermore, CE was able to verify that the extraction and purification process adopted is not carried out under drastic conditions capable of inducing fructose removal from the polysaccharide backbone.

3.3.3 CE Analysis of E. coli K5 Polysaccharide (Heparosan)

Capillary electrophoresis (electrokinetic chromatography with sodium dodecyl sulfate) was also applied for the determination of another bacteria polysaccharide produced by the uropathogenic *E. coli* K5 [62] (Fig. 3.10). This natural polysaccharide,

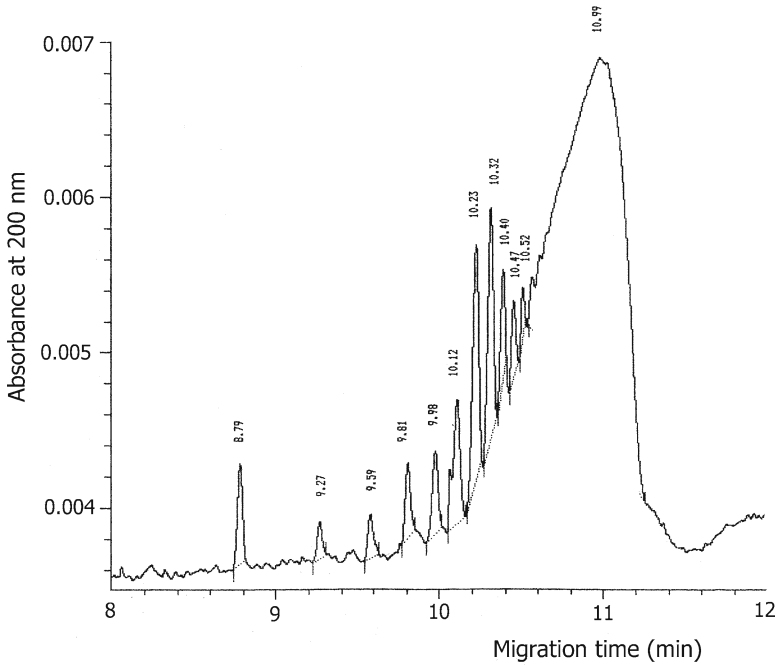

Fig. 3.10 CE electropherogram of the K5 polymer. The K5 polysaccharide species with a migration time of 8.79 min corresponds to a decasaccharide (modified from [62])

having the structure of a desulfo-heparin composed of -4)-alphaGlcUA-(1,4)-alpha-GlcNAc-(1-, was separated and qualitatively and quantitatively determined within 20 min on an uncoated fused-silica capillary using normal polarity at 20 kV and detection at 200 nm. A linear relationship (correlation coefficient greater than 0.99) was found for the polymer over a wide range of concentrations, from approximately 60 to 1,500 ng, with a detection sensitivity of less than approximately 60 ng. Furthermore, this qualitative and quantitative CE approach was applied to the K5 extraction and purification process from cultured bacteria in several stages. CE was also able to separate several molecular species mainly due to the presence of polysaccharides of distinct and increasing mean chain lengths. A linear relationship was found for migration time and log molecular mass of different K5 polysaccharide species, and this model was used to calculate the molecular mass of the main K5 species producing a result of approximately 17,000.

3.4 Conclusion

The separative power of CE reduces pretreatment steps, making it useful for a variety of applications, such as in quality control in the pharmaceutical industry, in disease diagnosis, in glycobiology, in carbohydrate biochemistry and structural characterization, and in the chemical synthesis of oligosaccharides. The informative capacity of on-line and off-line CE-MS (and tandem mass) allows the assessment of the identity of partially unresolved peaks, making this mode of detection particularly advantageous.

The microheterogeneity of the LPS molecules expressed by a broad range of gram-negative bacteria can be characterized by CE-MS and CE-MS/MS. Applications of on-line CE tandem mass spectrometry open the door to elucidating structural detail of isomeric oligosaccharides in complex LPS mixtures. On-line CE-MS also provides additional strategies to improve detection limits by desalting and sample stacking, which has been successfully applied to probe oligosaccharide LPS glycoform populations of bacteria isolated from an in vivo source directly by a MS-based techniques without the need for further passage.

Improvements in CE separation and sensitivity have been achieved by derivatization with suitable charged tags having fluorescence properties. Furthermore, derivatization can aid peak assignment during MS/MS experiments, providing additional information on the structure of the saccharides under investigation. However, CE-MS has some limitations in analysis of complex natural polysaccharides. First, CE, while generally requiring small amounts of samples, requires high concentrations that are often difficult to obtain from biological samples. Second, CE-MS interfaces are not as well developed as the LC-MS interface, and they are available from a limited number of commercial equipment suppliers and often have poor sensitivity. Third, MS often has some limitations in the analysis of charged carbohydrates compared to other biopolymers. In fact, sulfate esters in GAGs are particularly labile, resulting in extensive fragmentation,

making the detection of molecular ions difficult, and the presence of adducts often also complicates analyses.

To overcome low sample loading capacity, different preconcentration strategies have been investigated. It should be pointed out that the nature of samples is the most important factor for choosing an appropriate preconcentration protocol. In the meantime, other preconcentration techniques need to be developed, especially for microscale analysis of LPS samples where the biological matrix is largely creating significant interference in sample analysis. Furthermore, the small injection volumes required by CE allow the development and optimization of analytical methods tailored for each specific sample. This feature allows the maximum exploitation of the CE-MS informative capacity for samples having microheterogeneities and available in small amounts. As a consequence, the most promising applications of on-line but also off-line CE-MS concern the structural characterization of the oligosaccharides generated from biomacromolecules, like complex polysaccharides or macromolecular associations.

Analysis of LPS samples has been done following *O*-deacylation or after mild acid hydrolysis. This results in loss of base or acid labile substituents. Therefore, there is a need to optimize methodologies for the analysis of intact LPS molecules. In the near future, automation and miniaturization is expected for high-throughput structural elucidation of carbohydrates in glycomics, in which the implementation of microfluidic devices and chip-based CE-MS technology will play a central role.

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Chapter 4
Capillary Electrophoresis and Its Microchip Format for the Analysis of Glycosaminoglycans

Kazuaki Kakehi and Yu-ki Matsuno

Abstract Glycosaminoglycans (GAGs), linear negatively charged polysaccharides, possess highly structural heterogeneity caused by modifications of amino and hydroxyl groups. Heterogeneity of molecular sizes is also an important feature. These characteristics participate in many cellular events and physiological and pathological processes. Capillary electrophoresis (CE) is one of the most powerful technologies for characterization of such heterogeneous macromolecules based on its high resolving performance. Glycosaminoglycans are generally analyzed by CE both as the intact form and as their enzymatic digested products. This chapter outlines the CE analysis of GAGs. Furthermore, microchip-based capillary electrophoresis for the analysis of GAGs is also introduced, which affords rapid analysis on a time scale of second. This technology has great potential as a tool for routine assessment of pharmaceutical preparations and for clinical diagnosis.

Keywords Capillary electrophoresis • Microchip • Polysaccharides • Glycosaminoglycans • Hyaluronic acid • Disaccharides • Oligosaccharides

Abbreviations

APTS	1-aminopyrene-3,6,8-trisulfonic acid
CE	capillary electrophoresis
CS/DS	chondroitin sulfate/dermatan sulfate
EDA	ethylenediamine
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GAG	glycosaminoglycan

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N. Volpi (ed.), *Capillary Electrophoresis of Carbohydrates: From Monosaccharides to Complex Polysaccharides*, DOI 10.1007/978-1-60761-875-1_4,

HA	hyaluronic acid
HP	heparin
HS	heparan sulfate
KS	keratan sulfate
LED	light-emitting diode
LIF	laser-induced fluorescence
MC	methylcellulose
ME	microchip electrophoresis
MECK	micellar electrokinetic chromatography
NeuAc	*N*-acetylneuraminic acid
OSCS	oversulfated chondroitin sulfate
PEG70000	polyethyleneglycol
PGs	proteoglycans
PMMA	polymethylmetacrylate
ssDNA	single-stranded DNA.

4.1 Introduction

Glycosaminoglycans (GAGs) are the family of linear, complex, negatively charged polysaccharides formed from repeating disaccharide units of uronic acid (glucuronic acid or iduronic acid) linked to hexosamines (glucosamine or galactosamine) (Fig. 4.1) [1]. Glycosaminoglycans include hyaluronic acid (or hyaluronan, HA), heparin (HP), heparan sulfate (HS), and chondroitin/dermatan sulfate (CS/DS). Keratan sulfate (KS) has galactose instead of uronic acids. GAGs are covalently linked to the core protein through the *O*-glycosidic linkage (some KS forms *N*-glycosidic linkage) to form proteoglycans (PGs), except for HA, which is considered to be present as free form. Although the backbone structures of GAGs are simple and linear polysaccharides, they acquire considerable structural variety by extensive modifications such as sulfation and uronate epimerization. Such modifications afford wide variety of domain structures with unique biological activities [2, 3].

Glycosaminoglycans are ubiquitously found on the cell surface and in extracellular matrices of animal tissues, and are not only structural component in connective tissue but also correlate with many cellular events and physiological and pathological processes [4, 5]. Cell-surface GAGs interact with a number of proteins such as growth factor families, growth factor receptors, cytokines, and chemokines [6, 7]. The binding of fibroblast growth factors (FGFs) to heparan sulfate proteoglycans is a typical example of GAGs–protein interaction. The binding is necessary for signaling by forming a trimolecular complex comprising the receptor, heparan sulfate, and FGF [8].

It has been believed that specific patterns of sulfation on the GAG backbone are necessary to mediate interactions with proteins. Therefore, structural analysis of GAGs and understanding of the interaction between GAGs and proteins are considered to lead potential therapeutic applications.

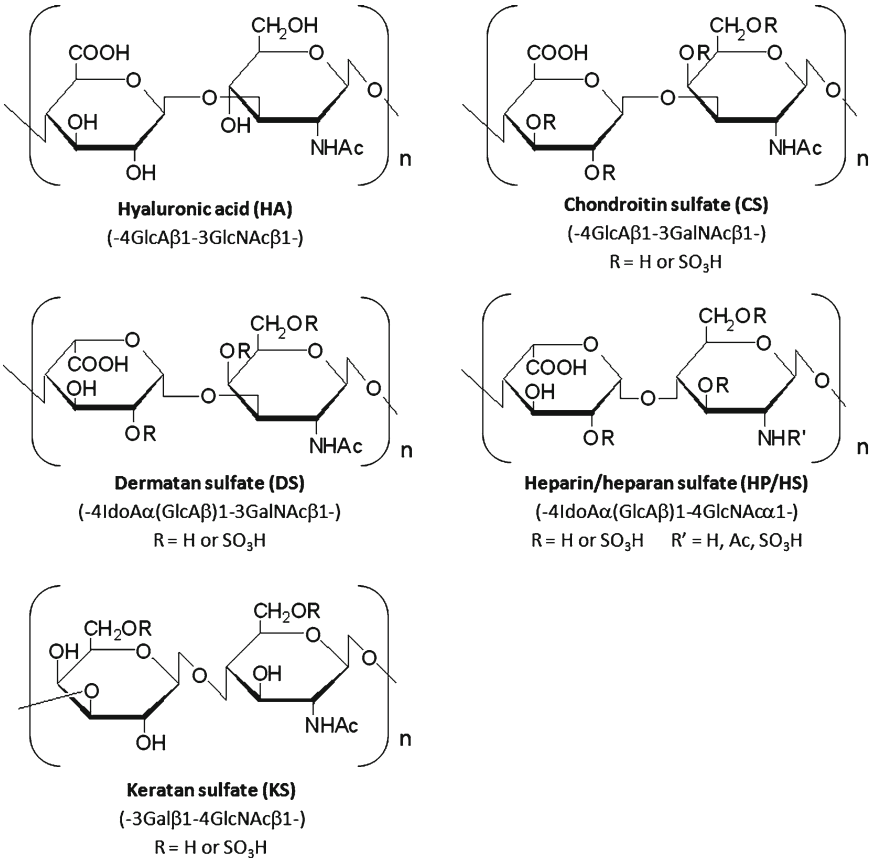


Fig. 4.1 Repeating disaccharide structures for glycosaminoglycans

Glycosaminoglycans, the highly negative-charged molecules, are good targets for analysis by electrophoresis approaches. Cellulose acetate membrane electrophoresis has been widely used for a long time for its separation and identification, and it is still used as a routine analytical method. Gel-based electrophoresis methods for GAGs analysis have also been reported [9–11]. Especially, capillary electrophoresis (CE) is a powerful tool for the analysis of GAGs and their enzymatic digested products because of its high resolving performance and wide variety of applications [12]. Microchip-based capillary electrophoresis, which is often simply called microchip electrophoresis (ME), has been recently applied to the rapid analysis of GAGs [13–16], and is an attractive technology in terms of rapidness, easy operation, and wide applicability to the analysis of various biological compounds. In addition, ME has great potential as a tool for routine analysis of pharmaceuticals, environmental analysis, and clinical diagnosis [17, 18].

This chapter describes an outline of CE analysis of GAGs, and discusses some recent works on the rapid analysis of GAGs using ME.

4.2 CE Analysis of GAGs

4.2.1 Analysis of Hyaluronic Acid and Its Oligosaccharides

Hyaluronic acid (HA) is a macromolecular substance having molecular masses of 10^5–10^7, but often exists as small fragments or oligosaccharides. It is composed from heterogeneous molecules having different numbers of disaccharide units. But the disaccharide units (-4GlcAβ1–3GlcNAcβ1-) are quite simple and not modified with sulfate or other functional groups. HA is used for various therapeutic purposes such as maintaining viscoelastic properties in synovial fluids and surgical ocular operations. The biological functions of HA are closely related to the molecular mass; thus, it is important to estimate the molecular mass and the amount of HA in biological samples or pharmaceutical preparations. HA samples having different molecular masses were quantitatively determined by CE in a bare fused silica capillary using a slightly acidic buffer (pH 4.0), and were successfully observed at different migration times in a buffer containing pullulan as a sieving matrix [19]. The different migration times and the peak broadening of HA samples were based on the size exclusion effect of the entangled matrix of pullulan. It is considered that pullulan makes up relatively "loose matrix" compared to those formed by other polymers. The method using the pullulan matrix is suitable for the discrimination of intact HA samples having different molecular size distributions [19]. Quantitative determination of HA using this method is also achieved, and calibration curves showed good linearity from 0.01 to 3.3 mg/mL for all HA samples from different sources.

Micellar electrokinetic chromatography (MEKC) using borate buffer containing sodium dodecyl sulfate (SDS) is often employed in CE analysis of HA and its oligosaccharides [12, 20], and applied to the separation of relatively small-size oligosaccharides (~30 mer) (Fig. 4.2a)

Addition of SDS in the running buffer affords the separation based on the difference in hydrophobicity of each oligosaccharide, and improves the resolution of peaks. The method is useful for monitoring the reaction course of HA by digestion with hyaluronidase [21, 22]. A mixture of HA oligosaccharides composed of more than 100 disaccharide units has been successfully separated in a buffer containing polyethyleneglycol (PEG70000) as a sieving matrix (Fig. 4.2b) [23]. In this mode of separation, HA octasaccharide is observed at the earliest migration time, and oligosaccharides having higher molecular masses than that of decamer are observed in the order of their molecular masses. In contrast, oligosaccharides smaller than octamer migrate in the reverse order of their molecular masses (Fig. 4.2c).

In the analysis of oligomers having different molecular masses in a buffer containing a neutral polymer, larger oligomers were assumed to migrate at a smaller velocity on the basis of the Ogston model or the reputation model [24]. However, these theories do not explain the reversal of the migration times of these oligomers. The authors indicate that this unusual migration pattern is closely related to the stereochemical structures of HA oligosaccharides and the fastest migrating oligosaccharide, octamer, is considered to be the minimum unit that forms the regular

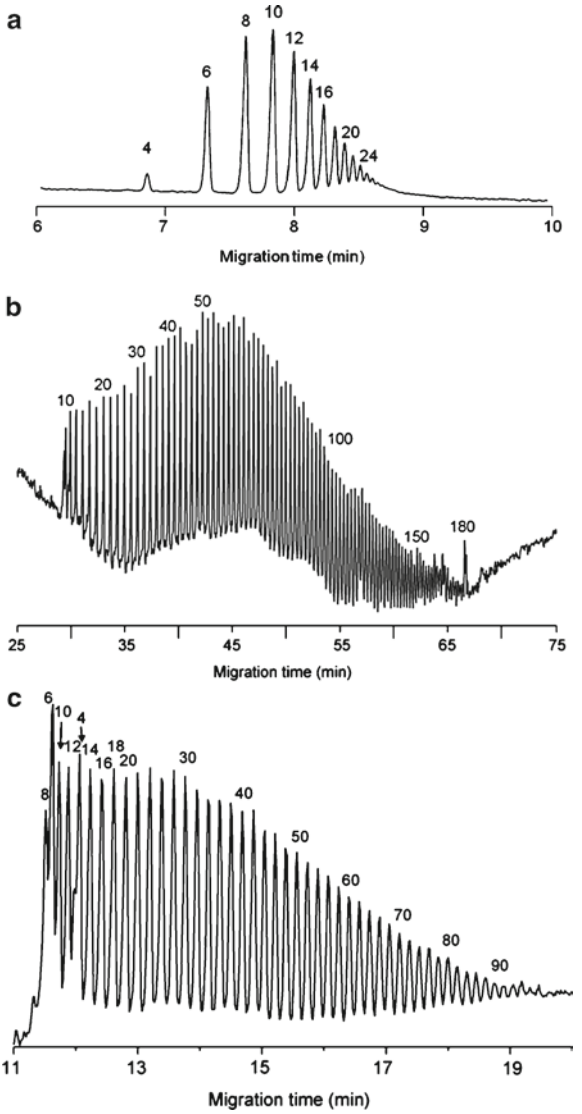


Fig. 4.2 Separation of HA oligomers (**a**) by CE using a buffer containing SDS (unpublished data). Separation of HA polymers (**b**) and oligomers (**c**) by CE using a buffer containing 10% PEG70000. The numbers indicates the degree of polymerizations. (Modified from ref. [23], with permission)

three-dimensional structure required for the biological function of HA oligosaccharides [23]. In the same report, the reverse order of migration of α2,8-linked *N*-acetylneuraminic acid (NeuAc) oligomers is also shown. The transition point of the reversal migration in NeuAc oligomers is a pentamer, and this

observation is also well correlated with their biological functions [23]. Mohanty et al. [25] reported similar results, in which anomalous migration is observed in the analysis of short sequences of single-stranded DNA (ssDNA) by polyacrylamide gel electrophoresis. They proposed that the electrophoretic mobility of small oligomers of ssDNA is described by $\mu = \mu 0 \exp(-R/\xi_{mesh})$

where μ is the mobility of the analyte ion, μ_0 is the mobility in free solution, R is the helix size, and ξ_{mesh} is the mesh spacing formed by the polymer matrix. However, the transition points were independent of the concentration of PEG for hyaluronic acid and NeuAc oligomers. The rigidity and conformation of the carbohydrate chains may be other factors responsible for such anomalous migration.

Hyaluronan oligosaccharides labeled with 1-aminopyrene-3,6,8-trisulfonic acid (APTS) were also well separated using a capillary filled with polyacrylamide gel [26]. The authors proposed the presence of certain conformational states of HA oligomers that are observed as additional peaks of the regular oligomers (referred to as the "satellite peaks"). This observation indirectly supports the complex and unusual rheological and viscoelastic properties of HA.

4.2.2 Analysis of Sulfated GAGs

Glycosaminoglycans other than HA are extremely heterogeneous with respect to both their molecular sizes and substitution patterns with sulfate groups. Direct analyses of intact sulfated GAGs have been reported, but most of CE analyses of sulfated GAGs are those of unsaturated disaccharides after digestion with various lyases.

4.2.2.1 Intact Sulfated GAGs

In general, intact GAGs have not been successfully analyzed with high sensitivity by CE because of the absence of a chromophore/fluorophore in GAG molecules. Several GAGs have been analyzed by CE with reverse polarity in an acidic buffer at pH 4.5 and at 240 nm based on the formation of copper (II) complex [27]. The copper (II)–GAG complex, which shows optical properties permitting ultraviolet (UV) detection, was applied to sensitive detection of GAGs by CE. Detection of the copper (II)–HP complex is the most sensitive among the GAGs tested, permitting the analysis of as little as 10^{-9} g as the injected amount. The copper (II)–GAG complexes show a broad peak having a distinctive migration time (Fig. 4.3). However, it is difficult to separate and discriminate the mixture of these different GAGs.

Partial separation of HP has been achieved using a buffer containing ethylenediamine (EDA) as an ion-pairing reagent (Fig. 4.3e) [28]. EDA interacts with the negative charges of the HP molecule selectively and reduces the electrophoretic mobility of HP. Partial separation of the extremely complex polysaccharides mixture was achieved.

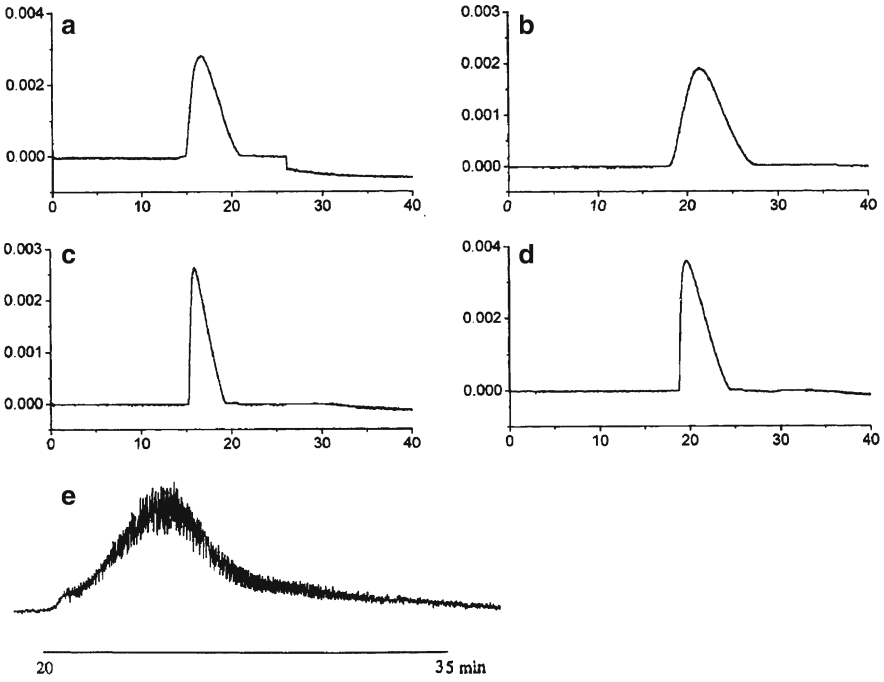


Fig. 4.3 Electropherograms of the copper (II)–GAG complexes. (**a**) HP, (**b**) HS, (**c**) CSA, (**d**) DS. (**e**) Partial separation of HP using a buffer containing EDA (modified from refs. [27, 28], with permission)

In the beginning of 2008, hundreds of cases of anaphylactic reaction as adverse effects were reported by the presence of contaminating oversulfated chondroitin sulfate (OSCS) in certain lots of HP pharmaceutical preparations [29]. The presence of OSCS in HP preparations was demonstrated by nuclear magnetic resonance (NMR) and CE methods proposed by the US Food and Drugs Administration (FDA). These methods also revealed the presence of significant amount of DS in several lots. Since then, several studies pursuing the determination of OSCS and DS in HP preparations have been reported using various technologies [30–32]. The conditions for CE analysis of HP preparations to detect these contaminating materials have been improved by some research groups [33–35], because the CE-based method proposed by FDA provides relatively poor resolution between OSCS and HP (Fig. 4.4a).

Kakehi's group [35] successfully separated HP and OSCS in a bare fused silica capillary using a high molarity buffer containing 1,000 mM Tris-phosphate buffer (pH 3.5). Due to high concentrations of the electrolyte, large current and high applied voltage hampered good reproducibility. Therefore, a narrow capillary (25 μm) and a constant current mode were used in the analysis. Although sensitivity of the CE method becomes somewhat lower due to short light path, the sensitivity is still higher than NMR method.

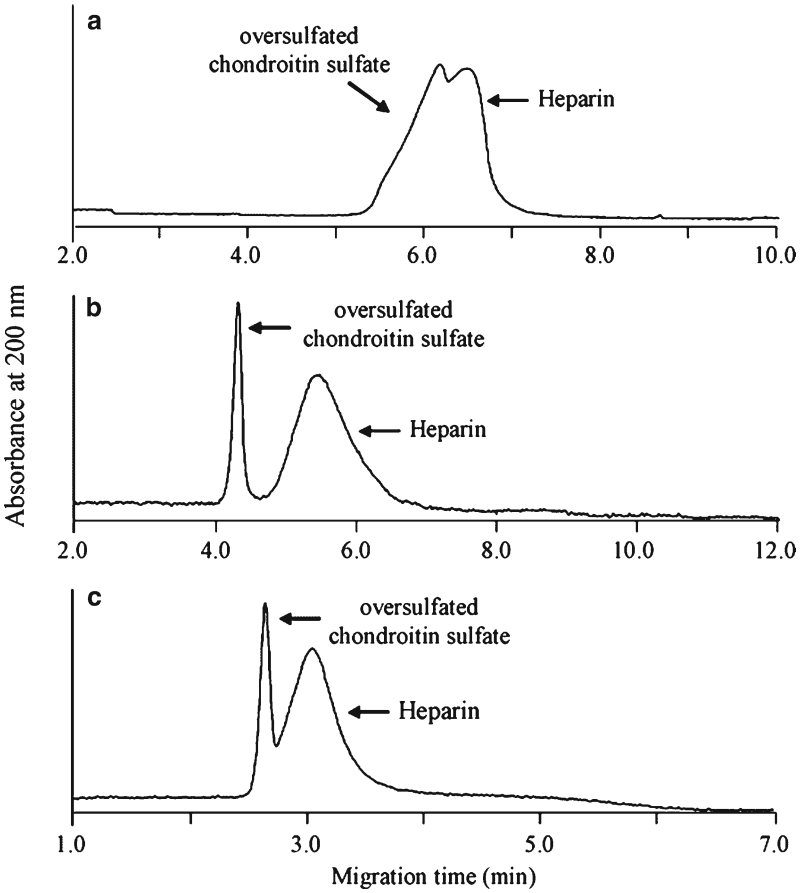


Fig. 4.4 Capillary electrophoresis analysis of a contaminated heparin sodium preparation by (**a**) FDA method, (**b**) OSCS method, and (**c**) DS method (from Kakoi et al. [35] with permission)

The contaminated lot of HP sodium preparation that actually caused the adverse reactions was analyzed by the optimized conditions, and the lot clearly showed a peak due to OSCS at 4.3 minutes (Fig. 4.4b). As shown in Fig. 4.4a, the FDA method showed incomplete resolution of HP and OSCS. Separation of DS and HP was also optimized using 100 mM Tris–phosphate buffer (pH 2.5) [35], although the DS peak was not observed in this preparation (Fig. 4.4c). Thus, the method will be an alternative to that by NMR or ion exchange chromatography.

4.2.2.2 Unsaturated Disaccharides

Analysis of the unsaturated disaccharides is one of the important approaches for the characterization of GAGs. To date, more than 50 important works on the CE analysis

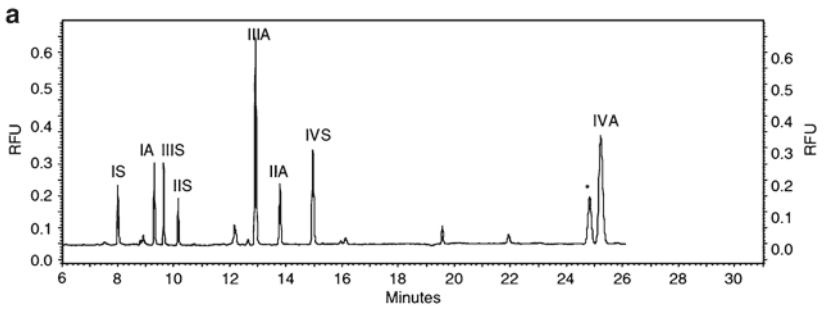
of GAGs-derived unsaturated disaccharides have been reported. The early papers on the separation of GAG-derived unsaturated disaccharides by CE appeared in 1991 [36, 37]. The analyses were performed using a normal polarity with UV detection at 232 nm, where the unsaturated disaccharides showed strong absorption. The unsaturated disaccharides have been also analyzed as some derivatives to improve the sensitivity [38–40]. 2-Aminoacridone (AMAC) is the most popular fluorescent tag for the analysis of GAGs-derived unsaturated disaccharides with laser-induced fluorescent (LIF) detection, because the tag is easily removed by the extraction with organic solvent such as chloroform [39, 41–44]. The methods were reported to be an alternative to the conventional approaches using high-performance liquid chromatography (HPLC) or slab gel electrophoresis. Improved workup for the disaccharides analysis using CE was recently reported [45]. These improvements include optimization of labeling reaction (i.e., reductive amination and cleanup procedure), and separation conditions. The original protocol can detect eight of the 12 HP/HS disaccharides, because HS disaccharides IH-IVH have an unsubstituted amino group at $GlcNH_2$ residues, which show poor recovery in derivatization (Fig. 4.5a). In contrast, 11 of the 12 disaccharides can be detected efficiently using the optimized method (Fig. 4.5b).

The IVH disaccharide is still absent, possibly due to the fact that it is a twitterion and the amine may be protonated, causing failure to migrate through the charged capillary. This report also showed robust and reproducible separation of ten CS/DS disaccharides and application to some biologically relevant proteoglycans from small samples of intact tissue. This improved CE-LIF method enables disaccharide quantification as low as 1 pmol [45].

4.3 Microchip Electrophoresis of GAGs

Microchip electrophoresis is a microchip-based capillary electrophoresis performed in a microchannel with a dimension of 10–100 μm fabricated on a small chip. Microchip electrophoresis was the earliest format of the "microfluidics" when it was first introduced [46], and has emerged as a powerful tool for rapid analysis of various biological compounds such as amino acids, peptides, proteins, and carbohydrates as well as nucleic acids [47–53]. This technology has been especially extended to the routine analysis of nucleic acids to reduce the analysis time and the overall cost required for the analysis. This has led to the production of some commercial instruments instead of CE or slab gel electrophoresis. In addition, micrototal analysis systems (μTAS, also called "lab on a chip") are growing rapidly and attracting much attention, because this concept promises to integrate the many steps required for enzyme reactions or immunoassays on a planar chip [54]. The technology has great potential as a tool for routine analysis of minute amount of clinical samples [55].

Heat dissipation in chip format is much better than that in a capillary format. Thus, a higher electric field can be applied across a much shorter separation channel, and microchip electrophoresis can be performed much faster on a time scale of second.



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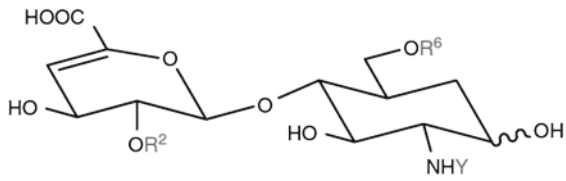

	Disaccharide	R^2	R^6	Y
IS*	ΔUA2S-GlcNS6S	SO_3	SO_3	SO_3
IIS*	ΔUA-GlcNS6S	H	SO_3	SO_3
IIIS*	ΔUA2S-GlcNS	SO_3	H	SO_3
IVS*	ΔUA-GlcNS	H	H	SO_3
IA*	ΔUA2S-GlcNAc6S	SO_3	SO_3	Ac
IIA*	ΔUA-GlcNAc6S	H	SO_3	Ac
IIIA*	ΔUA2S-GlcNAc	SO_3	H	Ac
IVA*	ΔUA-GlcNAc	H	H	Ac
IH	ΔUA2S-GlcN6S	SO_3	SO_3	H
IIH	ΔUA-GlcN6S	H	SO_3	H
IIIH	ΔUA2S-GlcN	SO_3	H	H
IVH*	ΔUA-GlcN	H	H	H

Fig. 4.5 CE-LIF electropherogram of 12 HP/HS disaccharides. (**a**) The original AMAC derivatization protocol. (**b**) The optimized AMAC derivatization protocol. (**c**) The structures of 12 HP/HS disaccharides (modified from ref. [45] with permission)

The basic considerations, principles, and the technological details are discussed elsewhere [56, 57]. Here, we introduce an application of microchip electrophoresis to the rapid analysis of GAGs.

4.3.1 Analysis of Di- and Oligosaccharides

Electrophoresis analysis of GAGs-derived di- and oligosaccharides on microchip format was first reported in 2004 using a commercially available instrument designed for routine analysis of DNA with a light-emitting diode (LED) detector for nucleic acids staining dyes [13]. The chip made of polymethylmethacrylate (PMMA) having a simple cross channel was used (Fig. 4.6)

Two-step electrokinetic injection was employed: (1) the sample flow is pulled into injection channel by an electric field; (2) the sample plug in the cross zone is brought to separation channel when the voltage is switched, and separation starts immediately. To detect GAG-derived di- and oligosaccharides in the apparatus specialized for DNA analysis (Excitation (Ex): 518 nm, Emission (Em): 605 nm), these oligosaccharides were derivatized with AMAC. AMAC shows good sensitivity even at this wavelength. In addition, the excess amount of the reagent can be easily removed by extraction with chloroform after derivatization by reductive amination in the presence of $NaBH_3CN$. AMAC-labeled oligosaccharides derived from HA and chondroitin sulfates have been separated rapidly within a few minutes by microchip electrophoresis using a channel of a very short separation path (30 mm) with excellent resolution. This rapid separation has been successfully applied to monitoring the enzyme reaction course such as hyaluronidase [13].

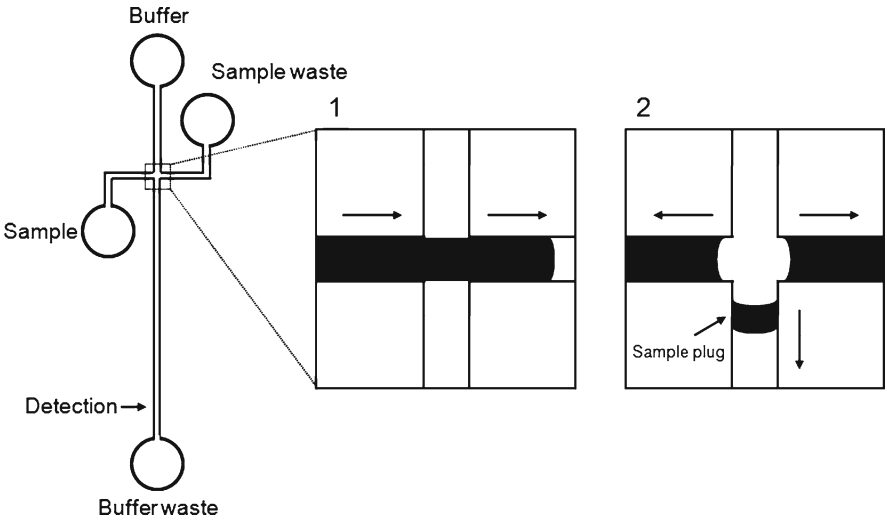

Fig. 4.6 Electrokinetic injection for microchip electrophoresis

The analysis of AMAC-labeled unsaturated disaccharides derived from chondroitin sulfates has also been performed. The mixture of Δdi-4S and Δdi-6S (monosulfated unsaturated disaccharide isomers) was resolved in 0.1 M Tris–borate buffer at pH 8.0 within 100 s, although baseline resolution could not be achieved [13]. Separation is probably due to competitive complex formation between carbohydrates/Tris and borate ions. Several CS samples were confirmed by this rapid separation method. AMAC-labeled unsaturated disaccharides derived from CSA, CSC, DS, and CSD were analyzed (Fig. 4.7)

CSA showed an abundant peak of ΔDi-4S at 100 s, with a small peak of ΔDi-6S at 95 s (Fig. 4.7a). CSC gave ΔDi-6S with a small peak of ΔDi-4S (Fig. 4.7b). DS showed only ΔDi-4S as the monosulfated unsaturated disaccharide (Fig. 4.7c). CSD gave almost equal amount of ΔDi-6S and ΔDi-4S at around 100 s (Fig. 4.7d). All CS samples also showed a peak due to unsaturated disaccharide having two sulfate groups at about 70 s. Especially, CSD gave a large amount of ΔDi-diS_D unit (Δdi-2S6S). This rapid detection could be performed even at 10 ng/mL of CSA at the signal-to-noise ratio of about 3 [13].

Zhang et al. [15] improved the separation of isomers of AMAC-labeled monosulfated unsaturated disaccharides (ΔDi-6S, ΔDi-4S, and ΔDi-UA2S) by ME using 1,4-dioxane as an additive in the running buffer and a combination of increasing the electric field and field amplified sample stacking (Fig. 4.8a). A mixture of monosulfated unsaturated disaccharide isomers was baseline-separated within 75 s. The authors

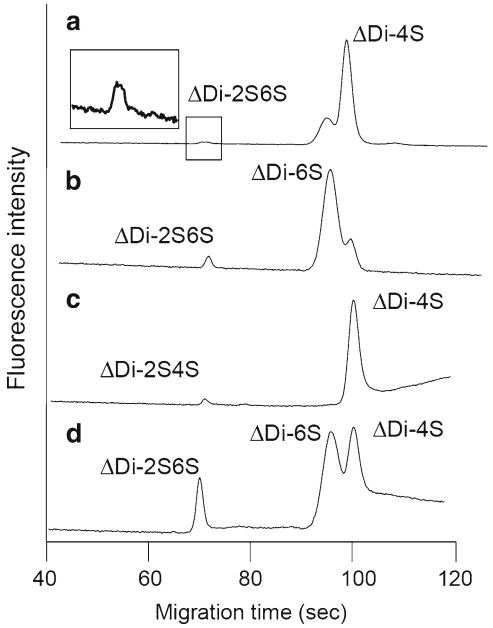

Fig. 4.7 Analysis of unsaturated disaccharides after digestion of chondroitin sulfates followed by labeling with AMAC. (**a**) CSA, (**b**) CSC, (**c**) DS, (**d**) CSD (from Matsuno et al. [13], with permission)

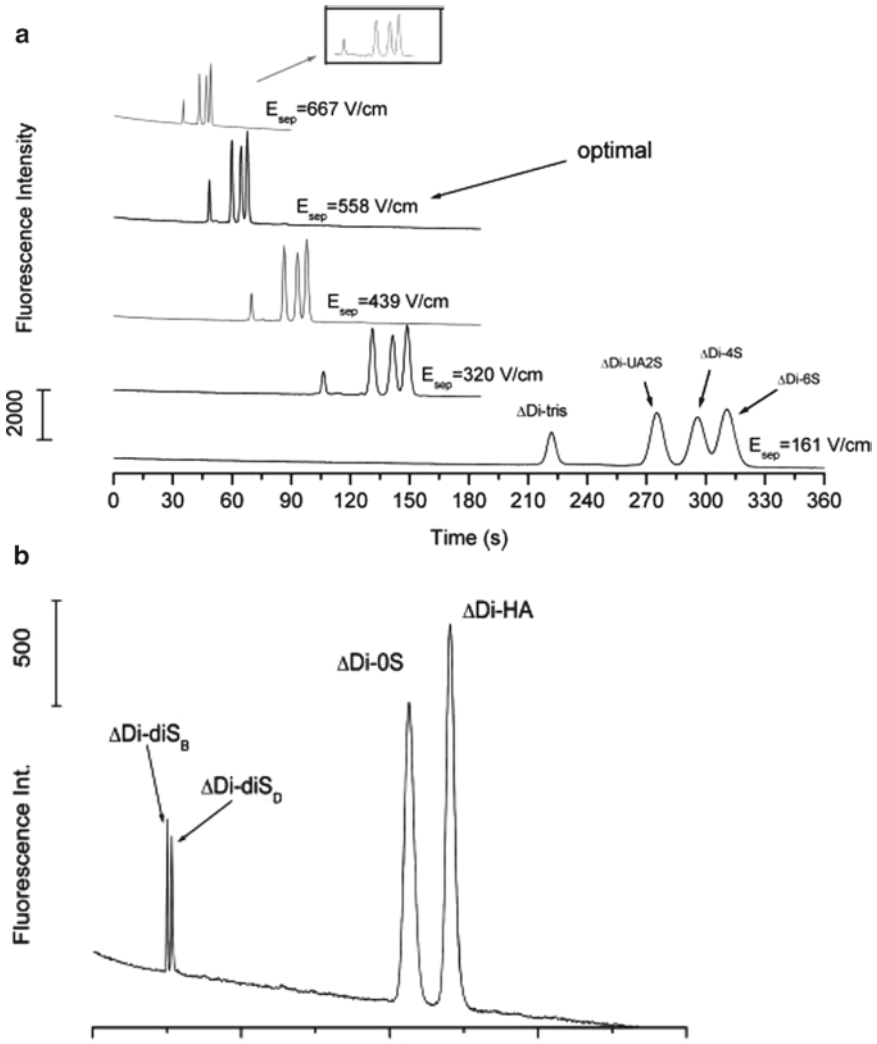

Fig. 4.8 Separation of isomers of AMAC-labeled (**a**) monosulfated unsaturated disaccharides (ΔDi-6S, ΔDi-4S, and ΔDi-UA2S), and (**b**) nonsulfated disaccharides (ΔDi-HA and ΔDi-0S) (modified from refs. [15, 16], with permission)

explained that the good separation of these isomers was based on the solvation effects of 1,4-dioxane to different steric conformation of these isomers. The addition of methylcellulose (MC) also contributes to high resolution due to suppression of electroosmotic flow (EOF) and analyte adsorption to the inner surface of the separation channel [15]. Furthermore, they enhanced the resolution of disulfated (ΔDi-diS_B and ΔDi-diS_D) and nonsulfated (ΔDi-HA and ΔDi-0S) isomers of unsaturated disaccharides using a PMMA chip modified with poly(vinylalcohol) [16] (Fig. 4.8b).

4.3.2 *Analysis of GAG Polysaccharides*

Rapid analysis of intact GAGs by ME was also developed [14], using a commercially available instrument for the analysis of DNA. Fluorescent dyes used for DNA detection were applied to the analysis of native GAGs. DNA forms fluorescent complexes by intercalation with such dyes. The detection of native GAGs using the intercalators seems to be a similar method for DNA detection. However, in the case of GAGs detection, the authors indicate that the molecular size of these dyes and their alkyl groups seem to be important for development of fluorescence [14].

Hyaluronic acid samples from three different sources were analyzed in the buffer containing ethidium bromide by ME, and the results were compared with those obtained by conventional cellulose acetate membrane electrophoresis (Fig. 4.9) The buffer used for ME is 0.1 M Tris–acetate buffer (pH 7.5) containing 0.001% ethidium bromide and 1% polyethyleneglycol 70,000 (PEG70000). In the case of analysis of HA, the addition of PEG70000 into the running buffer is effective in obtaining clear peaks of HA. This is probably due to the reduction of the hydrophobic interaction of HA to the inner wall of the microchannel on the PMMA chip.

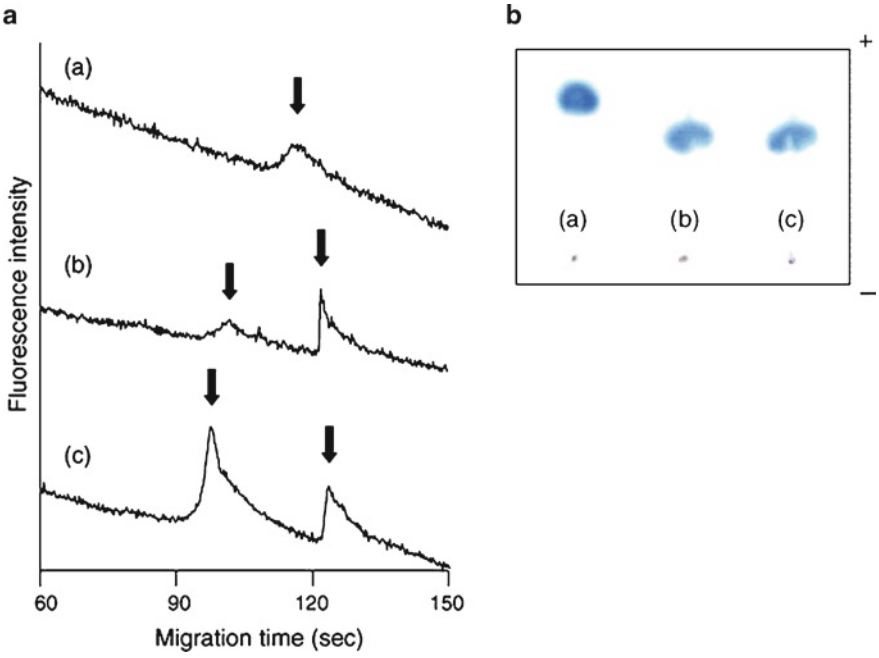

Fig. 4.9 Analysis of HA preparations. (**a**) Microchip electrophoresis: (a) low molecular mass HA (40,000–60,000 Da) derived from pig skin, **(b)** HA from rooster comb having molecular size of 800,000 Da, and (c) HA from *Streptococcus zooepidemicus* having molecular size of higher than 1,000,000 Da. *Arrows* indicate the peak of HA. **(b)** Analysis by cellulose acetate membrane electrophoresis (from Matsuno et al. [14], with permission)

The low molecular mass preparation of HA from pig skin (about 40,000–60,000 Da) showed a small peak at 110 s (Fig. 4.9A[a]). In contrast, high molecular mass HA preparations from rooster comb (about 800,000 Da) and from *Streptococcus zooepidemicus* (higher than 1,000,000 Da) showed two peaks at 95 and 125 s (Fig. 4.9A[b] and [c], respectively). The peak observed at 95 s was more abundant in HA from *S. zooepidemicus* than that observed in HA from rooster comb. However, these HA preparations showed a single spot when analyzed by cellulose acetate membrane electrophoresis, and the low molecular mass HA from pig skin migrated faster than others, probably because of low viscosity (Fig. 4.9B).

These observations can be explained by the following suggestive reports. Computer simulation and energy calculation indicate that HA molecules form a twofold helix [58, 59]. These observations indicate that the intercalator molecules occupy the positions in the hydrophobic cavity of the helix and show enhancement of fluorescence. Low-molecular-mass HA preparation (i.e., HA preparation having low viscosity) occasionally shows single filaments, but HA of high molecular masses forms networks and shows an irregular honeycomb structure under observation using rotary shadowing and electron microscopy [58]. Therefore, HA molecules of low molecular masses derived from pig skin showed only a small peak at 110 s. In contrast, HA molecules having larger molecular masses contain a network form as well as single filaments in solution, and showed two peaks, as shown in Fig. 4.9A(b) and (c). A large peak at 95 s as well as a smaller one at 125 s suggested that both HA preparations derived from rooster comb and *S. zooepidemicus* have two different molecular forms, as reported by Scott et al. [58].

Some typical GAGs were also analyzed by this technique (Fig. 4.10), and their detection limits are summarized in Table 4.1. Heparin was observed at about 60 s (Fig. 4.10a), and a solution at 0.25 mg/mL was detected with signal to noise ratio of about 3. A preparation of low molecular HP was also observed at about 60 s (data not shown). Sensitivity was not as high as that observed for HP, and the detection limit was 1.0 mg/mL, with a signal-to-noise ratio of about 5. This observation on low sensitivity of low molecular heparin is well correlated with that observed in the analysis of HA. HS was observed at 80 s (Fig. 4.10b). The slow migration was due to the lower content of sulfate groups than HP, and the broad peak indicated that carbohydrate chains of HS were highly heterogeneous. Preparations of CSA, CSC, and DS were also analyzed in a similar manner and observed at about 65 s (Fig. 4.10c–e). Detection limits of CSA, CSC, and DS were 0.1, 0.1, and 0.2 mg/mL, respectively.

The present microchip device requires 10 μL of sample solution for analysis. Therefore, sensitivity was at the microgram level, which was comparable with that using cellulose acetate membrane. This technology is also applicable to the rapid analysis of some acidic polysaccharides such as colominic acid, dextran sulfate, and fucoidan. Detection limits of these acidic polysaccharides were also 1.0–2.0 μg as the injected amount [14].

Figure 4.11 shows the rapid analysis of some pharmaceutical HP preparations on microchip. Preparations (a) and (b) are the products from pig intestine, and the preparation (c) is a low-molecular-weight HP preparation. Preparations (a) and (b) gave incompletely resolved two peaks at 60 and 65 s, but the low-molecular-weight

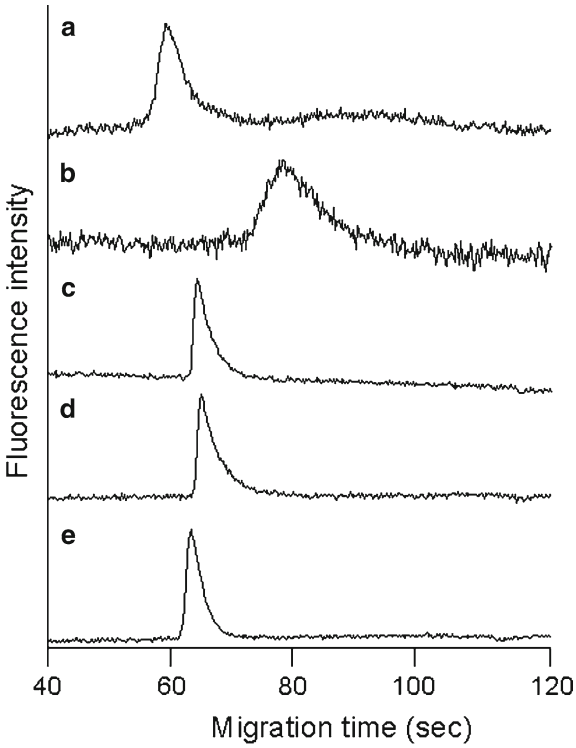

Fig. 4.10 Microchip electrophoresis of typical sulfated GAGs. (**a**) HP, (**b**) HS, (**c**) CSA, (**d**) CSC, and (**e**) DS were analyzed in 0.1 M Tris–HCl buffer containing 0.001% ethidium bromide (unpublished data)

Table 4.1 Lower-detection limit of GAG samples

GAGs	Origin	Detection limit (μg/10 μL)
HA	Pig skin	10
HA	Rooster comb	1.0
HA	*S. zooepidemicus*	1.0
HP	Porcine intestine	2.5
HP (low Mw)	Porcine intestine	10
HS	Bovine kidney	5.0
CSA	Whale cartilage	1.0
CSC	Shark cartilage	1.0
DS	Pig skin	2.0

preparation showed a peak at 60 s. However, in the analysis by cellulose acetate membrane electrophoresis, preparations (a) and (b) were observed as single spot at the same position with that of a standard HP sample. These results indicate that HP preparations (a) and (b) have higher heterogeneities than low-molecular-weight

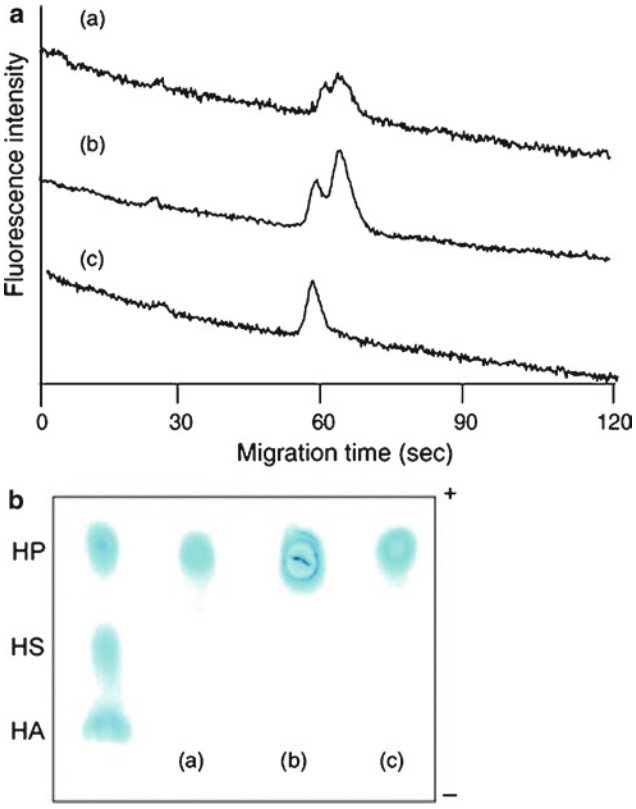

Fig. 4.11 Analysis of pharmaceutical preparations of HP. (**a**) Microchip electrophoresis. (**b**) Cellulose acetate membrane electrophoresis. (a) and (b) are HP preparations from pig intestine, and (c) is the low-molecular-weight HP preparations

heparin preparations. From these observations, rapid analysis of GAGs using ME will be a useful tool for routine analysis of pharmaceutical preparations.

4.4 Conclusion

This chapter outlined the CE analysis of GAGs, and then introduced the rapid analysis of GAGs using microchip electrophoresis with some examples from selected papers. In the past two decades, CE has been demonstrating its usefulness for GAGs analysis in terms of resolution, sensitivity, and throughput. As mentioned above, GAGs are extremely heterogeneous macromolecules, and separation of native (intact form) GAGs is still a challenging task even by CE. However, it is clear that CE is the most powerful technology for the separation and characterization of GAGs at present. Furthermore, recent advances in hyphenation studies of

CE and mass spectrometry are also promising for the analysis of GAGs, although the topic is not included in this chapter. The use of mass spectrometry combined with CE is one of the means for facilitating the characterization of GAGs.

Microchip electrophoresis is promising as a tool for the routine analysis of pharmaceutical preparations and for clinical diagnosis because of its rapidness, easy operation, and cost saving. Although the analysis of GAGs by ME for practical use is still in the early stages of development, the applicability and advantages of this technology have been demonstrated, as shown in this chapter.

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Chapter 5
Extracellular Polysaccharides in Microbial Biofilm and Their Influence on the Electrophoretic Properties of Microbial Cells

Filip Ruzicka, Marie Horka, and Veronika Hola

Abstract The surfaces of biofilm-positive microorganisms are usually covered with biofilm-specific extracellular polysaccharide substances that play a key role in a biofilm formation and function [1,2]. The presence of this substance on the surface can affect the physicochemical properties of the bacterial cell, including the cell-surface hydrophobicity and surface charge. The differences in the surface charges lead to the different isoelectric points and the different electromigration characteristics of biofilm-positive and biofilm-negative cells. For this reason, the use of the capillary electrophoresis, especially capillary isoelectric focusing (CIEF) and capillary zone electrophoresis (CZE) [3,4], appears to be the appropriate method for the detection of biofilm positivity in a particular strain, as well as for the separation of biofilm-positive and biofilm-negative strains.

Keywords Capillary electrophoresis • Capillary isoelectric focusing • Capillary zone electrophoresis • Microbial biofilm • Bacteria • Polymeric matrix • *Pseudomonas* biofilm • Candida biofilms • Lactobacilli

Abbreviations

CE	capillary electrophoresis
CIEF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
EPM	extracellular polymeric matrix
HOPAB	polyethyleneglycol 3-(2-hydroxy-5-*n*-octylphenylazo)-benzoate
LIF	laser-induced fluorescence
PB-PEG	polyethyleneglycol 4-(1-pyrene)-butanoate
PEG	polyethyleneglycol
PEO	poly(ethylene oxide)

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pI	isoelectric point
PIA	polysaccharides intercellular adhesion
PNAG	poly-*N*-acetylglucosamine
PS/A	polysaccharides/adhesion
SAA	slime-associated antigen

5.1 Biofilm: The Mode of Bacterial Life

In addition to the planctonic way of living, microbes are able to adhere to surfaces and to form organized communities, a so-called biofilm, which colonize surfaces available in nature. These communities are beneficial to humans as microflora colonizing surfaces of the human body. They also form an integral part of many biotechnologies, including industrial production and sewage water treatment [5]. But the growth of a biofilm form brings many problems. In industry, the problems are related to the corrosion of industrial structures. The developed biofilm is the cause of the turbulent flow of liquid in pipelines. It also reduces the internal diameter of these pipelines. In heat exchangers it forms a heat-insulating layer. Biofilms are also a s erious problem in waterworks and drinking water distribution systems, where these biofilms can become a source of pathogenic bacteria (e.g., *Legionella, Mycobacteri`um, Vibrio, Aeromonas*) 6].

In medicine, biofilms are investigated, especially in connection with dental plaque [7], and the formation of the biofilm focuses on the surfaces of implants or biological surfaces (e.g., native valve endocarditis). These formations are the sources of serious chronic infections, and a biofilm is considered a key virulence factor [8–10]. The growth of the biofilm form facilitates the processes of adhesion and colonization of microbial cells. It also protects the cells from attack by the immunity system as well as from the effect of antibiotics. Therefore, it is difficult to eradicate these formations through conservative therapy, which results in the chronic character of biofilm infections and in the poor response to treatment [1, 10].

The phenotype of biofilm cells differs from that of planctonic cells, particularly in its surface characteristics, which are determined by the expression of the specific genes, that is, the gene-encoded adhesins and the genes responsible for the synthesis of polysaccharides that form a surface layer, so-called glycocalyx, on the surface of microorganisms [10, 11].

5.1.1 Extracellular Polymeric Matrix

Extracellular polymeric matrix (EPM), colloquially called slime, is a key building component of biofilms and is responsible for most of their physical, chemical, and biological properties [1]. EPM is a jelly-like, slimy substance containing up to 97% water. It can consist of many kinds of polymeric substances, especially polysaccharides, proteins, and extracellular DNA [12, 13]. The extracellular polysaccharides

form a dominant part of the extracellular matrix and play an important role in biofilm formation and behavior [13]. The best-known biofilm-associated extracellular polysaccharides include alginate (*Pseudomonas aeruginosa*), polysaccharide intercellular adhesin (staphylococci), and glucans and fructans (streptococci and lactobacilli). Their physicochemical properties can vary greatly, depending on the type of monomer units, the glycosidic linkages, and the presence of substitutions [11–13].

At the first phase of biofilm formation, EPM usually participates in the primary adhesion of the cells to a surface; later it takes part in the aggregation of bacterial cells and also in the formation of microcolonies. EPM also shows a substantial influence on the ability of biofilms to resist the adverse effects of the external environment. For example, in a host's body it significantly reduces the effect of antimicrobials used in therapy, as well as the action of the immunity system. EPM also protects bacterial cells from opsonization and from the impact of the complement system. It also protects cells from phagocytosis [1, 11, 12].

The recognition of the structure of EPM that surrounds microbial cells in biofilms is very important for our understanding of the mechanisms of biofilm formation as well as for the explanation of its abilities to protect bacterial cells from the effects of the immunity system and also from the effect of some antibiotics. The detection of the presence of EPM, especially extracellular polysaccharides, may help differentiate the microorganisms that are able to grow in the biofilm form from the microorganisms that are biofilm-negative [3, 4].

5.1.2 Extracellular Polysaccharide Matrix in Staphylococci

A typical example of the biofilm-associated extracellular polysaccharide that plays an important role in biofilm formation is staphylococcal extracellular polysaccharide substance. This substance was described for the first time as so-called capsular polysaccharide/adhesin (PS/A) responsible for the adhesion of *Staphylococcus epidermidis* cells [14]. Its main component is poly-*N*-acetylglucosamine (PNAG). It also emerged that the chemical structure of PS/A corresponded with the structure of the polysaccharide intercellular adhesin (PIA) that had been identified as a key component enabling the aggregation of staphylococcal cells and thus also facilitating accumulation in biofilm [15]. Baldassari et al. [16] found that the staphylococcal extracellular polysaccharide, described by Christensen et al. [17] as slime-associated antigen (SAA), chemically corresponded to the composition of PIA. Thus, all these substances are practically the same entities that are encoded by the same genes, *ica* operone genes [18]. EPM produced by the strains of *Staphylococcus aureus* [19], and possibly by other staphylococci, has a similar structure to PIA.

Currently, the basic structure of these polysaccharide substances is considered to be composed of the linear homopolymer *N*-acetylglucosamine linked with a β-1,6 glycosidic linkage whose subunits are randomly deacetyled 16% (±10%) [20]. Deacetylated PNAG is positively charged due to free amino groups that are essential for the correct function of *EPM* [18]. By PIA production, the staphylococci can efficiently change the electrostatic properties of their cell surface [20].

The cationic character of PIA enables the firm adhesion of this substance to negatively charged bacterial cell surfaces. So it is essential to localize the substance on the surface of staphylococcal cells. The substance is also responsible for bacterial aggregation. It was proved that the loss of the cationic character of the nondeacetyled PNAG at the isogenic *ica*B-deletion mutant *S. epidermis* reduces the ability to adhere on artificial surfaces, to form biofilms, to resist phagocytosis of neutrophiles, to resist antibacterial peptides (humane β-defensin 3), and to persist on catheters inserted into experimental animals. This implies that the enzymatic modification of PNAG through deacetylation is a crucial moment in the PIA synthesis that determines its correct function. A partial deacetylation of PNAG is also a necessary condition for its solubility in water, in contrast to fully acetyled PNAG that coagulates in water [20].

5.1.3 Extracellular Matrix in Pseudomonas Biofilm and Alginate

Alginate, one of the best-known extracellular polysaccharide substances, was isolated from bacteria (*P. aeruginosa*) for the first time in 1966 [21]. Its production was ascertained also in other pseudomonads and later in different bacteria too, for example, in the genus *Azotobacter* [22].

Alginate is high-molecular-weight, acetylated polymer composed of nonrepetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids. Separate subunits form homopolymeric structures (polymannuronate or polyguluronate) or heteropolymers. As a rule, these polymers are O-acetylated on the second or third position of the individual subunits [22]. The properties and the level of the polymer's viscosity are defined by the chemical composition, the level of acetylation, the presence of the negative charge, and the molecule size. The alginate of *P. aeruginosa* does not contain bigger blocks of polyguluronate and thus forms relatively flexible, liquid gels. At the same time, the high percentage of O-acetylation of this polymer increases its ability to bind water [23].

Alginate plays an important role as a virulence factor in these bacteria and in the formation of the pseudomonas biofilm layer. The mucoid strains of *P. aeruginosa* that produce alginate cause serious chronic pneumonia in patients with cystic fibrosis. These strains are also often isolated from other chronic infections, for example, infections of the inner ear or urinary tract infections [24, 25]. However, alginate production is widespread among the strains from the external environment, too. It facilitates the plant pathogens to colonize and infect plants [1].

5.1.4 Extracellular Polysaccharides in Candida Biofilms

The composition of yeast extracellular matrix and the mechanisms by which it is produced are not fully known. It was found that the biofilm extracellular matrix contained significantly less total carbohydrate (41%) and proteins, but had a higher

proportion of glucose, in contrast to the extracellular polymeric material obtained from planctonic culture. Galactose (3.1%) and mannose (9.0%) were also present in the biofilm extracellular matrix [26]. There are also substantial differences in the chemical composition among different species. For example, the major component in *Candida albicans* matrix was glucose (32%), whereas in *Candida tropicalis* matrix it was hexosamine (27%) [27].

The EPM of the yeast biofilm probably originates from the yeast cell surface, and it contains the major structural component of yeast cell wall, especially β-1,3 glucan and mannoproteins, responsible for yeast adhesion [28–30].

One of the important features of biofilm yeast cells is the change of the surface character, including cell-surface hydrophobicity and the surface charge, in comparison with biofilm-negative cells. The changes of the composition of the cell-surface polysaccharides can be responsible for this difference. Particularly, the acid-labile mannan from hydrophobic cells has a different structure and composition than that from hydrophilic cells [31, 32].

The character of the yeast extracellular matrix also can be influenced by the growth medium and culture conditions. For example, *C. albicans* produced a markedly higher quantity of the EPM in aerobic or dynamic culture conditions compared with anaerobic/static conditions [29, 33].

5.1.5 *Production of Extracellular Polysaccharides in Streptococci, Lactobacilli, and Multispecies Oral Biofilms*

The best-investigated multispecies biofilm communities are oral biofilms. The synthesis of extracellular polysaccharides, especially of glucans and fructans, is a key component of the sucrose-dependent colonization of teeth surfaces by oral bacteria. The production of polysaccharides by oral streptococci and lactobacilli essentially influences the formation of dental plaque. These polysaccharides are involved in the bacterial adherence. They regulate the diffusion of substances through the layer of a plaque. They can serve as extracellular energy reservoirs, and they are essential for the cariogenic effect of dental plaque [34–36].

Glucans are important in determining the permeability properties and the adhesiveness of dental plaque [37]. The glucans synthesized with glucansucrase enzymes differ in a number of characteristics, for example, the type of glucosidic linkages, the degree and the type of branching, the length of glucan chains, the molecular mass, and the conformation of the polymers [38]. All these properties strongly contribute to specific polysaccharide properties, such as solubility, rheology, and other physical characteristics. On the basis of the main glucosidic linkages, four types of α-glucans are recognized: dextran, mutan, alternan, and reuteran, [38, 39]. Reuteran, produced by *Lactobacillus reuteri,* contains mainly α-(1,4), but also α-(1,6) glucosidic linkages, and α-(1,4) and α-(1,6) branching points [40]. Dextran consists of α-(1,6) linkages with some branching points at position 2, 3, or 4. Dextrans are produced by the genera *Streptococcus*, *Lactobacillus,* and *Leuconostoc* [41, 42]. Mutan polymers

are mainly produced by various streptococci (*Streptococcus mutans*, *Streptococcus downei*, *Streptococcus sobrinus*, *Streptococcus salivarius*) [65], but also by *Lactobacillus reuteri*. Most of glucosidic linkages (over 85%) in mutan polysaccharides are formed by α-(1,3) bonds. Alternan is an α-glucan polymer from *Leuconostoc mesenteroides* composed of alternating α-(1,6) and α-(1,3) glucosidic linkages [43].

Oral biofilm bacteria produce also two types of fructans: levans, which consist mainly of α-(2,6)-linked fructose residues (with occasionally present α-(2,1)-linked branches), and inulin-type fructans, with α-(2,1)-linked fructose residues, with α-(2,6)-linked branches [44]. Inulosucrase enzymes are present only in lactic acid bacteria (e.g., streptococci and lactobacilli), while levansucrase enzymes are widely distributed in both gram-positive and gram-negative bacteria, although they show low similarity [45].

The presence and production of different types of polysaccharides is species and strain dependent. There are well-described differences in the production of water-soluble polysaccharides (fructans) and water-insoluble polysaccharides (glucans) in the groups of mutans streptococci isolated from caries-active patients and caries-free patients. According to Khoo et al. [36], caries-active mutans streptococci isolates produced significantly more water-insoluble polysaccharides than the caries-free ones, while no statistical difference was found in water-soluble polysaccharides between these two groups. This indicated that the production of water-insoluble polysaccharides may contribute to the increased caries activity. The complexity of the whole process of dental caries development illustrates the fact that the presence of higher levels of sucrose leads to significantly higher numbers of lactobacilli in the biofilm matrix of water-insoluble polysaccharides [46].

The presence of other polysaccharides may help the stabilization of streptococcal chains in oral biofilm, for example, the supramolecular organization involving a polyanionic glycan in a streptoccocal capsule (hyaluronan) that forms parallel arrays. These formations are based on hyaluronan tertiary structures (β-sheet-like), which are stabilized by hydrophobic and hydrogen bonds. The hyaluronan tertiary structures in aqueous solutions resist shear stress. Thus, supramolecular hyaluronan wrapping covering many cells probably stabilizes chains of bacterial cells [47].

5.2 Capillary Electrophoresis of Microbial Cells

5.2.1 The Surface Charge of Microorganisms

Generally as a result of the dissociation or protonation of carboxyl, phosphate, and amino groups in a three-dimensional surface of the ion-penetrable layer, the cells have a surface charge that depends on the pH of the solution [48–50]. The charged surface leads to the formation of an electric double layer between the solid surface

and the surrounding liquid that is characterized by the electrokinetic potential, ζ (zeta potential) [50]. The zeta potential is defined as the electric potential at the hydrodynamic plane of shear that is generally assumed to be located within several nanometers of the particle surfaces [51]. However, for microorganisms it is difficult to define the location of the surface or the plane of shear due to the inherent morphological heterogeneity of their surface.

The zeta potential is often assumed to be equal to the diffuse double layer potential ψ_d. The Gouy-Chapman theory [50, 52] describes the value of ψ_d for spherical particles of the radius a as a function of the distance r from the center of the sphere:

$$\Psi \mathrm{d} = \Psi a \frac{a}{r} e^{(a\text{-}r)\kappa} \tag{5.1}$$

with Ψ_a being the potential at the surface ($r=a$) and κ the diffuse double layer thickness determined by the composition of the solution through [49, 50] (the Debye-Hueckel parameter [53]):

$$\hat{e} = \sqrt{\frac{e0^2 \Sigma z_i^2 n_i}{\varepsilon_{\varepsilon 0} kT}} \tag{5.2}$$

Here e_0 is the electric charge, z_i the charge number of ionic species i, n_i is the concentration of the species in the bulk solution far from the surface, ε the dielectric constant, ε_0 the permittivity of vacuum, k the Boltzmann constant, and T the absolute temperature. The thickness of the double layer is $1/\kappa$ [50, 53] and is inversely proportional to the square root of the ionic strength of the solution I,

$$1/\kappa = \frac{0.304}{\sqrt{I}} \tag{5.3}$$

The bacterial cell surface charge strongly changes in response to environmental factors, thereby contributing to the dynamic nature of bacterial cell surfaces [50]. The existence of the electric double layer leads to the so-called electrokinetic phenomena [49], electrophoresis, and electroosmosis, when an electric field E is applied. In an aqueous phase at a given pH and constantion strength, the electrophoretic mobilities μ are defined as

$$\mu = \frac{2}{3} \frac{\zeta \varepsilon_{\varepsilon 0}}{\eta} f(\kappa a) \tag{5.4}$$

where η is the viscosity of the solution, which can be expressed quantitatively for spherical particles according the general theory developed by Henry [49, 52] but disregards the function $f(\kappa a)$, which is related to the double layer thickness, $1/\kappa$,

and the core radius of the particle, *a*. The suspended microorganisms move with a velocity v [49, 50],

$$v = \mu E \tag{5.5}$$

The pH at which the electrophoretic mobility is zero is referred to as the isoelectric point (pI). In the absence of specific adsorption, the pI is determined by the balance between anionic and cationic acid/base groups at the cell surface and can be used to obtain insight into the molecular composition of their surface [54]. The isoelectric point of most bacteria is in the pH range of 1.5 to 4.5 [55] with a few exceptions [50, 55, 56].

Experimental studies have shown the importance of electric double-layer interactions in bacterial adhesion [57, 58]. The bacteria may have an ion-penetrable surface layer, soft and hard, which may result from encapsulation or fibrillation of the bacterial cell surface, or a traditional, ion-impenetrable bacterial cell surface. In electrophoretically soft bacteria, the hydrodynamic plane of shear is not located outside but inside the ion-penetrable layer, close to the ion-impenetrable core of the bacterium. The hard and soft ion-penetrable bacteria demonstrate distinctly different electrophoretic velocities in an applied electric field.

5.2.2 The Isoelectric Point of Microorganisms

Although the cell-surface hydrophobicity is a major parameter indicating affinities of cells to the surface, other properties are also of importance [59–61]. Recently the isoelectric point of bacterium was used as the measure of the ability of bacterial surface polymers to inhibit adhesion. Sulfate groups are rarely reported to occur in bacterial cell surfaces, which leaves the following acid/base couples as the most probable ones to be involved in the charging of microbial surfaces [54]: phosphate either in phosphodiester bridges (R-O-HPO_2-O-R/R-O-PO^-_2-O-R) as in teichoic acids or at the end of a polymer (R-H_2PO_4/R-$HPOa_4$) as in phospholipids ($pK_a = 2.1$) [54, 62]; protein- or peptidoglycan-associated COOH/COO^- ($4 \leq pK_a \leq 5$); polysaccharide-associated COOH/COO^- ($pK_a = 7.2$) [54]; protonated phosphate (R-HPO^-_4/R-PO^{2-}_4); and peptidoglycan or protein associated ammonium (R-NH^+_3/R-NH_2) ($9.0 \leq pK_a \leq 9.8$).

The isoelectric point of bacterium is a more appropriate parameter than the electrophoretic mobility, μ, for predicting the steric properties of cell surface polymers and their consequences for cell adhesion [54]. The interpretation of the electrophoretic mobility in terms of zeta-potential and electrokinetic charge requires information about the mobile charge inside the bacterial wall [63].

5.2.3 Capillary Electromigration Techniques

As is apparent from the preceding chapters, the mobilities of the charged cells are comparable to those of low-molecular-mass organic or inorganic ions. The difference in mobility or pI allow for the separation and characterization of microorganisms [49].

The selection of appropriate separation techniques is limited by the size of the separated microorganisms. One of the most important aspects of the capillary electromigration techniques, capillary electrophoresis (CE) in free solution, often capillary zone electrophoresis (CZE) or capillary isoelectric focusing (CIEF), is the absence of the rigid porous gel structure that restricts the migration of large entities and the possibility of their on-line detection [64]. The second advantage of CE techniques for the identification and detection of microorganisms lies in their short separation times compared to conventional microbiological assays (e.g., 1 day). Reports that demonstrate the clear advantages of capillary electrophoretic techniques for the analysis of microorganisms have begun to appear [3, 48, 49, 65–68].

5.2.3.1 Capillary Surface Modification

Microorganisms that secrete extracellular polysaccharide substance [69] tend to adhere strongly on the inner surface of the fused silica capillary [70, 71]. Poly(ethylene oxide) coatings have been shown to reduce the adhesion of different microbial strains and species due to the decrease of the Lifshitz–van der Waals attraction [70] and thus are promising as coatings to prevent the biomaterial-centered infection of medical implants [69]. Poly(ethylene oxide) (PEO) chains are attached to a surface and project into the surrounding medium and reduce protein adhesion by forming a steric barrier between the protein molecule and the surface [70]. PEO-coatings have also been investigated for their ability to prevent bacterial adhesion [72–74].

In the capillary format, the adsorption of the bioanalytes onto the capillary wall may increase the zone distortion [75–78]. Simultaneously, it is necessary to minimize the strong electro-osmotic flow on the uncoated fused silica capillary. The suggested solutions include the dynamic modification of the inner capillary surface [79, 80] by soluble polymers [76, 78, 81–87], such as hydroxymethylpropyl cellulose, hydroxypropyl cellulose, or polyvinyl alcohol, or static modification of the capillaries by silanizing reagents [81, 82, 88–90] or the sol-gel technique [91]; a number of polymeric materials were used as replaceable coating agents [79, 90, 92–96]. The use of the polymeric capillaries for CIEF [83] and other electrophoretic techniques [97], for example, from polytetrafluorethylene [98, 99], was another option.

The lifetime of the polymeric film on the inner surface of the capillary is often shortened by its degradation due to strong acids and bases used as background electrolytes in CZE or as the anolyte and the catholyte in CIEF [79]. At the same time, the adsorbed microorganisms must be washed out from the wall of the capillary prior to the subsequent run. The rinsing procedure between individual runs was shown to have a strong effect on reproducibility [100]. Therefore, the application of the dynamically modified, uncoated fused silica capillary seems to be the simplest solution. The poly(ethylene glycol) (PEG) belongs to the group of the hydrophilic uncharged polymers used for the dynamic coating of the capillary and for the modifying of electro-osmotic flow (EOF) [101]. The PEG chains attached to surfaces are reported to reduce the protein and microbial adhesion [71–73]. The application of a diluted PEG solution was demonstrated to increase the efficiency of the separation of the bacteria by CZE [77, 93, 102–106].

5.2.3.2 Detection of the Microorganisms in Capillary Electrophoresis

The detection of microorganisms is based on the light scattering in ultraviolet (UV) or in the visible region of the spectrum usually at the wavelengths 210, 214 [102, 103, 107], or 280 nm [77]. It is easily possible to quantify on-line mixed microbial cultures by electrophoretic techniques [107, 108]. However, it is not sensitive enough to detect low concentrations of microorganisms [109]. The infectious doses of pathogenic microorganisms can be very low, on the order of tens to hundreds of cells. Thus, the limits of the detection have to be improved by the on-line or off-line bioanalytes labeling. Both covalent and noncovalent bonding [77, 92, 110–116] are recommended for the purpose of the fluorometric or laser-induced fluorescence (LIF) detection [105, 111–118]. The commonly used derivatization of biopolymers may cause problems in the interpretation of electrophoretic separation record [119]. They can improve the detection limits, and change the acido-basic properties [119, 120] and mobilities when compared to native samples [119].

For improvements in detectability [65, 118, 121], the Armstrong group [118, 121] used a sweeping method based on the injection of a microbial sample, a buffer space plug, and a blocking agent plug. Using this concentration approach, the authors were able to detect single microorganisms in a commercial CE-LIF instrument [118].

The nonionogenic tenside, poly(ethylene glycol) 4-(1-pyrene)-butanoate (PB-PEG) [123–125], or poly(ethylene glycol) 3-(2-hydroxy-5-*n*-octylphenylazo)-benzoate (HOPAB) [4] were used as a buffer additive for the dynamic modification of microorganisms during their CZE or CIEF separation with fluorometric and UV detection, respectively. The detectability of the cells was improved.

5.2.3.3 CZE of Microorganisms

Hjerten et al. [126] were the first to show the possibility of electrophoretically separating and UV detecting bacteria such as *Lactobacillus casei* in the capillary. Likewise, the fraction of the bacteria – *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae,* and *S. aureus*, or different developmental stages of these bacteria [127] were separated also by CE. Sonohara et al. [128] found that gram-negative *Escherichia coli* that have a lipopolysaccharide surface layer are more negatively charged than the gram-positive *Streptococcus aureus* covered by a peptidoglycan layer at different pH and ionic strength. On CZE, the electrophoretic mobilities of the bacteria *Pseudomonas* species–(*Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas denitrificans), E. coli* K-12 [129–131], *Acetobacter pasterianus*, *Bifidobacterium longum*, *Serratia marcescens*, *Acetobacter aceti*, and yeast–*(Saccharomyces cerevisiae)*, at different pH and ionic strength [129, 130], were determined. Broad bands, common phenomena in CE microbes, and the aggregation of the cells [130] were observed [49, 129, 130]. The mobility was expressed as the function of the potential at the boundary between the medium and the surface region as the function of the ionic strength [49, 130]. A rather high mobility of the microbial cells must be caused by

a high charge density, or ζ potential, respectively [129]. On the model of bacteria *Pseudomonas* species, the authors [129] concluded that the best pH range for the bacterial CZE separations was between pH 7 and 10, where the mobilities changed very little.

The high-efficiency CZE was used to the separation of the mixture of microorganisms, P. fluorescens, Enterobacter aerogenes, Micrococcus luteus, S. cerevisiae [77] or Lactobacillus acidophilus and Bifidobacterium infantis [103] in health products or E. coli and Staphylococcus saprophyticus in urine matrix [104, 132] by adding poly(ethylene oxide) to the background electrolyte. Sharp peaks were detected, and the migration time of the bacteria could be altered by varying concentration of poly(ethylene oxide). The brief sonication before the separation was recommended as the prevention of the clusters aggregation. It was found a linear relationship between the electrophoretic mobility and the number of cells in the aggregates [77, 102, 133]. The cells of S. cerevisiae and E. coli were moved electrokinetically in microfluidic systems [134, 135].

Armstrong et al. [103, 105] used CZE with LIF detection or connected to a CCD camera [93, 106] for the evaluation of cell viability. SYTO 9 was used here for the labeling of all the bacteria, while propidium iodide was used only for the labeling of the bacteria with damaged membranes. Shintani et al. [109] used CE-LIF of labeled *Salmonella enteritidis* and *Salmonella typhimurium* by SYTO 9, and they detected as few as three cells per injection from culture. Likewise, the aggregation of labeled *B. infantis* by SYTO 9 and monitored by the use of a microscope with an intensified CCD camera was studied by Zheng and Yeung [136]. Kourkine et al. [137] used CZE-LIF and the immunofluorescence staining for the detection of *E. coli*. Pyrenebutanoate, a fluorescent amphiphilic probe, was suggested as a capillary zone electrophoresis buffer additive for the dynamic modification of the cells from the mixed cultures of microorganisms *E. coli*, *C. albicans*, *E. faecalis,* and *S. epidermidis* [138]. The cells were concentrated, and separated by CZE using UV excitation for the on-column fluorometric detection. The detection sensitivity for the microorganisms on the order of one to tens of injected cells was achieved.

Poly(ethylene glycol) pyrenebutanoate was used as a nonionogenic tenside for a dynamic modification of yeast cells (biofilm-negative), *C. albicans, Candida glabrata, Candida kefyr, Candida krusei, Candida lusitaniae, Candida parapsilosis, C. tropicalis, Candida zeylanoides, Geotrichum candidum, S. cerevisiae, Trichosporon asahii,* and *Yarrowia lipolytica,* separated by CZE with fluorescence detection [124]. This dye played a dual role as both dynamic coating and fluorescent reagent, making it possible to detect down to ten cells. *Pseudomonas* species, native or dynamically modified by poly(ethylene glycol) pyrenebutanoate, were separated by CZE with UV or fluorometric detection. Narrow peaks were detected too [139].

Likewise, the HOPAB was prepared and used as a buffer additive for the dynamic modification of microorganisms including *E. coli*, *S. epidermidis*, and the yeasts during a capillary electrophoresis [4].

When using CE to analyze microorganisms in real samples, the complex backgrounds can make the identification of targeted microorganisms impossible. The selective detection of *S. aureus* in the presence of other microorganisms was reported by Gao

et al. [67]. They used mAb-coated latex particles to enrich for *S. aureus* and then fluorescent immunolabeling of the bound *S. aureus* for their selective detection by CE-LIF. The presence of the other microorganisms was not detected here.

5.2.3.4 CIEF of Microorganisms

Several reports have demonstrated successful separation of microorganisms based on their pI by CIEF [49, 64, 77, 140–143], such as *Serratia rubidae*, *Pseudomonas putida,* and *E. coli* [77, 144] or yeast cells [145]. Liu et al. [146] reported promising results using CIEF followed by the whole-column imaging detection. One particular dynamic coating based on poly(ethylene glycol) molecular weight 4,000 was used for CIEF of microorganisms and the yeasts *S. cerevisiae, Candida albicans, Candida parapsilosis* (biofilm-negative), *E. coli, S. aureus, S. agalactiae, E. faecalis, S. epidermidis* (biofilm-negative) that were UV detected after focusing [147].

Later, poly(ethylene glycol) pyrenebutanoate was used for the labeling of microorganisms before their CIEF separation [125]. This technique was used when analyzing different species – *E. coli, S. epidermidis, Proteus vulgaris, E. faecalis, Stenotrophomonas maltophilia,* yeast strains, *C. albicans, C. krusei, C. parapsilosis, C. glabrata, C. tropicalis,* and *S. cerevisiae* [125] – or the pathogenic species of the genus *Clavibacter, Xanthomonas,* and *Pseudomonas* sp. [123, 139]. They were reproducibly focused and separated by the suggested technique. Using UV excitation for the on-column fluorometric detection, the minimum detectable amount was down to ten cells injected into a separation capillary. The chromophoric nonionogenic tenside HOPAB [4] was used for the labeling of the microorganisms *E. coli* and *S. epidermidis* (biofilm-negative), and the strains of yeast cells *C. albicans* and *C. parapsilosis* (biofilm-negative) during CIEF with UV detection at 326 nm. A minimum detectable amount has been assessed lower than 100 cells injected into a separation capillary. The values of the isoelectric points of proteins and microorganisms labeled this way were found comparable with the pI of the native compounds and were not host specific.

5.3 Capillary Electromigration Techniques: A Useful Tool for the Detection of Biofilm Formation, an Important Virulence Factor in Microorganisms

The increasing incidence of the infections associated with a biofilm formation shows the necessity of a fast and reliable method for the proof of this virulence factor. As mentioned above, the detection of the ability to form a biofilm in a particular clinical strain is important for the prognosis of the infection as well as for the choice of an appropriate therapy. The most commonly used methods are based on the cultivation on appropriate culture surfaces, for example, microtiter plates, and on the subsequent demonstration of the formed biofilm layer. Unfortunately, these methods are time consuming and their results can be easily influenced by a change in culture conditions.

Unlike the biofilm-negative cells, the biofilm-positive cells are surrounded with an extracellular polysaccharide substance. This substance may lead to the change of the surface charge and thus to the change of the isoelectric point in biofilm-positive cells. Therefore, one of the promising ways of detection of biofilm formation is the evaluation of the differences in physicochemical properties of the cell surface, including the surface charge, between the biofilm-positive and the biofilm-negative strains.

The difference between the biofilm-positive and the biofilm-negative *S. epidermidis* strains was revealed by means of the CIEF [66] (Fig. 5.1). Afterward, the differences in the pI of the biofilm-positive, pI = 2.6, and the biofilm-negative,

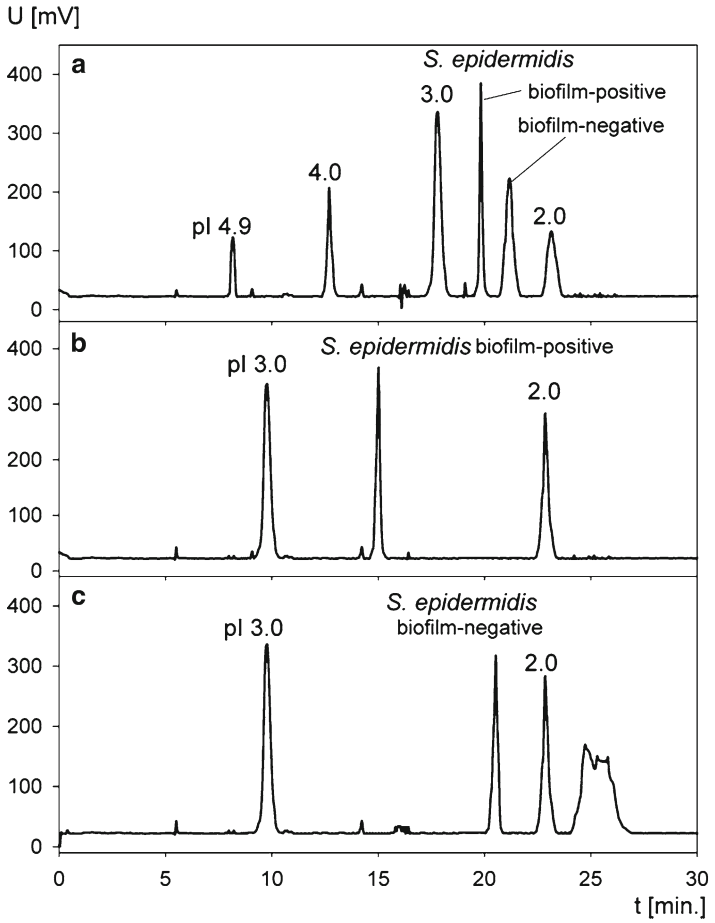

Fig. 5.1 The capillary isoelectric focusing (CIEF) of the biofilm-positive and biofilm-negative *Staphylococcus epidermidis* strains in the pH gradient 2 to 4.9 (electropherogram **a**) and 2 to 3 (electropherogram **b** and **c**). For CIEF of the biofilm-positive *S. epidermidis* strain, the strain was sonicated for 30 s (electropherogram B) and for 5 minutes (electropherogram **c**). Other CIEF conditions: fused silica capillary, 0.1 mm inner diameter, length 270 mm; applied voltage (–) 20 kV; wavelength, $\lambda = 280$ nm; the anolyte, 0.1 mol L^{-1} H_3PO_4, the catholyte, 2×10^{-2} mol L^{-1} NaOH, in both the anolyte and the catholyte was dissolved 2% (w/v) PEG 4,000

pI = 2.3, *S. epidermidis* strains were also demonstrated on the larger group of clinical isolates (73 strains). The results indicated the possibility of using CIEF for the determination of this virulence factor in various *S. epidermidis* strains [3]. The premise that PIA is responsible for such differences in pI among staphylococcal strains supported the finding that the strains that were originally focused as biofilm-positive bacteria start to focus as biofilm-negative bacteria after sufficient sonication.

The mechanical impact of ultrasound probably causes the removal of a slimy surface layer from these bacteria. The electrophoretic "softness" has no connection to the mechanical properties of the surface layer. However, atomic force has indicated correspondence between mechanical and electrophoretic "softness" of two tested streptococcal strains [148].

Also, the differences in the electrophoretic mobility between the biofilm-positive and the biofilm-negative microorganisms labeled by HOPAB, *C. parapsilosis* and *S. epidermidis*, were assessed by means of CZE (Fig. 5.2). The biofilm-positive and the biofilm-negative strains significantly differed in their electrophoretic mobility in both *C. parapsilosis* and *S. epidermidis* strains [4].

The rapid separation and the identification of the different yeast species or their lysates, based on IEF, were introduced [132]. The isoelectric points of the biofilm-positive and the biofilm-negative *C. parapsilosis* and *C. tropicalis* strains were determined by CIEF with UV detection in the acidic pH gradient (Fig. 5.3). The differences between their isoelectric points were up to 0.3 U of pI.

These differences point to the existence of chemical structures on the surface of yeast cells, especially cell-wall glycoproteins and extracellular matrix that are specific for the biofilm-positive strains. The biofilm formation in yeasts is in relation

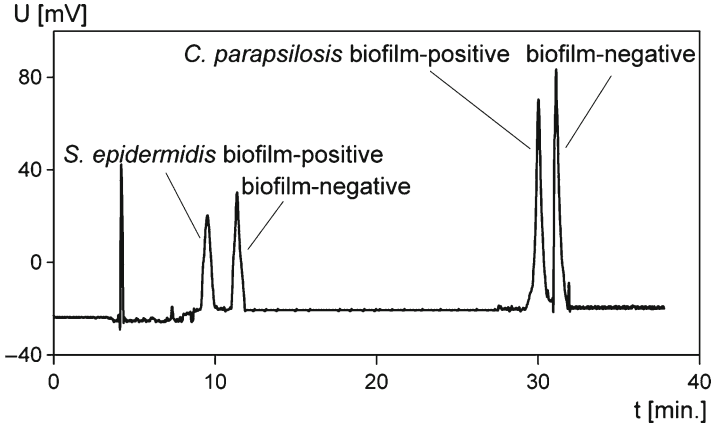

Fig. 5.2 The capillary zone electrophoresis of the biofilm-positive and the biofilm-negative *Candida parapsilosis* and *Staphylococcus epidermidis* strains labeled by the non_ionogenic tenside HOPAB [4] with UV detection. Other capillary zone electrophoresis (CZE) conditions: fused silica capillary 0.1 mm inner diameter, length 320 mm, 170 mm to the detection cell; applied voltage (–) 20 kV; wavelength, $\lambda = 326$ nm; background electrolyte, 1×10^{-2} %(w/v) HOPAB, 3 %(v/v) EtOH, 1.5×10^{-3} mol L^{-1} phosphate buffer, pH 5.5

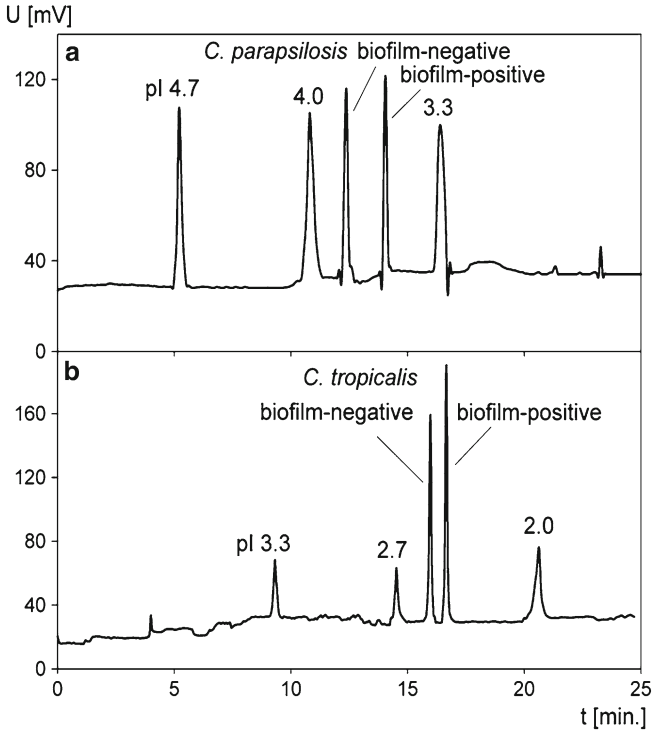

Fig. 5.3 The separation of the biofilm-positive and the biofilm-negative *Candida parapsilosis* strains in the pH gradient 3.3 to 4.7 (**a**) and the biofilm-positive and the biofilm-negative *Candida tropicalis* strains in the pH gradient 2 to 3.3 (**b**) by CIEF with UV detection. Other CIEF conditions: fused silica capillary 0.1 mm inner diameter, length 350 mm, 200 mm to the detection cell; applied voltage (–) 20 kV; wavelength, $\lambda = 280$ nm; the anolyte, 0.1×10^{-1} mol L^{-1} H_3PO_4, and the catholyte, 4×10^{-2} mol L^{-1} NaOH, in both was dissolved 1 %(v/v) EtOH, 0.6 %(w/v) PEG 10000; pI markers, pI, 2.0, 2.7, 3.3, 4.0, and 4.7. (From Horká et al. [132], with permission)

to cell-surface hydrophobicity. Masuoka and Hazen [32] assume that there is correlation between the hydrophobicity of the *C. albicans* cells and the changes in the acid-labile, phosphodiester-linked β-1,2-oligomannoside components of the N-linked glycans of the cell wall mannoprotein. The acid-labile mannan from hydrophobic cells is composed of larger oligosaccharides than that from hydrophilic cells [31]. It seems that the changes in wall protein glycosylation [32] can contribute to the difference between the isoelectric points of yeast strains.

The isoelectric points of tested microorganisms corresponded well with their ability to form a biofilm. Therefore, CIEF seems to be useful for the evaluation of the differences between the physicochemical properties of the biofilm-positive and the biofilm-negative microorganisms, particularly in *S. epidermidis* and yeasts [3]. In comparison with other electrokinetic techniques, such as capillary zone electrophoresis, the results of CIEF are not affected by the aggregation of staphylococcal cells, and the sonication of the sample cannot be too long.

The assessment of the pI by means of CIEF is very fast; it takes about 10–15 min. Another advantage is the relative independence of the microbial pIs on the measurement conditions [114]. Moreover, this technique can be used for the separation and the quantification of yeast cells in the samples, and it enables also the demonstrating of mixed culture. CIEF has great potential for further development, especially in miniaturization of the CIEF format to a microfluidic system or microchip device. In this way, this method enables automatization and further reduction of the analysis time [149].

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Chapter 6
Capillary Electrophoresis Applied to Polysaccharide Characterization

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Abstract Capillary electrophoresis is a consolidated analytical approach for the structural characterization of polysaccharide mono- and oligomer constituents, as demonstrated in this chapter, which surveys several applications of this technique on chemically and enzymatically degraded polysaccharides, covering the last 10 to 12 years. Capillary electrophoresis is also demonstrated to be highly reliable for determination of polysaccharides in biological samples, as it analyzes quite complex matrices even without any pretreatment, a distinctive feature with respect to other separative strategies. The versatility of this technique is clearly demonstrated by its potential in evaluating macromolecular features of polysaccharides, such as size (molecular weight, chain length), chain rigidity, charge density, and chemical modifications.

Keywords Capillary electrophoresis • Polysaccharides • Glycosaminoglycans • Biomedical applications • Biological applications • Degradation mechanisms

Abbreviations

ABEE	4-amino-benzoic ethyl ester
ABN	4-aminobenzonitrile
AGU	anhydrogalacturonic
AMAC	2-aminoacridone
APTS	8-aminopyrene-1,3,6-trisulfonate
bFGF	basic fibroblast growth factor
CE	capillary electrophoresis
CID-VE	collision-induced dissociation conducted at variable energies
DE	degree of methylesterification

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DP	degree of polymerization
ELFSE	end-label free solution electrophoresis
EPG	*endo*-polygalacturonic
FACE	fluorophore-assisted carbohydrate electrophoresis
GPC	gel permeation chromatography
HG	homogalacturonan
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
ILIF	indirect laser-induced fluorescence
LPGC	low-pressure gel permeation chromatography
MECK-UV	micellar electrokinetic capillary chromatography with UV detection
PACE	carbohydrate gel electrophoresis
PG	polygalacturonase
RG	rhamnogalacturonic
RGI	rhamnogalacturonan
SEC	size exclusion chromatography
SF	synovial fluid
TEC	transient entanglement coupling
XGA	xylogalacturonan

6.1 Introduction

The variety and complexity of polysaccharidic structures produced by nature, especially by the vegetal world and bacteria, renders the characterization of their structures a particularly difficult challenge, but also an essential process, necessary to correctly assign the composition of the polymer in terms of monosaccharidic units and stereochemistry of linkages.

Chemical or enzymatic degradation is a useful tool for structural characterization of polysaccharides. Depending on the reaction conditions, the products of such process can vary from high-molecular-weight chains to monosaccharidic constituents. Moreover, the polysaccharide treatment can be tuned for preserving or not preserving specific structural features (e.g., occurrence of substituents). A complete degradation down to the monosaccharide level is often a convenient tool for quantitative analysis of polysaccharides or glycoconjugates when no qualified macromolecular standard is available [e.g., 1]. Moreover, the analysis of specific monosaccharidic constituents is useful for identification of polysaccharides from complex sources [2].

In addition to composition and linkage stereochemistry, it is important to study polysaccharides as intact macromolecules, since the biological roles of these relevant biopolymers are also associated with their average dimension, shape, and charge. To achieve a complete characterization of polysaccharides, it is therefore necessary to apply several analytical approaches, depending on the specific aspect to be investigated.

Typically, chromatographic techniques [3] are particularly suited for mono- and oligosaccharide composition studies (liquid chromatography, such as high-performance anion-exchange chromatography with pulsed amperometric detection [HPAEC-PAD], or reverse-phase ion pair chromatography with ultraviolet [UV] or fluorescence detection), as well as for studies on linkage positions (gas chromatography coupled with mass spectrometry). Size-exclusion chromatography, especially when combined with light-scattering detection, can be used for size and shape evaluation of intact polysaccharides. All these approaches, despite belonging to the same category (i.e., chromatographic techniques), are based on quite different separation/detection principles that require dedicated columns and eluents, which are needed in significant amounts.

As an alternative separative technique, capillary electrophoresis (CE) has significant features that warrant attention. It is extremely versatile, in that it can perform analyses, with the same capillary, from mono- to polysaccharides, merely changing/optimizing the separation buffer composition, which is requested in minimal volumes (typically 10 to 15 mL per day). Note that this versatility is combined with high separation selectivity, which is competitive with one of the most advanced liquid chromatographic techniques; therefore, CE has been successfully used for structural studies of degraded polysaccharides, as will be demonstrated in the examples given in this chapter. Moreover, CE is increasingly being used for macromolecular characterization, and some relevant examples of this kind of applications are provided later in the text. Finally, CE requires minimal amounts of sample, and this feature, combined with the poor buffer consumption, allows method optimization.

On the other hand, CE does not always provide the same sensitivity as chromatographic techniques, especially with respect to HPAEC-PAD, but derivatization with suitable chromophores or fluorophores (compatible with the most common detectors used for CE, UV, and laser-induced fluorescence) is necessary to have a competitive signal-to-noise ratio. Nevertheless, derivatization is useful for achieving a quantitative picture of saccharide distribution (provided that the derivatization yield is the same for all the sample constituents), while HPAEC-PAD might not be able to provide the same information, as described in detail in Chap. 7, which also provides a general comparison between CE and high-performance liquid chromatography (HPLC).

This chapter surveys the applications of CE for characterization of polysaccharides before and after degradation. In particular, the following topics are discussed: structural studies via degradation mechanisms; studies of macromolecular features; CE for practical uses; and CE in biomedical and biological application fields. When appropriate, CE performance is compared to other analytical techniques.

6.2 Structural Studies Via Degradation Mechanisms

There are several reports on the use of CE for the determination of the monomer composition of polysaccharides, especially from plant sources. The ability of this technique to distinguish different monosaccharides makes it very useful for these

applications. In addition, since it is always necessary to hydrolyze a polysaccharide in order to analyze its oligo- and monosaccharide composition, an advantage of CE is that it does not require further purifications of the crude hydrolyzed. The enzymatic or chemical, either acid or basic, hydrolysis is generally the preferred route to degrade the polysaccharide chain, but, depending on the matrix, other strategies are also reported, such as methanolysis, reductive hydrolysis, and the hydrothermal treatment of wood, as reported in the work of Kabel and coworkers [4]. The aim of these reports is to elucidate the chemical composition of complex carbohydrate-based polymers such as carrageenans [5], acetylated and arabinosylated xylanes [4], and complex heteropolysaccharides [6, 7].

If CE is recognized as a useful tool for the qualitative and quantitative determination of the monosaccharides present in a polymer, it is generally assumed that to assess the stereochemistry and sequence of glycosidic bonds, is necessary to turn to other techniques, such as nuclear magnetic resonance (NMR), gas chromatography, and mass spectrometry (MS), or, if available, the on-line coupling of different methods, as demonstrated, for example, in the report of Che and coworkers [8] for the analysis of oligosaccharides derived from acid hydrolysis of dextran. There, the on-line use of CE-QIT-MS (QIT: Quadrupole Ion Trap) allowed for the identification of oligosaccharides differing by one single monomer as well as by their quantification with sensitivity on the order of picomole units.

There are, however, some interesting examples showing the great contribution that CE makes to reconstructing the linkage sequence, to evaluate the extent of substituents along the backbone as well as their distribution pattern.

Jacobs and coworkers [9] studied the distribution of the 4-*O*-methylglucuronic residues along xylan chains isolated from wood meal from different sources (spruce, pine, larch, aspen, and birch) after alkali extraction of holocellulose. Carbohydrate composition of enzymatically hydrolyzed xylans was determined in terms of 4-*O*-methylglucuronic/xylan ratios by capillary zone electrophoresis (CZE) after derivatization with 4-amino-benzoic ethyl ester (ABEE) in sodium acetate at pH 4 as the running buffer. Moreover, CZE performed on partially acid-hydrolyzed xylans provided also reliable information concerning the distribution of uronic acid substituents in the original xylan samples. Information on the saccharide sequence was obtained by exploiting the known greater stability toward acid hydrolysis that the linkage between xylose units and the 4-*O*-methylglucuronic acid substituents shows with respect to both the $\beta(1\rightarrow4)$ linkage of the backbone and the $\alpha(1\rightarrow3)$ linkage of the furanosidic arabinose. Therefore, by modulating hydrolysis conditions, CE provided basilar structural information that, further refined by coupling with other techniques, led to the identification of saccharidic sequences [9].

Separation and identification of different glycotopes belonging to the same family may be extremely relevant in the biological field. This is certainly the case with polysialic acid, of which more than 30 different types are now known as the "glyco" portion of glycoproteins found in a wide variety of animal species. Cheng and coworkers [10] performed a comparative structural study of polysialic chains from diverse sources. Analysis were performed on three different series of $\alpha2\rightarrow8$-linked oligomers containing $(\rightarrow8\text{Neu5Ac}\alpha2\rightarrow)n$, $(\rightarrow8\text{Neu5Gc}\alpha2\rightarrow)n$, $(\rightarrow8\text{KDN}\alpha2\rightarrow)n$, and an

$\alpha 2 \rightarrow 5\text{-}O_{\text{glycolyl}}$-linked chain, that is, $(\rightarrow 5\text{-}O_{\text{glycolyl}}\text{-Neu5Gc}\alpha 2\rightarrow)n$ obtained by controlled acid hydrolysis. During the analysis it was also possible to determine a different characteristic of $(\rightarrow 8\text{Neu5Ac}\alpha 2\rightarrow)n$ and $(\rightarrow 5\text{-}O_{\text{glycolyl}}\text{-Neu5Gc}\alpha 2\rightarrow)n$ chains that enables them to undergo intramolecular esterification. By means of micellar electrokinetic capillary chromatography (100 mM sodium dodecyl sulfate [SDS] and 100 mM sodium bicarbonate at pH 8.0) using direct UV detection at 200 nm, the separation of three sets of homologous $\alpha 2 \rightarrow 8$-linked oligononulosonic acids was achieved by varying the degree of polymerization (DP). Three series of $(\rightarrow 8\text{Sia}\alpha 2\rightarrow)n$ with identical DP (e.g., 1–5) were successfully separated from each other and the resolution improved with DP. Moreover, it was also possible to separate two series of isomers of oligo/polyNeu5Gc differing in the interketosidic linkage.

Starch might seem to be an easy polymer to analyze since it is composed only of glucose units linked via either α-1,4 or α-1,6 glycosidic bonds. But the complexity of chain arrangement, coupled with a high degree of polymerization and high polydispersity, makes the characterization anything but an easy task. Indeed, the starch complex mixture, composed of amylose (α-1,4 poly-glucan) chains of relatively short length, bearing few α-1,6 linked glucosidic branches, and of long amylopectin (α-1,4 poly-glucan) chains with a high frequency of α-1,6-linked glucose branches, renders its structural characterization very challenging. To understand more about the biosynthesis processes as well as to relate the polymer structure to the biological functions and to the physicochemical properties, structural information beyond the simple amylopectin/amylose ratio is needed.

Morell and co-workers [11] studied isoamylase debranched starch and glycogen samples, and characterized them in terms of DP and the degree of α-1,6 branches calculated from the chain length distribution of the released oligosaccharides, with DPs ranging from 3 to 85. Further structural information on the monomer and disaccharide populations was obtained by using specific enzymes in a sequence (i.e., β-amylase digestion followed by isoamylase and pullulanase debranching). After 8-aminopyrene-1,3,6-trisulfonate (APTS) labeling, the released oligosaccharides were analyzed using three different fluorophore-assisted carbohydrate electrophoresis (FACE) methods (i.e., polyacrylamide slab gels, polyacrylamide gel electrophoresis using a DNA sequencer, and CE). CE with laser-induced fluorescence (LIF) detection turned out to provide the highest resolution and the best reproducibility for the separation of oligosaccharides with DPs from 1 to 100. The capillary used for FACE-CE analysis was a 50-μm-diameter eCAP™ neutral coated capillary with the supplied carbohydrate separation gel buffer.

Capillary electrophoresis also has been widely used to study pectins, typically consisting of 1,4-linked galacturonic acid units, whose complexity derives from the presence of minor quantities of neutral monosaccharides and from a variable extent of uronic esterification. CE has been shown to be a suitable technique to characterize pectin structure in terms of degree of esterification (DE) and esterification pattern by correlating the electrophoretic mobility with the linear polymer charge density after sample de-esterification.

In a preliminary study by Zhong et al. [12], three pectins were examined – two with low calcium sensitivity, taken to imply a random charge distribution, and one

with high calcium sensitivity, taken to imply a blockwise charge distribution. Pectins were de-esterified using the enzyme pectin esterase from *Aspergillus* to obtain fractions of varying DE. After preliminary experiments carried out at pH 3.0, pH 7.0 and reverse polarity were chosen as preferable separation conditions. Within the range of DE studied (30–80%), the electrophoretic mobility scaled linearly with the residue average charge $z = -(100 - DE)/100$. In addition, it was shown that, for a fixed charge, mobility increases with decreasing ionic strength, but does not depend significantly on whether the pectin charge distribution is blockwise or random. CE was shown to provide a simple and rapid method for separation and quantitative detection of pectins having different degrees of esterification, and to have the potential of quantifying directly the polydispersity of charge to size ratio.

Later, Zhong et al. [13] tested the CE method on pectin samples with variable content of neutral sugars, and investigated the possibility of obtaining intermolecular DE distribution directly from the shape of the CE signals. Specifically, fractions of varying DE were obtained via de-esterification using the enzyme pectin esterase of *Aspergillus*, and CE analysis was performed as previously reported [12]. The degree of esterification of 11 pectins from different sources (citrus, apple, and beet), having a different amounts of anhydrogalacturonic acid (AGU) (86.2 ± 63.2%), was quantified by CE (50 mM phosphate buffer at pH 7 and UV-detection at 192 nm) and showed to be in excellent agreement with the results obtained by the titration method, irrespective of pectin type and neutral sugar content. The method also allowed determination of the intermolecular DE distributions from the CE peak shape, being the peak width mainly determined by the polydispersity of the mass-to-charge ratio within a particular pectin sample. Both DE and DE distribution were obtained from CE in much shorter time than from ion-exclusion chromatography (IEC) or size exclusion chromatography (SEC).

In line with these findings is the work of Williams et al. [14], in which control of the methyl ester distribution, a highly esterified sample (90%), was first effected by treating a commercial sample with methanolic sulfuric acid and then de-esterifying it by base saponification or enzymatic de-methylation, leading to a random or block-wise distribution, respectively. Here, it is clearly shown that electrophoretic mobility of pectins is largely determined by the chain-averaged charge density, irrespective of how that charge is distributed. For chains with lengths in excess of about 15 residues, a symmetrical scaling of charge and hydrodynamic friction coefficient with the degree of polymerization (DP) was found, demonstrating that larger polymeric chains, regardless of their DP, elute based on their average charge density, and therefore that each CE migration time marks species with a unique degree of methylesterification. Peak shapes thus reflect the intermolecular methylesterification distribution of the sample.

Recently, Williams and coworkers [15] reported further investigations aimed at correlating CE signals with both intra- and intermolecular methylesterification patterns. This work is in fact focused on the assessment of electrophoretic methods for the study of inter- and intramolecular distribution of methylesterification in pectins. Analyzed samples were (1) homogalacturonan (HG), from citrus pectin, de-esterified, hydrolyzed in 0.1 M HCl and re-esterified in different extents with

CH_3I; (2) rhamnogalacturonan (RG) from *Arabidopsis thaliana*; and (3) various pectins. CE analyses were carried out in phosphate buffer at pH 7.0. Electrophoretic mobility was plotted against the fraction of sugar ring charge, z, and against the typical dimensionless polyelectrolytic linear charge density parameter ξ. The comparison between HG and RG showed that the nature of linkage between sugar rings does not have a large impact on electrophoretic mobility. A universal electrophoretic mobility dependence on charge density was generated for the investigated polygalacturonans. For homogalacturonan substrates with random intramolecular distribution of esterification, it was shown that the experimentally extracted intermolecular distributions agree well with the predictions of calculations based on the binomial theorem, demonstrating that the intermolecular distribution of the methylesterification contains information on the intramolecular pattern. For randomly esterified samples, then, the width of the intermolecular distribution may allow an estimation of the molecular weight.

In the work of Goubet et al. [16], four pectin samples with different degrees of methylesterification were treated with an *endo*-polygalaturonase (EPG), and the obtained oligogalacturonides were analyzed by two different strategies: CE and "polysaccharide analysis using carbohydrate gel electrophoresis" (this method is sometimes referred to as PACE, not to be confused with the acronym of pressure-assisted capillary electrophoresis). The degree of methylesterification was controlled via treatment with a pectin methyl esterase of fungal origin, and titration with NaOH. CE analysis was performed in a fused-silica capillary with a phosphate buffer at pH 7.0, UV absorbance at 191 nm, and applying a voltage of 20 kV in a positive modality. The comparison between the two techniques was successful, in particular regarding the relative amounts of the different pectin species.

Another method to analyze pectin oligomers is proposed by Coenen et al. [17], who describe the separation by CE-MS of complex mixtures of rhamnogalacturonan (RGI), xylogalacturonan (XGA), and homogalacturonan (HG) oligomers in apple pectin. Specific enzymes were used on diverse pectic substrates: XGA was degraded by XGA hydrolases from *Aspergillus tubingensis*, polygalacturonic acid was digested by endopolygalacturonase from *Kluyveromyces fragilis*, and apple modified hairy regions (MHRs) were degraded by rhamnogalacturonan hydrolases from *A. aculeatus*. Moreover the last substrate was degraded also by controlled acid hydrolysis, originating a mixture of HG, RGI, and XGA oligomers. All the oligomers were derivatized with APTS and separated on a polyvinyl alcohol (PVA)-coated capillary. Separation was carried out in reverse polarity at 30 kV, with LIF detection ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm), with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide and 0.3% formic acid at pH 2.98 or with 25 mM sodium acetate buffer containing 0.4% polyethylene oxide at pH 4.75. The addition of polyethylene slowed the sugars' oligomer migration. In such a manner, complex mixtures also could be separated and identified.

Similar to pectins are the charged polysaccharides analyzed by Wiedmer and coworkers [18] in their report from 2000. In this work CE was applied to investigate the ability of polygalacturonic acid to complex different metal ions. According to

the molecular-sieving principle, a successful separation of fluorescently labeled polygalacturonic acid oligomers with DPs of up to about 30 was achieved using a linear poly(acrylamide) (LPA) (home)-coated capillary and a running buffer (24 mM citric acid and 2 M urea at pH 3) containing 4% of linear polyacrylamide. The technique of choice to study the oligomer–metal ion interaction was instead the end-label free solution electrophoresis (ELFSE), for which, in contrast to gel CE, pH limitations were not encountered. With the ELFSE technique the oligosaccharide relative mobility was measured as a function of increasing metal ion concentration. For moderately large oligomers, the strongest binding resulted with calcium and cadmium ions and the smallest one with magnesium ions.

In determining the fine structure of polysaccharides, enzymes have a crucial role. In the following studies, enzymatic strategies in combination with CE analytical tools were applied for this purpose.

Cellulose derivatives were characterized by means of a mixture of enzymes; in the work of Stefansson [19], cellulose solutions were incubated with four cellulases (from *Penicillium funiculosum*, *Aspergillus niger*, *Trichoderma reesei*, and *Trichoderma viride*) and one hemicellulase from *A. niger*. In some cases the citrate/Tris running buffer was added by charged surfactants such as aminodextran. Data from enzymatic and acid hydrolysis was combined, resulting in carboxymethylated celluloses of different substitution degrees [19].

Cellulase digestion was also used compared with a β-mannanase action to cleave a glucomannan polymeric chain produced by *A. konjac*. The obtained dimers and trimers were successively analyzed with NMR, MS, and CE, achieving useful information such as the exclusion of the existence of large block structures and the composition of the glucomannan polymer chain of random mannose and glucose sequences. CE experiments on digested derivatized with 4-aminobenzonitrile (ABN) were performed in fused-silica columns with a running buffer of 100 mM borate, pH 9.2, applying a voltage of 15 kV and UV detection at 285 nm [20].

Glycosaminoglycans (GAGs) constitute a large family of polysaccharides of very different composition that are often difficult to identify. Their biological importance as fundamental components of the extracellular matrix is demonstrated and justified by the impressive number of reports that address their structure [e.g., 21–25]. We cite only a few examples of the extensive research published in the literature, to give a brief overview of the effort required to develop capillary methodologies as well as enzymatic reaction strategies in the study of this important polysaccharide family. The examples reported below demonstrate that CE is a valuable alternative to conventional HPLC [26] for the analysis of oligosaccharides resulting from enzymatic digestion of glycosaminoglycans.

A CZE method for the separation of all the disaccharides obtained by digestion with lyase of chondroitin sulfate, dermatan sulfate, and hyaluronic acid in a single analysis was developed by Al-Hakim and Linhardt [27]. After digestion with chondroitin ABC lyase, samples were analyzed in CE using 10 mM sodium borate, 50 mM boric acid at pH 8.8, with a UV detection at 232 nm. Here, to improve sugar separation, the well-known ability of borate to increase sugar's net charge through vicinal diols complexation is exploited.

The characterization of hyaluronic acid and its derivatives can imply either the use of hyaluronidases from animal origin, which hydrolyzes the β-1,4-glycosidic bond between GlcNAc and glucuronic acid, or the hyaluronan (HA) lyases, hyaluronidases from bacterial sources that through a β-elimination reaction lead to unsaturated oligosaccharides that are easily detectable by conventional spectrophotometric methods. Fragments obtained from a bacterial hyaluronidase (HA lyase from *Streptococcus agalatiae*) were analyzed by Kühn et al. [28] using a CE-MS coupled system. In this procedure the separation was performed in a coated fused-silica capillary, with 40 mM ammonium acetate at pH 9.0 as the running buffer and a separation voltage of 30 kV in normal polarity. The advantage is that this method achieves separation and online identification of various hyaluronic acid oligomers up to 16-mers, which is unachievable in CE-UV systems.

The use of heparin lyase enzymes from *Flavobacterium heparinium* has facilitated heparin and heparan sulfate analysis. Heparin lyases are able to cleave the glycosidic linkage between the glucosamine and uronic acid residues via a β-elimination reaction, producing an unsaturated bond. Three classes of heparin lyase enzymes are known (heparin lyase I–III), and each of them has a different specificity with respect to chain cleavage and sulfation pattern. CE is very suitable for oligosaccharides derived from heparin and heparan sulfate because of their strongly anionic character. The combination of the three mentioned enzymes was used in the report of Karamanos and coworkers [29], which describes a CE method for the determination of the sulfation pattern of heparin and heparan sulfate oligosaccharides. The applied method (in reverse modality [20 kV] using 15 mM sodium orthophosphate buffered at pH 3.50 with phosphoric acid in an uncoated fused-silica capillary) allowed 12 non-mono-, di-, and tri-sulfated Δ-disaccharides to be separated and determined.

The same enzymes mixture was used also by Ruiz-Calero and coworkers [30] to characterize heparin using a CE-MS method. In this case a volatile 30-mM formic-acid solution adjusted to pH 3.20 with ammonium hydroxide was chosen as the running buffer for its compatibility with MS injection. The electrophoretic separation occurred in an uncoated fused-silica capillary applying a voltage of 30 kV in reverse modality. Analytic parameters were also calculated. The authors reported a lower limit of detection (LOD) of CE_MS_MS compared with the CE-UV method for most of the disaccharides and good run-to-run precision.

Ruiz-Calero and coworkers [31] reported as well a careful optimization of the separation procedure applied to seven substituted hexoses composing the GAGs. Analysis was performed by CE using alkaline fluorescein-based electrolytes, and optimal separation was achieved in co-electro-osmotic flow and using the rather unusual indirect laser-induced fluorescence (ILIF) detection. The separation was optimized in terms of pH, capillary length, fluorescein concentration, and addition of organic solvents. The co-electro-osmotic conditions were achieved by adding the polycationic surfactant hexadimethrine bromide to the electrolyte buffer. The method proved to be suitable also for the determination of hexosamine content in hydrolyzed sulfated GAGs mixture and for the evaluation of minor contamination of dermatan sulfate in heparin.

6.3 Studies of Macromolecular Features

The above reported examples suggest the potentiality of CE for the characterization of complex polysaccharides, but applications of this technique are even wider, since it has been exploited for the study of subtle structural properties of charged and uncharged polysaccharides, such as molecular weight, molecular weight distribution, the ability of interactions with ions, and the degree and distribution of chemical modifications along the chain.

In the last decade, several efforts have been made to overcome the long-known applicability limit of CE to the macromolecular and physicochemical study of large molecules in terms, for example, of molecular weight and mass distribution. As far as partially depolymerized samples are concerned, CE strategies provide useful quantitative evaluation of polysaccharide characteristics.

Campa et al. [32] reported on the application of micellar electrokinetic capillary chromatography with UV detection (MEKC-UV) to the quantitative evaluation of the average degree of polymerization (DP) and distribution of oligosaccharides in partially acid hydrolyzed mannuronans in comparison with different techniques, namely ^{1}H-NMR, electrospray ionization mass spectrometry. The investigation showed that MEKC-UV allowed the determination of oligomer distribution and appropriate statistical averages of samples with a DP up to 10. This represents a significant new development, as it enables calculation of the quantity of individual oligomers of nominal DP by direct analysis of a defined oligomer mixture. The apparently unfavorable derivatization needed for MEKC-UV (and not for HPAEC-PAD) has the advantage of providing a direct indication of the oligomer amount, on the basis of the chromophore response; in comparison, in HPAEC-PAD the electrochemical response was demonstrated to vary as a function of DP.

A limit of resolution of about 90 DP was found [33] for CE analysis performed using a polyacrylamide-coated capillary and LIF detection on fluorescently labeled dextran samples. The average molecular weight determined for two dextran fractions by CE was shown also to be sensibly lower (almost two times) than the one measured by SEC-LS (LS: light scattering) (i.e., 2,500 by CE compared with 4,510 by SEC, and 3,719 by CE compared with 6,567 by SEC), suggesting a chain length dependence of the labeling efficiency.

Modeling low DP polymer migration data obtained from CE may lead to determining chain characteristics. Ruddick and Goodall [34] described a simple analytical model relating the electrophoretic mobility to the size and conformation of a polymer chain. Based on the Debye-Hückel-Henry theory and on the assumption of freely rotating chain, it is shown that the mobility extrapolated to zero ionic strength varies linearly with the inverse square root of the degree of polymerization of 8-aminonaphthalene-1,3,6-trisulfonate (ANTS)-labeled dextran and amylose. Being the slope inversely related to the square root of the characteristic ratio of the chain (C_{∞}), a value of 3.5 was obtained for the characteristic ratio of amylose, whereas a more extended chain conformation was deduced from dextran data in agreement with gel permeation chromatography (GPC) results. The DP ranged from

11 to 35 for amylose and from 12 to 41 for dextran. CE analysis was performed using a fused-silica capillary in phosphate background electrolyte of concentrations varying from 20 to 100 mM and containing 10 mM at −5 kV applied voltage.

Unfortunately, only a limited number of investigations have done systematic studies of the electrophoretic behavior in free solution of polyelectrolytes, in particular of charged polysaccharides. Most of the CE applications have focused on the search for separation efficiency and on the development of analysis strategies more than on the knowledge of absolute mobility to be related to physicochemical parameters of polyelectrolytes, underestimating in this way the role that CE might play as a tool for basic macromolecular investigation in terms, for example, of charge density, chain length, and chain rigidity dependence of the mobility. In contrast with the routine use of free solution electrophoresis to characterize particle surface charge so to obtain microstructural information with microscopic or molecular models, theoretical models for the mobility of polyions are insufficiently tested or limitedly developed due to the shortage of systematic studies in simple electrolyte controlled conditions.

In the work of Cleland [35], the experimentally observed electrophoretic mobilities for tryptophane-labeled hyaluronans and chondroitin 4-sulfate were compared to those theoretically predicted for a worm-like chain with discrete charge sites distributed along the chain axis [36]. Good agreement was found between the predicted and the experimental mobilities measured in constant ionic strength and varying pH buffers. A chain-length dependence of the mobility on attaining the asymptotic value was also predicted by the model. The limiting chain length, however, was far beyond the oligomer dimensions (>200 hyaluronan disaccharide units), and the approach to the asymptote was shown to occur, as would be expected, at a higher chain length as the ionic strength decreased. This high dimension is in contrast with most of the experimentally reported findings from which, however, a uniformly clear picture of the limiting chain length for mobility discrimination has not yet been assessed, nor is it clear how inherent macromolecular features may influence it.

Kim and coworkers [37] attempted to correlate the linear charge density of charged polysaccharides with the free solution mobility dispersity. The mobility of the sulfated polysaccharide fucoidan (M_w 9,000 g/mol) from brown algae was studied by CE in UV detection, and the results obtained by varying pH, ionic strength, and counterion conditions were compared with those of a heparin sample with a molar mass of 15,800 g/mol. Measurements were performed using a fused-silica capillary and either a positive or negative voltage of 30 kV. Electropherograms were converted into absorbance versus mobility curves, and the electrophoretic behavior was compared with the electrophoretic mobility dispersity. However, linear correlations between the measured μ and the charge density parameter ξ were not established, as the latter was above the limiting value for counterion condensation, and the complex electrolyte buffer was likely too high in concentration.

As for mobility versus charge density, it is worth noting that in the work of Williams et al. [15] cited above, a universal electrophoretic mobility versus charge relationship was derived from CE measurements in free solution on several galacturonic-containing polymers.

The recently published approach for the evaluation of the polymer effective charge from the detection sensitivity in CE using indirect UV detection might turn out to be of great interest for the characterization of charged biopolymers [38]. Here, the quantity of a probe contained in the buffer displaced by the charged polymer is related to the effective charge per monomer. The method was applied to synthetic charged homopolymers as well as to block- and random co-polymers with molar masses ranging from 10^4 to 10^5 g/mol, depending on the type. The experimentally obtained values for the linear charge density parameter were compared with those calculated using the established counterion condensation theory.

As mentioned above, the free solution mobility of sufficiently long and uniformly charged chains is independent of the chain length; for a length greater than the so-called free-draining limit, the friction increase with length parallels the increase of the effective charge, leading to a constant charge-to-mass ratio. The end-labeled free-solution capillary electrophoresis (ELFSE) method has been developed to restore size-dependent mobility, making use of a monodisperse label that modifies the charge-to-friction ratio of the polyelectrolyte, overcoming the free-draining behavior. A hydrodynamically large label may impose a frictional drag sufficiently high to enable long charged chains to be separated. Besides being monodispersed, the drag labels should present water solubility and a unique site for the main chain anchorage. The developed method has been especially applied to study DNA fragments [e.g., 39].

The electrophoresis separation of polyelectrolytes of varying length by ELFSE has been studied by Grass and coworkers [40], who, by using a molecular dynamics simulation model, characterized the drag coefficient of different label types, namely, linear or branched polymers and micelles. The hydrodynamic sizes, which for branched polymers are coupled with the stiffening of the polyelectrolyte chain induced by lateral branches, are apparently governing the frictional drag.

To characterize higher molar mass samples of broader polydispersity, the sieving matrix principle has been exported from SEC and applied to CE experiments. Due to the reduced half-life, the chemically cross-linked matrices directly polymerized in the capillary were soon substituted for separate DNA and proteins by physically entangled matrices [41]. Gel CE is a particular useful tool in the case of large and uniformly charged polyelectrolytes, which having a constant mass-to-charge ratio and tend to migrate in free solution with size-independent velocities.

Kakehi's group experimented with pullulan [42] and polyethylenglycol [43–45] as the sieving medium for the study of charged (degraded) polysaccharides. Kakehi and coworkers [43] developed an analytical CE method for the determination of the molecular masses of acidic polysaccharides composed of simple repeating units, using a NeuAc homopolymer as a model. The authors achieved the resolution of preparations of NeuAc polymers up to more than 100 monomer units, showing, however, an increasingly poorer resolution for NeuAc molar masses greater than 29,000 g/mol [45]. From the separation profiles of NeuAc, a relationship between electrophoretic mobility and molecular mass was found, and it was used to obtain molecular masses of higher polymers, taking into account that those determined by

CE are closely related to the average molecular weight. The established method, when applied to the examination of polysulfate esters of hyaluronic acid, leads to an understandable underestimation of the molecular weights in comparison with those determined by gel filtration. The analytical conditions used during the experiments were as follows: fused silica capillary coated with dimethylpolysiloxane (buffer, 50 mM Tris–borate [pH 8.5] containing 10% polyethylene glycol, with a molecular mass of 70,000).

Novotny's group [46] used a polyacrylamide-coated capillary, filled with highly viscous polyacrylamide entangled matrix, and LIF detection to achieve a monomer resolution up to at least 80 kDa for an intact hyaluronan sample of 258 kDa molecular weight. Various hyaluronan oligomeric fractions from either enzymatically or ultrasonically cleavage were also analyzed. Interestingly, the authors found the presence of a series of satellite peaks, whose intensity is pH dependent. They suggested that this peak-splitting phenomenon is a hint of different conformations present in solution.

The same authors [47] have also explored the possibility of using aminodextran as an effective electrolyte additive for a size-dependent separation of hyaluronate oligomers in capillary gel electrophoresis. In this case, positively charged aminodextran is used to complex HA oligomers to an extent that proportionally increases with the molecular weight (i.e., with the number of charges along the chain). Using a polyacrylamide-coated capillary and a linear polyacrylamide/citric acid/Tris/aminodextran running buffer, the uniformly charged polymers were separated with high selectivity. The size-dependent separation could be modulated by varying the concentration of the added gel, the ionic strength, and the pH of the buffer, enabling polymer chain separation up to 70,000 g/mol.

To induce a size-dependent migration so as to extend CE application to larger polysaccharides, Stefansson [48] analyzed by CE the molecular weight and molecular mass distribution of fractions of fluorescently labeled alginates. By varying the concentration of linear polyacrylamide, Stefansson was able to separate alginate fractions in the range of megadaltons, and to obtain a more subtle resolution of structural variants. Indeed, content and block length of guluronic residues were evaluated by selective complexation of calcium ions added to the buffer.

Other strategies include the modification of the polysaccharide mobility achieved by the action of differently charged additives included in the buffer. In this manner, electrophoretic migration can be induced in otherwise uncharged polysaccharide by absorbing a charged additive or can be modulated by ion pairing in cases of highly charged chains [49].

Depending on the nature of the participating amphiphile, and taking into account that migration velocity and selectively are strongly influenced by both concentration and charge number of the involved species, the separation of chemically modified cellulose mixtures could be achieved [50]. Several isotherm models were applied to interpret the mobility dependence on surfactant concentration apparently governed by a complex balance between hydrophobic and polar interactions. Measurements were done using LIF detection and a 4% linear polyacrylamide-containing buffer on APTS-labeled polysaccharides.

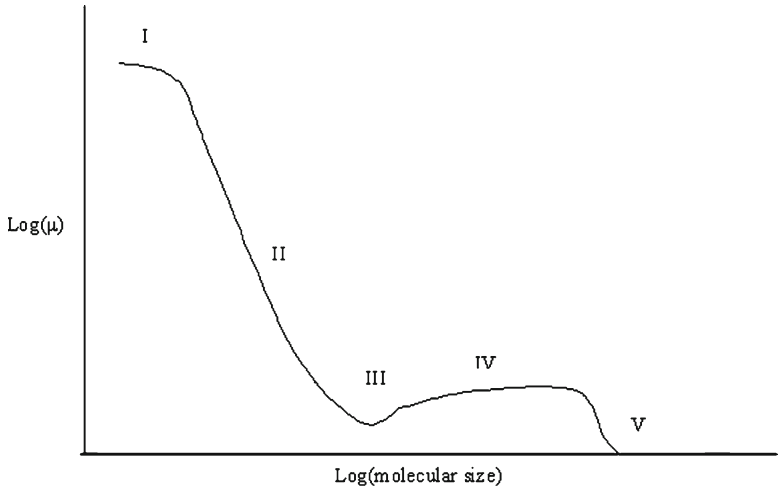

Fig. 6.1 Schematic approximate representation of polymer migration in sieving matrices

An important aspect of gel electrophoresis concerns the influence that the concentration and size of the polymer used as sieving medium have on the migration behavior of the investigated chain induced by an applied electric field. It is now apparent that for charged polymer separation, a concentration (C) greater than the overlap concentration (C^*), so that an highly entangled network is formed, is not strictly needed. In all cases a nonlinear dependence of polymer-related mobility on molar mass is found. A common method to depict the several mechanisms involved in the migration of a sample chain through a polymer transient network is shown in Fig. 6.1, in which I is the Ogston region, where the mobility decreases exponentially with the polymer size; II is the reptation region, where the sample chain "snaking" through the polymer network is modeled by the encountered physical obstacles (entanglements), and an approximate inverse dependence of mobility versus size is found; III is the reptation trapping, where the sample chain becomes trapped in a U-shaped conformation entanglement with sieving chains, slowing down its motion; IV is the region where the reptating reference chain is (partially) oriented in the direction of the electric field; and V is the complete trapping region, where sample chain aggregation leading to knot formation with sieving chains is supposed to occur.

To elucidate the mechanism of separation in CE, techniques such as linear dichroism, fluorescence video microscopy [e.g., 51, 52], and epifluorescent video microscopy have been used, with DNA as the sample chain. Carlsson and Jonsson [51] quantified the orientation and the orientation dynamics of a long DNA (164 Kbp) chain in solutions of linear polyacrylamide for field strengths up to 150 V/cm. Interestingly enough, it was shown that even an amount of polyacrylamide ($M_w = 18 \times 10^6$ g/mol) as small as 0.003% was sufficient to increase the orientation of the DNA chain in pure buffer solution by a factor of 100.

The transition between the transient entanglement coupling (TEC) migration mode of fluorescently labeled DNA (48 Kbp) and the full reptation mode has been investigated by Chiesl and coworkers [52] by epifluorescent video microscopy. The deformation response of a single chain to either the occasionally encountered constraints (TEC) or to snaking through an entangled network, followed by a microchip electrophoretic run, can be related to universal polymer solution characteristics.

The biological importance of DNA certainly justifies the extensive research published in literature, in order to achieve a more generalized picture; however, investigations on a wider spectrum of chain conformation would certainly be desirable.

6.4 Capillary Electrophoresis for Practical Uses

This section presents a brief overview of the practical uses of CE.

The high selectivity of CE is exploited to support the synthesis of *O*-sulfated hyaluro-oligosaccharides. After partial degradation of hyaluronic acid with a bacterial hyaluronidase, the digested matter was size-fractionated by low-pressure gel permeation chromatography (LPGC) and desalted. Then, to confirm the purity and sugar length of each oligosaccharide, a pivotal condition preceding the successive functionalization, CE separation was performed using the CZE method (fused silica capillary, in normal polarity mode using a mixture of 40 mM disodium phosphate, 40 mM SDS, and 10 mM tetraborate pH 9.0) [53].

Several applications for CE were found also in quality control of commercial products of the pharmaceutical and food industries in order to determine the polysaccharidic components and to identify contaminants.

In the work of Volpi [54], a micellar electrokinetic CE procedure, specifically developed to determine the total alginic acid content of both liquid and solid pharmaceutical preparations after treatment with alginate lyase from *Flavobacterium* sp. is presented. The endo-cleaving activity of the enzyme produces C4–C5 unsaturated oligomers with a DP ranging from 1 to 6 that can be readily detected with high sensitivity due to their strong absorption at 230 nm. After enzyme incubation, samples were centrifuged and analyzed by the MEKC method using a fused-silica capillary, 10 mM sodium borate/50 mM SDS at pH 9.0 as the running buffer and an applied voltage of 20 kV in normal polarity. The enzymatic degradation products were detected and quantified as a single peak and with a sensitivity greater than in previously reported methods. Furthermore, the analysis, performed in a rather short time, did not require elaborate and time-consuming pretreatment steps.

The high resolution power of CE has been applied for the determination of chitosan of relatively high molecular weight (200,000 g/mol). Under optimized conditions (fused-silica capillary with 100 mM triethylamine-phosphate buffer at pH 2), using UV detection, this method was suitable for rapid profiling and screening of chitosan as a food additive in commercial products [55].

A galactomannan produced from *Cassia tora* is widely used in European food industries, blended with carrageenan and guar. CE methodologies can be applied to evaluate the adulteration of *C. tora* gum with guar or *Cassia occidentalis*. A series of CE methods were developed to distinguish galactomannan components in several gums in order to detect product adulteration and substitution [56].

Matsuno and coworkers [57], using CZE analysis (50 mM sodium tetraborate buffer at pH 9.3 containing 100 mM SDS; voltage 20 kV, UV absorbance at 200 nm) of various commercially available hyaluronan samples, were able to evaluate, in some of them, the presence of dermatan sulfate as a contaminant that inhibited the testicular hyaluronidase digestion of hyaluronic acid. The same samples were instead readily degraded by bacterial HAses. The contamination was confirmed by the digestion that occurred with chondroitinase ABC, and the relative abundance of DS in HA samples was obtained through labeling of disaccharides with a fluorescent tag AMAC (2-aminoacridone) and LIF detection (λ_{ex} = 488 nm, λ_{em} = 520 nm). The digestion products were analyzed in a DB-1 capillary with a dimethylpolysiloxane-modified inner surface, at 20 kV in normal mode and using a 100 mM Tris buffer adjusted to pH 8.0 with boric acid and containing 1% PEG70000. The inhibition effect of DS, apparently specific to mammalian testicular HAse but that might be effective also in endogenous HAases located in human skin or cartilage, is of particular interest for the biomedical and industrial applications. A proper mixture of HA and DS may lengthen the half-life of hyaluronic acid in HA-based pharmaceutical preparations.

Contamination in a commercial enzyme preparation was detected by Williams and Benen [58] in a study of the enzymatic digestion of pectic substrates with different polygalacturonase (PG) samples. From the different patterns of oligogalacturonides obtained from a polygalacturonic sample and two pectins digested with pure endo-PG II or a commercial PG in comparison with those obtained from fungal or plant polymethylesterase incubation, it was deduced that a nonconventionally known *exo*-acting methylesterase that attacks the oligogalacturonide's nonreducing end was present in the commercially available enzyme preparation. A CE method developed for the separation and quantification of oligogalacturonides residues was applied. The analysis was performed using a 50-mM phosphate buffer at pH 7.0 in a fused silica capillary with an applied voltage of 20 kV and UV detection at 191 nm.

The availability of recombinant enzymes facilitates the study of substrates of great complexity for which highly pure enzymes, not always commercially available, are certainly needed. This is the case reported by Bauer and coworkers [59], who expressed 74 recombinant enzymes to facilitate the analysis of the structure and composition of plant cell wall polysaccharides. Here, the product mixtures obtained by polysaccharide digestion with these enzymes were analyzed either by CE after their labeling with ANTS or APTS, or by matrix-assisted laser desorption ionization-Time Of Flight (MALDI)–TOF-MS after desalting. The CE analysis was performed using carbohydrate separation buffer (Beckman–Coulter), LIF detection (λ_{ex} = 488 nm, λ_{em} = 520 nm), and an applied voltage of 25 kV.

6.5 Capillary Electrophoresis in Biomedical and Biological Application Fields

Resolution power and sensitivity coupled with low sample consumption and rapidity of analysis render CE a very versatile analytical tool, as demonstrated by its multiform applications that may also include analysis of very complex matrices without particular pretreatments. Examples are given by the quantitative analysis of hyaluronan in vitreous humor and in synovial fluid (SF) using CE [60, 61]. Vitreous humor samples from bovine and human sources were analyzed both as intact samples and after digestion with testicular hyaluronidase. All samples where thawed, centrifuged, and filtered before CE analysis was performed in the UV detection mode using a 50 mM disodium hydrogen phosphate/40 mM SDS/10 mM sodium tetraborate running buffer at pH 9. The presence of SDS as the protein complexing agent was found to be essential to prevent protein migration times from being in the time window of interest [60]. In a similar manner, CE was applied to the study of SF from human joint sources [61]. To relate the abundance of hyaluronan in SFs to the disease state, human SF samples from normal and diseased joints were analyzed by applying the same CE procedure previously established.

Capillary electrophoresis turned out to be a useful analytical tool to investigate enzyme activity in crude biological samples. The incubation of a known amount of hyaluronic acid with an aliquot of crude venom sample was followed in time by CZE analysis using an uncoated silica capillary and a 40 mM borate/40 mM SDS running buffer at pH 9.8 in positive mode (20 kV). The most important advantage of the applied methodology was that hyaluronidase activity could be detected by measuring changes in hyaluronan signal directly on an undigested and intact HA peak. Other advantages are the methodology's rapidity and sensitivity, and the absence of any sample pretreatment [62].

A contribution of CE in the diagnosis of a neoplasm is presented by Karamanos et al. [29], who proposed a procedure to quantify the hyaluronan present in pleural effusion from mesothelioma patients to be compared with the amount present in effusions of nonmesothelioma patients. After precipitation with ethanol, the GAGs mixtures were digested with chondroitinase ABC and AC, the undegraded and undesired components removed following the ultrafiltration of the digest by centrifugation. The profile of the HA- and galactosaminoglycan-derived mixture was then analyzed both by CE and HPLC. The analysis of hyaluronan disaccharides was performed using an uncoated fused-silica capillary under the separation conditions previously described. Determination of HA in effusion was based on the analysis of its nonsulfated degraded species produced by digestion with chondroitinases and on a separation with sulfated and nonsulfated Δ-disaccharides species from the enzymatic cleavage of chondroitin sulfate and dermatan sulfate, which was essential for the quantification of HA in clinical samples. The results indicated that the CE procedure could be used as a valid alternative to HPLC. Analytical parameters such as sensitivity, linearity, and limit of detection were also reported [63].

It is now apparent that CE methodologies are very important tools for the study of biological samples, with a special concern for the analysis of glycosaminoglycans, which are involved in a variety of important biological events.

A method for the structural analysis of heparin and heparan sulfate in biological samples has been proposed and applied by Karamanos's group [64] to analyze heparin and heparan sulfate derived from bovine kidney and porcine intestinal mucose. Here, after polysaccharide digestion with an equi-unit mixture of heparin lyases I–III, the obtained Δ-disaccharides were labeled with 2-aminoacridone fluorophore. All 12 known non-mono-, di-, and trisulfated Δ-disaccharides were resolved in a single run using a 50-mM phosphate buffer at pH 3.5. Furthermore, with LIF detection, a sensitivity 100-fold higher than UV detection at 233 nm was achieved.

The CE-LIF approaches have been tested with various biological samples including crude cartilage extract [65] and juvenile bovine cartilage [66] to validate the quantification of proteoglycan-derived disaccharides obtained by the liquid chromatography–mass spectrometry (LC–MS) methods.

The optimization of the above-reported methods led to a fast and reproducible CE-LIF procedure applicable to both pure standards and disaccharides derived from lyase depolymerization of polysaccharides in biological extracts [67]. The procedure entails sample purification and removal of the excess labeling agent to improve the sensitivity. Given the limited amount of GAGs present in biological samples and that only small samples of intact tissue are available, to obtain a sufficiently high sensitivity is in fact the major concern in performing this type of analysis. Polysaccharides extracted from intact juvenile bovine and adult human cartilage explants were digested by three chondroitin lyase enzymes (chondroitinase ABC, chondroitinase ACI, and chondroitinase B), whereas heparin and heparan sulfate from bovine kidney were digested with a mix of heparin lyases I–III. The obtained Δ-disaccharides were labeled with AMAC (2-aminoacridone) and analyzed by CE with LIF detection ($\lambda_{ex} = 425$ nm, $\lambda_{em} = 530$ nm) using uncoated fused-silica capillary, 50 mM phosphate buffer at pH 3.5, and in reverse polarity at 30 kV. The applied conditions allowed for sample component separation based on Δ-disaccharides charge density. To improve both the sensitivity of detection and the signal-to-noise ratio, the carbohydrate samples were purified via cellulose microspin columns to remove the excess of the fluorescent label (AMAC). In this manner the improved CE-LIF method enabled the quantification of biologically relevant GAG-derived disaccharides of concentrations as low as 0.01 μg, comparable to that normally found in mammalian tissues [67].

The high resolving power of CE when combined with the specificity of an enzyme action may provide useful information on ligand–receptor interactions generally occurring through the involvement of specific structural domains of the partners. Physical interaction of cell adhesion molecules with the extracellular matrix (ECM) can turn cell signaling pathways on or off. Also, ECM components can sequester growth factors or hormones by binding, making them unavailable to turn cell signaling on or off. As components of the ECM, heparin and heparan sulfate are known to be involved in the extracellular regulation of cellular functions,

and heparan sulfate proteoglycans on the cell surface are recognized as a versatile coordinator of cellular functions, modulating the action of a large number of extracellular ligands. The sulfation patterns of distinct oligosaccharide domains within heparin and heparan sulfate determine their binding affinity with basic fibroblast growth factor (bFGF). Combining CE technique with the specificity of the cleaving action of heparin lyases I–III, the ability of differently sulfated oligosaccharides to bind bFGF has been investigated [68]. Oligosaccharide mixtures from enzyme-catalyzed depolymerization were analyzed by CZE using an uncoated fused silica capillary with a 50-mM sodium phosphate buffer at pH 3.5 in reverse polarity (30 kV). After incubation with bFGF the oligosaccharides were again analyzed in order to identify those specifically interacting with bFGF.

The identification of the biologically active sequence was the aim also of Zamfir and coworkers [69], who studied a new protocol for the structural analysis of decorin-derived CS/DS oligosaccharides able to interact with FGF-2. A solid-phase binding assay showed the ability of the intact proteoglycan ^{35}S-sulfate-labeled decorin from human skin fibroblast to bind to FGF-2 in a manner very similar to that shown by ^{35}S-sulfate GAGs. The GAG chains, released from decorin via β-elimination, were digested with chondroitin B lyase and the saccharide mixture purified by GPC. Taking into account that the binding sequence may be present in those chondroitin B lyase-resistant oligosaccharides that are larger than hexamers, an elucidation was attempted of the full complement of chondroitin lyase-resistant structures for which an interaction with FGF-2 was shown. This was done by a three-stage method based on CE fragments separation, electrospray ion (ESI)–MS screening, and MS/MS sequencing with a novel approach of collision-induced dissociation conducted at variable energies (CID-VE). The separation in CE was achieved on fused silica capillary with UV detection at 214 nm, at 25 kV in normal polarity, and with a running buffer of 50 mM ammonium acetate, pH 12.0, in water/MeOH 40:60 [v/v] that is compatible with ESI–MS analysis requirements [69].

6.6 Conclusion

This chapter's survey of applications of CE demonstrates the reliability and the consequent consolidated use of this technique for structural studies of polysaccharides after appropriate degradation. The analysis of polysaccharides in biological samples is also extremely appealing due to the capability of analyzing complex matrices even without pretreatment. On the other hand, the characterization of intact polysaccharides can still be considered a growing field, especially considering the evaluation of macromolecular features, which, despite the need to be consolidated with orthogonal approaches, show the potential of CE in assessing molecular weight, molecular weight distribution, the ability of interactions with ions, and the degree and the distribution of chemical modifications along the chain, as well as charge density, chain length, and chain rigidity.

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Chapter 7
Capillary Electrophoresis for Monitoring Natural and Synthetic Processes of Saccharide-Bearing Molecules

Marco Rossi, Amelia Gamini, and Cristiana Campa

Abstract A survey of the more recent applications and developments of capillary electrophoresis (CE) to study reactions involving saccharide-bearing molecules is presented. Selected studies are cited to highlight the potentialities as well as the versatility of capillary electrophoresis. CE strategies that are applied to monitoring the synthesis of carbohydrate-based molecules are discussed. One strategy is the monitoring of mono- and oligosaccharides synthesis; another is the preparation of glycoconjugates, including those bearing nonsaccharidic backbone. The biological implications are also discussed, such as the action of enzymes on carbohydrate-based species, particularly on polysaccharides, in terms of kinetic parameters, enzyme activity, and substrate specificity evaluation, as well as active site identification. Studies showing how capillary electrophoretic strategies are applied to investigate naturally occurring processes, pursuing the ultimate goal of monitoring single cell reactions, are also presented.

Keywords Capillary electrophoresis • Polysaccharides • Monosaccharides • Oligosaccharides • Enzymes • Enzyme activity

Abbreviations

ABEE	ethyl 4-amino benzoate
ANTS	8-aminonaphthalene-1,3,6-trisulphonic acid
BGE	background electrolytes
BTH	bovine testicular hyaluronidase
C^4D	capacitively coupled contactless conductivity detector
CE	capillary electrophoresis
CZE	capillary zone electrophoresis

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DS	degree of substitution
EPG	endopolygalacturonase
ESI-MS	pulsed amperometry, on-line electrospray mass spectrometry
Fa	Forssman antigen
FACE	fluorophore-assisted carbohydrate electrophoresis
FCHASE	6-(fluorescein-5-carboxamido) hexanoic acid succimidyl ester
Fmoc	*N*-fluorenyl-methoxycarbonyl
HA	hyaluronic acid
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	high-performance liquid chromatography
HSA	human serum albumin
MALDI	matrix-assisted laser desorption ionization
MECK	micellar electrokinetic chromatography
MU	methylumbelliferyl
NMR	nuclear magnetic resonance
PAD	pulsed amperometric detection
PAGE	polyacrylamide gel electrophoresis
SAX-HPLC	strong-anion exchange–HPLC
SEC	size exclusion chromatography
S/N	signal-to-noise
T/H	tetra-/hexa-saccharide
TLC	thin-layer chromatography
TMR	tetramethylrhodamine

7.1 Introduction

Over the last years, noticeable efforts have been devoted to developing strategies for the synthesis of oligosaccharides and glycoconjugates. Indeed, several biologically active carbohydrates cannot be easily extracted from natural sources, due to the low occurring amount or the difficult purification process. Moreover, synthetic strategies are developed for modifying carbohydrates with functional reactive groups that can be used for further modifications. For instance, carbohydrates can be conjugated to a polymeric carrier (e.g., a protein, a synthetic polymer, or a polysaccharide). Such carbohydrate-containing macromolecular assemblies allow the occurrence of the "cluster effect," which induces a high local concentration of the molecules linked to the polymeric backbone, thus further enhancing their biological activity [1–5]. Additionally, several polysaccharides display functional groups suited for clusterization of non-saccharidic compounds, with the advantages associated with a natural, biocompatible carrier [6, 7]. The synthesis of carbohydrate-containing products often exploits the action of enzymes, which, in adequate conditions, are able to transfer sugar moieties on appropriate acceptors

[8, 9]. This is one of the reasons why there is great interest in deepening our knowledge of the enzymatic processes in which carbohydrates are involved. The study of enzymes' mode of action, moreover, can support a better understanding of carbohydrate's multiple roles, some of which are not yet completely understood.

All this information is extremely useful for designing new and effective synthetic strategies and for scouting for new active substances to be used in the pharmaceutical and dietary industries.

In this context, the availability of reliable characterization tools for process monitoring is extremely important. For carbohydrate-involving routes, the complexity of the reaction mixtures is not the only challenge; the characterization of saccharidic compounds is an additional issue to be faced, considering the lack of strong chromophores, the multiplicity of biological role, and the minute structural variants (substituents and position of hydroxyl groups, anomeric configuration, linkage position, and so on), as described in the literature [10].

Due to the nature of the samples, separation techniques are best suited to approach the analytical problem (i.e., monitoring of complex reaction mixtures). Among them, high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and capillary electrophoresis (CE) are the most popular, due to their compatibility with water-soluble, nonvolatile compounds, such as carbohydrates. For the most useful automated techniques, HPLC and CE, on-line detection methods include, for instance, ultraviolet (UV), fluorescence, laser induced for CE (after opportune derivatization), pulsed amperometry, and on-line electrosprayion–mass spectrometry (ESI–MS).

Capillary electrophoresis has increasingly been used for monitoring carbohydrate-involving processes, both natural and lab-induced. Indeed, this technique is highly versatile, allowing the effective separation of analytes with very different structure and size. Moreover, it may be applied to crude samples; since no stationary phase is present in the separation capillary, the "clogging" or "matrix-analyte competition" risk is not present. Finally, CE is applied to very small sample volumes, and this aspect is extremely useful when dealing with reactions involving precious components, such as enzymes, synthetic oligosaccharides, and glycoconjugates.

Among liquid chromatographic techniques, a very effective analytical approach for carbohydrate process monitoring is high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which, despite the higher injection volumes with respect to CE, allows extremely selective and sensitive analysis of oligosaccharides without sample derivatization [11, 12]. However, despite the cited advantages of HPAEC-PAD, CE can be the technique of choice in many instances. With HPAEC, MS interfacing is not straightforward due to the presence of nonvolatile salts in the eluent; CE, in contrast, allows the use of volatile buffers (suited for MS detection) without losing its good separation selectivity and without the need of desalting, as demonstrated by some examples in this chapter and as recently reviewed [13].

Another interesting feature is that PAD detection is dependent on the oligosaccharide structure/size [12, 14, 15], while in CE–UV or CE-laser-induced fluorescence (LIF) the electropherogram allows the visualization of the real molar distribution of the oligomers, provided that the derivatization yield is the same for all the investigated compounds [16, 17]. Finally, unlike HPAEC-PAD, which is suited for the analysis of oligosaccharide but unable to provide for separation of macromolecules, CE allows the effective separation of compounds showing very different size and structure within the same session, as demonstrated by some of the examples below. Note that when PAD detection is combined with size exclusion chromatography (SEC) [Dionex Corporation (2001) Application Note 83, Size-Exclusion Chromatography of Polysaccharides with Pulsed Amperometric Detection (PAD)], polysaccharides can be successfully characterized, but in this case the selectivity for low-molecular-weight mixture components, unlike in CE, is lost.

Several reviews have used CE for the analysis of carbohydrates [10, 18–21], but, to our knowledge, no survey has applied this technique to natural and lab-induced reactions involving carbohydrates (from mono-oligosaccharide to macromolecular size). This chapter, therefore, discusses the study of such processes by CE, showing several applications of this technique:

1. Monitoring of synthesis (both chemical and enzymatic) of short saccharidic compounds (glycosides and oligosaccharides).
2. Preparing glycoconjugates, including macromolecules with a polysaccharidic backbone decorated with either saccharidic or nonsaccharidic small molecules, and polymers different from polysaccharides (proteins, synthetic polymers) conjugated with saccharidic moieties.
3. Evaluating processes involving enzymes acting on carbohydrates (with special care to polysaccharides).
4. Studying naturally occurring processes and reactions in single cells.

When necessary, CE will be compared with other analytical techniques.

7.2 Synthesis Involving Mono- and Oligo-saccharide Species

The modern chemist has many different analytical tools for monitoring both chemical and enzymatic reactions. The large number of recently published reports indicates that capillary electrophoresis is increasingly used for the direct analysis of the products of a synthesis. Let us focus, then, on the characterization of reactions involving mono- and oligosaccharides.

The studies of reactions using glycosyltransferases are usually carried out using (1) radiolabeled sugar nucleotides when the enzyme has weak activities, (2) spectrophotometric assays based on the release of fluorophores, (3) HPLC or TLC using a fluorescently labeled sugar acceptor, and (4) CE as a valid alternative [22–25].

The transglycolytic enzymatic synthesis of the Tn antigen (GalNAc-α-*O*-Ser), a tumor-associated carbohydrate, has been reported by Coslovi and coworkers [26].

A micellar electrokinetic chromatography (MEKC) method (with a borax–sodium dodecyl sulfate [SDS] buffer) and product derivatization with *N*-fluorenylmethoxycarbonyl (Fmoc) have been used for monitoring the kinetics of the transglycosylation reaction and for the setting up of the reaction optimization. Moreover, the CE analysis results were useful for investigating the role of the bulkiness of the serine-protecting groups on the yield.

A similar MEKC approach (borax–SDS buffer), but with the use of 4-aminobenzonitrile (ABN) as the derivatizing agent, has been reported by Campa et al. [27] for the transglycolytic synthesis of a trisaccharide (Glcα1-4Glcα1-6Glc) and a tetrasaccharide (Glcα1-4Glcα1-4Glcα1-6Glc) structurally related to pullulan. In this case, CE turned out to be a powerful technique for the structural characterization of the product formed and for the consequent comparison of two different enzyme performances, allowing the identification of the proper enzyme and the right synthetic strategy. The separation of oligosaccharides corresponding to the malto/isomalto series can be also successfully achieved by liquid chromatography, such as HPAEC-PAD [28], or reverse-phase chromatography of *per-O*-benzoyl derivatives [10], but an advantage of CE is that it requires minimal sample amounts, therefore being the technique of choice when monitoring reactions involving precious reactants such as enzymes or complex carbohydrates.

A number of papers report the use of CE for the characterization of galactosyltransferase activities. Snow et al. [29] report a CE-LIF application for the monitoring of the β1,4-galactosyltransferase enzymatic activity of recombinant proteins involving a fluorescent glycopeptides acceptor. The proposed method enabled the separation of lactose/glucose analogues resulting from the use of Uridine Diphosphate Galactose (UDP-Gal)as the activated donor molecule, thanks to the addition of hexamethylene diamine [an electro-osmotic flow (EOF) modulating agent] to the borate buffer. This approach, which eliminates the need to overexpress or purify the recombinant protein, is potentially applicable to the enzymatic analysis of different glycosyltransferases. CE-LIF [separation of the 6-(fluorescein-5-carboxamido) hexanoic acid succimidyl ester (FCHASE)-labeled products with phosphate-SDS-borate buffer] has been used also for the characterization of galactosyltransferase activities of three enzymes, and sequentially used in the Forssman antigen (Fa) synthesis [30].

A CE-LIF application has been used for the evaluation of synthetic UDP-Gal analogues as donors for three retaining configuration galactosyltransferases [31]; for this example, the rate of transfer from the modified donors was obtained with an injection of 3.1 of tetramethylrhodamine (TMR)-labeled oligosaccharide reaction products; a buffer composed by phosphate–sodium borate–SDS–phenyl boronic acid was used for the MEKC method. Similarly, Tsuruta et al. [32] reported the use of CE-LIF for the evaluation of two guanosine diphosphate (GDP)-5-thiosugars as glycosyl donors for mannosyltransferase.

For monitoring glycosyltransferase activity, some interesting CE assay methods have been reported elsewhere [33].

Both CE-LIF and CE-MS have been used to characterize complex gluco-oligosaccharides mixtures of two regioisomer series (degree of polymerization [DP]: 2→9) synthesized with the use of a transglycosidase and with glucose or

maltose as acceptors [34]. Under acidic buffer conditions (acetic acid and triethylamine), the 8-aminopyrene-1,3,6-trisulfonate (APTS)-gluco-oligosaccharides of each series were separated, enabling unambiguous size determination with CE-MS. However, neither these acidic conditions nor the alkaline buffer system could be adapted for the separation of the gluco-oligosaccharide regioisomers arising from the two combined series. In contrast, increased resolution was observed in an alkaline lithium borate buffer, using differential complexation of the regioisomers with the borate anions. The authors also investigated the ability of CE to resolve several glucose disaccharide regioisomers in order to validate separation of compounds that present the various linkage types generated by the enzymatic reactions.

A significant example regarding the monitoring of a chemical synthesis has been reported by Campa and colleagues [35]: the synthesis of some glycosylamines was carried out by treatment of the corresponding reducing sugars with ammonium hydrogen carbonate in concentrated ammonia. The reaction mixtures were preliminarily analyzed with capillary zone electrophoresis (CZE) with indirect UV detection (buffer sorbate-TTAB buffer) and HPAEC-PAD methods for the identification of the products and for clarifying the origin of some side products. It is important to provide an accurate evaluation of product yields in order to guide studies for reaction optimization. The quantification of the products, precluded by CZE and HPAEC, has been accomplished only by MEKC analysis of the corresponding Fmoc derivatives. Another example has been reported by Dreef-Tromp et al. [36], regarding the preparation of heparan sulfate-like oligomers via solid-phase methodologies with the use of polyethyleneglycol (PEG). A reverse CE-UV method (5-sulfosalicylic acid buffer) was employed for the determination of the identity and purity of the synthesized compounds.

Capillary electrophoresis is sometimes used as a complementary analytical technique with respect to HPLC. There is a report on how CE separation conditions could be exported to reverse-phase (RP) HPLC for the purification and quantitative isolation of products in nonpurified synthesis mixtures [37]. Among the saccharidic compounds, *O*- and *C*-allyl-glycosides are particularly challenging for CE, because of the lack of both native charges and anomeric carbon available for derivatizing agents. In this work, the CE buffer compositions have been optimized, exploiting specific interactions between analytes and buffer additives such as borate ion and SDS, in order to modulate the separation of α and β anomers. The data show that MEKC analysis provides good and selective separation of all anomeric couples, whereas CZE turns out to give satisfactory separation for *C*-allyl galactosides and *O*-allyl glucosides. These results demonstrated a simple route for the isolation of hard to synthesize pure β anomers, using RP-HPLC, which is known to provide separation selectivity similar to that of MEKC.

Regarding the comparison of CE with other analytical techniques, a crucial example was reported some years ago [38]: a fused silica capillary and borate–SDS buffer were used for monitoring chemical modification of trisulfated disaccharides, obtained by enzymatic depolymerization of heparin, and for the preparation of sulfated sucrose derivatives. These highly charged compounds preclude the use of TLC for monitoring reactions; furthermore, the high resolution power of CE permits

the separation of complex mixtures otherwise unachievable by a low-resolution technique such as TLC.

Heparin is an effective and well-established agent for thrombosis treatment. Recently, a novel class of synthetic sulfated bis-lactobionic acid amides were designed as important heparin-like pharmaceuticals. CE was used as a method for controlling the production of these preparations, for analyzing the purity of the final products, as well as for checking the decomposition of the species in solution [39]. The separation of the analytes, differing for the number of sulfate groups, was attempted with the use of electromigration injection, polyacrylamide-coated capillary, and background electrolytes (BGEs) of various compositions. The best results were obtained with acetate buffer with the addition of calcium lactate. It was shown that complex-forming equilibria between the analytes and bivalent cations present in the BGE provided for the selectivity necessary for the separation. When applied to old solutions, this method of analysis enabled also the identification of inorganic species such as nitrates and sulfates.

There are some cases in which CE has been used for a preliminary classification of the reaction products, and then the task of a complete characterization was done by chromatographic analysis. This was the case in the work reported by Splechtna et al. [40] regarding the preparation of prebiotic galacto-oligosaccharides from lactose using transgalactosylation capability of β-galactosidases; a phosphoric acid buffer-based CE method was used for a preliminary classification of mono-, di-, tri-, and tetrasaccharides, while HPAEC-PAD ensured the complete separation and identification of the signals. A similar strategy was used for the investigation of how the hydrolysis of lactose operated by two extremely thermostable β-glycosidases is affected by transgalactosylation [41]; in this case, the CE analysis was conducted upon aminopyridine derivatization of the reaction mixtures.

There are several reports of the usefulness of CE also for monitoring degradation reactions. In 2001, Yu et al. [42] reported the application of CE-UV to the characterization of the products resulting from lactonization and acid-catalyzed hydrolysis of α2,8-linked oligosialic acids. These two reactions are competitive under acidic conditions; the authors studied the settings that allowed both reactions to proceed, and the product mixtures were analyzed by CZE with a phosphate buffer and UV detection. In another case, CE turned out to be a good choice for the quantification of *N*-acetylglucosamine and five *N*-acetyl-chitooligosaccharides (C2-C6) produced after a chitinase reaction [43]. The detection of the underivatized oligosaccharides was directly performed with capacitively coupled contactless conductivity detector (C^4D) and a separation buffer composed of NaOH with 10% acetonitrile as the background electrolyte. In a strongly alkaline medium, the negatively charged sugars have lower mobility than hydroxide (the major anion component of the electrolyte buffer), enabling conductivity detection. The addition of acetonitrile improved the resolution of adjacent peaks; moreover, the use of CH_3CN is also important to increase the signal-to-noise (S/N) ratio because it reduces the conductivity of the electrolyte, diminishing the joule effect. The limit of the resulting detection is about 3 μM, and the data indicated that the larger the oligosaccharides, the higher the sensitivity.

In another paper [44], the purity of a series of large oligosaccharides (DP: 10→16), enzymatically prepared from acharan sulfate, were investigated with MEKC-UV (disodium phosphate/SDS/tetraborate buffer) and polyacrylamide gel electrophoresis (PAGE) on crude reaction mixture, obtaining consistent results.

There are some very particular and interesting examples of CE application to directly monitor the proceeds of a reaction course. Taylor and coauthors [45] reported the use of a borax–SDS buffered method to monitor β-glucuronidase hydrolysis conditions of drug glucuronide and sulfate conjugates. One of the major advantages of CE over chromatographic separation techniques for drug metabolism studies is that phase II drug metabolites, such as glucuronide and sulfate conjugates, may be analyzed directly and simultaneously with the parent drug [46]. The peculiarity of the proposed method is that the reactions were directly carried out in the autosampler vials with incubation in a thermostated CE autosampler tray; the proceeding of the course of the reactions was followed with sequential runs. The only drawback of this approach is that the measurable rate of reaction is limited by the number of runs that can be performed within the time required to hydrolyze the substrate.

Interesting work was also recently done by Urban et al. [47]: an enzymatic assay of glucose oxidase using 1,4-benzoquinone as the electron acceptor. The reaction and the assay were directly conducted in the separation capillary used for the analysis; in the transient engagement (plug–plug) mode, enzyme and substrate plugs migrate in the capillary until they merge, and the reaction is initiated, giving rise to the product signals. This method is called electrophoretically mediated microanalysis (EMMA), and it was introduced by Bao and Regnier [48] in 1992. The injection sequence was as follows: (1) glucose oxidase; (2) BGE made of phosphate buffer containing glucose and sulfated β-cyclodextrin; and (3) 1,4-benzoquinone. In contrast to many other assays, this method does not require peroxidase as a coupling enzyme.

Finally, the literature reports also an application of direct coupling of CE and nuclear magnetic resonance (NMR) spectroscopy [49]. A crude adenosine dinucleotide, synthesized by solid-phase chemistry, was analyzed by continuous-flow CE-NMR. The developed capillary NMR interface also enabled acquiring two-dimensional CE-NMR spectra using a stop-flow mode, turning off the applied potential in the capillary entering the NMR probe. The electrolyte consisted of glycine buffer in deuterium oxide, and the separation conditions were optimized, taking into account the demands of NMR spectroscopy.

It is worth mentioning also, as an example, the application of CE to the indirect monitoring of a reaction via characterization of compounds released upon product degradation. Similarly to the synthesis of large saccharidic compounds, the indirect monitoring of a reaction has been also applied to oligosaccharides. A typical example was reported in 2003 by Ren et al. [50], who proposed a CE method using a fused silica capillary, phosphate buffer, and UV detection for monitoring the degradation of a sialic acid [α-(2-5)Neu5Gc] octamer, catalyzed by a neuraminidase. In this way the quantification of the released repeating units provided additional information about the original synthetic oligomeric structure.

7.3 Glycoconjugate Preparation

The potential of CE can be exploited also in the analysis of carbohydrate-containing macromolecules, in order to monitor their synthesis as well as to characterize the composition of the reaction mixtures. The literature concerning this specific application is not particularly extensive, but often CE offers some relevant advantages over other techniques; this is particularly true for large macromolecules, which are difficult to characterize, for example, by NMR. What renders CE a rather unique methodology is its ability to monitor species in an extremely wide range of molecular weights, allowing the detection of small and large molecules in the same electrophoretic run. This ability makes it very powerful in monitoring the derivatization and modification of polymers.

We now focus our discussion on two major families of saccharide-bearing macromolecules: those having a polysaccharidic backbone decorated with either saccharidic or nonsaccharidic small molecules, and those with a nonsaccharidic backbone conjugated with saccharidic moieties.

7.3.1 Saccharidic Backbone

In this context, CE is generally used to characterize the final product and determine the polymer degree of substitution (DS) after partial or total hydrolysis.

One example of partial hydrolysis is given in the work of Coradini and co-workers [7], in which the degree of substitution of a sample of hyaluronic acid partially esterified with butyric acid was calculated from the amount of free butyric acid released upon alkaline hydrolysis of the ester groups.

A similar example involving hyaluronic acid is reported by Pravata and co-workers [51]; the aim of their work was the synthesis of hyaluronans grafted to a variable extent with lactic acid. After the synthesis, the modified polymers were hydrolyzed in 1 M NaOH at 37°C, yielding free lactate. The hydrolyzed polymer was analyzed by CE (tetraborate buffer 25 mM at pH 8.9), which showed the disappearance of the modified polysaccharide signal and the appearance of a new peak, due to the released lactate. A problem to overcome was the high viscosity of the polymer solution, a rather common problem when dealing with polysaccharides, which may perturb the electrophoretic signals. To obtain an accurate quantitative determination of the free lactate, the use of sodium cholate as an internal standard was then necessary. The molecular weight of the analyzed polysaccharides ranged from 22×10^3 to 960×10^3 $gmol^{-1}$; for polymers such as hyaluronic acid, whose mass-to-charge ratio does not depend on the molecular weight, it is normally not possible to distinguish between different degrees of polymerization since all the polymers display the same electrophoretic behavior. This work shows very clearly the versatility of the CE technique; in a single analysis it is possible to follow the signal of the polysaccharide and that of free lactate, two molecules very different

in size. No other technique could give information on both molecules with this accuracy.

Tselios and coworkers [52] present another excellent example of the potentiality of this technique. They used CE during different steps of the synthesis: to monitor the cyclization reaction of the peptide and the oxidation of the polysaccharide (mannan), and finally to verify the formation of the product, obtained by conjugation of oxidized mannan to the cyclic peptide. In this case the absence of the signal of the free peptide was indicative of the complete consumption of the reagent. The purity of the cyclic peptide was monitored in a 30-mM phosphate buffer (pH 3.0). Analysis of mannan oxidation was performed in a 25-mM borate buffer (pH 9.3), while the formation of the mannan-conjugated peptide analogue was followed in a 50-mM phosphate buffer, pH 5.1, after optimization of analysis conditions. At this pH it was possible to separate the peaks of mannan, oxidized mannan, cyclic peptide, and mannan-conjugated peptide.

There are several works reporting on the complete hydrolysis of the macromolecule, including the saccharidic backbone, and the analysis of the released mixture of monosaccharides, usually after derivatization with a chromo- or fluorofore.

For example, in this way it was possible to confirm the synthesis of methyl derivatives of galactan, unusually derivatized in position 3 [53]. After controlled depolymerization, the obtained mixture of monosaccharides, derivatized with 4-aminobenzoic acid, was analyzed (150 mM borate buffer, pH 10) by co-injection with a standard set (rhamnose, xylose, arabinose, glucose, and galacturonic acid), and demonstrated the presence of methylated sugars. In this case, however, the lack of modified sugars as the standard required further NMR analysis.

In a similar way, Lazik and coworkers [54] monitored the reaction of carboxymethylation of starch; after reaction, the polymer was completely hydrolyzed and the monomer composition analyzed after derivatization with 4-aminobenzonitrile. For the analysis a 150-mM borate buffer, pH 10, was used. The substitution at different hydroxyl groups caused the formation of different borate complexes, amplifying the separation of the carboxymethyl-glucose regioisomers. The authors state that the separation efficiency of the mono- and disubstituted glucose ethers was superior to that obtained by gas chromatography.

Finally, an interesting application is reported by Lamb and coworkers [55], who characterized vaccines consisting of *Streptococcus pneumoniae* or *Neisseria meningiditis* polysaccharide of different serotypes covalently linked to formaldehyde-inactivated diphtheria toxoid carrier protein. Their aim was to resolve the vaccine from free diphtheria toxoid carrier protein, in order to monitor the purity of the product. In this case the best method to resolve protein and polysaccharide peaks resulted in a micellar CE (with sodium borate and SDS as running buffer). This study suggests that CE may be a useful tool also for the study of protein–polysaccharide conjugate vaccines as well as for monitoring their synthesis.

Note that all the analyses reported in this section were performed with the same uncoated silica capillary, notwithstanding the variety of samples.

7.3.2 *Non-saccharidic Backbone*

Glycopolymers also include macromolecules of natural or synthetic origin decorated with saccharidic moieties. One example of the application of CE to the analysis of synthetic glycopolymers is the work of Donati and coworkers [2], in which the synthetic polymer HSMA [poly(styrene-*co*-maleic acid)] was conjugated via amidic bond to different glycosylamines. After treatment of the conjugate with 3 M NaOH at 70°C for 1.5 h, the amount of sugar released was estimated at different degradation times, allowing the determination of the degree of substitution and the kinetic profiling of the hydrolysis reaction. In this case the hydrolyzed mixtures were analyzed without any further derivatization; instead, the analysis of the released sugars was performed with indirect UV detection, using as buffer 6 mM sorbate at pH 12.5.

There are more examples of the analysis of glycopolymers obtained after chemical glycosylation of protein substrate. The study of Maillard reaction products in milk proteins and lactose is a key point of the dietary industry, and there are several works reporting the study of this reaction with capillary electrophoresis. For example, Zhang and coworkers [56] investigated the effects of glycation of fibrinogen and human serum albumin (HSA) through Maillard reactions, to study the consequences of a diet enriched with glucosamine (a common dietary supplement to treat osteoarthritis). CE (20 mM sodium tetraborate, pH 9) proved to be "a rapid, sensitive, and powerful technique" for separating the glycation products formed by glucosamine and fibrinogen or HSA from glucosamine autocondensation products. Similarly, in the work of Moreno and coworkers [57], the products of the Maillard reactions between the ovine caseinomacropeptide and lactose were monitored, as a function of temperature and time. In this case, CE performed on the intact protein revealed the presence in the mixture of the modified proteins, as products of the Maillard reaction. The degree of substitution was instead determined by mass spectrometry and RP-HPLC. Otte and coworkers [58] reported in 1998 an extensive study of the reaction mixtures at different pH, and in the presence or absence of additives in the electrophoretic buffer. These analyses provided some hints about alterations of the protein conformation after the glycation reaction.

7.4 Saccharidic Species as Enzyme Substrate: Studies on Enzyme Activity and Mechanism of Action

Capillary electrophoresis is widely used to study mechanisms and kinetics of enzyme reactions, and most of the studied enzymes are the same as those employed in degradation reactions finalized to the structural investigation of polysaccharides.

The transglycosidase activity of a hydrolytic enzyme is frequently exploited for synthetic purposes; therefore, there has been a great effort dedicated to the enhancement of the desired activity through direct enzyme evolution.

A typical case has been recently reported [59] that discussed the evolution of an α-L-fucosidase into a α-L-transfucosidase. CE and NMR have been used for monitoring both transferase and hydrolytic kinetics related to the synthesis of fucooligosaccharides of biological value. Separation was done in the electroendosmotic mode with the use of simple conditions: a borax buffer and UV detection for the identification of the products of the reaction between *p*NP-αFuc (donor) and *p*NP-βGal (acceptor). A similar approach has been employed as a screening methodology for the direct evolution of glycosidases into transgalactosidases [60]. CE contributes to the characterization of enzymes that have lost most of their hydrolytic activity while keeping their transferase ability; the evolution of substrate (*o*NPGal, cellobiose, and maltose) and the concentrations of products were monitored with the use of xylose as the internal standard and sodium phosphate as the separation buffer, after a derivatization reaction with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS).

The combination of NMR and CE, together with ESI–MS, was also used for studying the mode of action of the recombinant C-5 epimerase AlgE4 encoded by the soil bacteria *Azotobacter vinelandii* and expressed in *Escherichia coli*, exhibiting a nonrandom mode of action when acting on mannuronan and alginates of various monomeric composition [61].

Pichia pastoris is a yeast frequently used as an expression system for the production of proteins or enzymes. Shao et al. [62] reported on work done on a recombinant *P. pastoris* strain that harbored three heterologous enzymes implicated in the synthesis of an α-galactosyltrisaccharide: a sucrose synthase, a UDP-glucose C4 epimerase, and an α-1,3-galactosyltransferase. The individual enzyme activities were assayed by CE.

The enzyme activity is generally characterized in terms of kinetic parameters and inhibition phenomena. We can mention, for example, some applications of CE in this field [63], such as the determination of K_m and V_{max} of the synthesis of a *N*-acetyllactosamine derivative, catalyzed by a galactosyltransferase. The proposed method involved the use of methylumbelliferyl (MU) glycoside of *N*-acetylglucosamine as the acceptor molecule, UDP-Gal as the donor, and sodium borate as the CZE running buffer; the presence of the MU residue allowed the use of the UV detector. After evaluation of the kinetic parameters, an inhibition test was also carried out using the only UDP molecule as an inhibitor. In 1998, Wu et al. [64] reported the use of CE-LIF for the quantification of 4-methylumbelliferone liberated from 4-methylumbelliferyl-β-D-glucuronide by β-glucuronidase; using an acetate buffer and fluorescence detector, the enzyme kinetics and enzyme activity in buffer and urine were fully determined. CE turned out to be a good technique for understanding the biological role of β-glucuronidase activity in various disease processes.

Capillary electrophoresis could be a useful tool in the choice of an enzyme: for example, the enzyme specificity varies widely between analogues glycosidases from different source. β-Galactosidases are the subject of a paper [65] in which the specificities of four different β-galactosidases have been studied by CZE with a borate buffer. After enzymatic hydrolysis, the mixtures of substrates and products

were tagged with ethyl 4-amino benzoate (ABEE) by reductive amination. The enzyme specificity has been evaluated monitoring the galactose released from various di- and oligosaccharides as well as a biantennary asialo *N*-glycan. The proposed method has proven to be highly useful in terms of speed and labor efficiency.

Another example has been reported by Desai et al. [66] and regards the elucidation of heparin lyases actions. Four chemically modified heparins were used with the aim of studying the substrate specificity of three heparin lyases (I, II, and III). The modified heparins include the *N*- and *O*-desulfated and the *N*-sulfated and *N*-acetylated derivatives and a heparin composed by L-galactopyranosyluronic acid residues. These heparins were digested with the three enzymes and the formed disaccharides were identified by CZE using the method developed by Al-Hakim and Linhardt [67]. From the analysis it was deduced that heparin lyase II presents a wide specificity, and it is able to act on all the chemically modified polymers, including substrates with linkages to unnatural α-L-galactopyranosyluronic acid residues. Heparin lyase III cleaves linkages that have reduced sulfation density and that contain β-D-glucopyranosyluronic acid residues. Heparin lyase I requires both *O*- and *N*-sulfation for the cleavage, demonstrating a higher level of specificity with respect to the other two enzymes.

The time course of heparin-like glycosaminoglycans degradation by heparin lyase I was examined also by Rhomberg and collaborators [68]. Heparin-derived sulfated hexa- to decasaccharides of known structure were subjected to purified heparinase I digestion and analyzed by means of matrix-assisted laser desorption ionization (MALDI) mass spectrometry and CE. The former technique enabled deducing the composition from molecular weight identification, whereas the latter provided a quantification of the fragments. After an optimization of the digestion buffer aimed at improving the compatibility with MALDI analysis, CE was performed in reverse polarity (30 kV) on uncoated silica capillaries, with UV detection at 230 nm. The running buffer was composed of 10 μM dextran sulfate, which is able to suppress unspecific interactions with the capillary wall, and 50 mM Tris/phosphoric acid at pH 2.5. In CE the identification of peaks was obtained using pure standards and MS data. The combination of these two techniques provided information on heparin lyase I activity, not only confirming its reduced rate of tetrasaccharide cleavage but also revealing the pattern to be predominantly exolytic.

Another strategy for understanding enzyme activity is the one used by Zhang and collaborators [69] to detect and characterize by CZE various pectic enzymes both in vitro and in vivo [69]. They employed purified oligomers of galacturonic acid (GalA) labeled at their reducing end with the fluorophore ANTS that was incubated in vitro, with bacterial or fungal endopolygalacturonase (EPG), with pectolyase, and in vivo with cotyledons. Enzymes activities of in vitro and in vivo hydrolysis were measured using capillary electrophoresis and a fused-silica capillary, a 0.1 M phosphate running buffer at pH 2.5, an applied voltage of 17 kV, and a fluorescent detection (λ_{ex} 364 nm, λ_{em} ≥440 nm). By comparing digestion patterns, substantial differences between the mode of action of fungal EPG and that of bacterial origin were observed, and the number of residues needed for the active site to bind was deduced. Tests in vivo, pursued by injecting labeled hexamer into the

intercellular spaces of a cotton cotyledon, demonstrated an in situ high degree of exogalacturonidase activity, but on increasing the incubation time, the same results as those observed in the in vitro experiments by using fungal EPG were achieved.

The former data were confirmed by studying the substrate specificity of fungal and bacterial EPGs [70] in CZE mode. The authors found that four or more adjacent galacturonic residues are the site of action of both enzymes, but there was a difference between the products obtained from pectic acid by exhaustive digestion with the two enzymes.

The kinetic study of the chondroitin sulfate (CS) degradation with ABC lyase was performed by Denuzière and coworkers [71] by means of CZE using an uncoated fused silica capillary, 100 mM borate buffer at pH 9.0, and a voltage of 20 kV at 45°C. The final aim of the work was to apply the CZE method to a comparison of the chondroitinase hydrolysis of CS alone or complexed with chitosan. The kinetics of depolymerizations were obtained by the quantitative determination of the ΔDi-4S and ΔDi-6S disaccharides released with time. At physiological pH, the understandably lower amount of disaccharides released by enzyme action from chondroitin–chitosan complexes led to the conclusion that chitosan is able to protect CS backbone from hydrolysis, making it unrecognizable to the enzyme.

By monitoring (in CE) the action pattern of polysaccharides lyases, it is also possible to obtain a theoretical model of the active sites and subsites of enzymes in use.

In the work of Kinoshita et al. [72], the optimization of an analytical separation in CE evolves in an enzymatic mechanism investigation of one of the most used enzymes in the HA digestion, the mammalian hyaluronidase. The ideal percentage of linear neutral polymer (PEG-70000) in the buffer was first searched in order to enhance the resolution of oligomers. A digested hyaluronan (HA) was then analyzed by means of a capillary column coated with dimethylpolysiloxane (DB-1) in a Tris–borate electrolyte containing an optimized concentration of linear neutral polymer (PEG-70000). The observation of the already known, although substantially unexplained, phenomenon of the reversal migration order of smaller oligomers [73] stimulated further exploration of the hyaluronidase action. The result is the investigation, once more supported by CE, of the mechanism underlying the recognition and hydrolysis/transglycosilation of purified hyaluronic acid oligomers by a mammalian hyaluronidase based on the electrophoretic behavior. With this aim small oligomers (4-, 6-, 8-, 10mer) were incubated with the enzyme and analyzed by CE as a function of reaction time and temperature. Transglycosylation, leading to larger oligomers than the starting ones, was found to be more predominant at low temperature (4°C), whereas, as expected, hydrolysis predominated at higher T (37°C). By coupling CE results with the fluorescence polarization data for studying the binding between 3-aminobenzoic-labeled oligomers and hyaluronidase, the authors proposed a specific model for the enzyme binding site. There, the partial overlap of separate recognition and cleavage sites combined with recognition subsites seemed to justify the patterns of hydrolysis and transglycosylation observed by CE. Analysis of oligomer digestion was performed in normal polarity using a fused-silica capillary and a running buffer of 50 mM sodium tetraborate and 100 mM SDS at pH 9.3.

A similar detailed analysis of the enzymatic reaction product is facilitated by the availability of recombinant enzymes. An example is reported by Hofinger et al. [74], in which hydrolysis and transglycosylation activities, minimum substrates, and reaction kinetics of recombinant human enzymes PH-20 and Hyal-1 are compared with those observed for the bovine testicular hyaluronidase (BTH). The characterization was performed by CZE (with uncoated fused silica capillary and 50 mM sodium phosphate/20 mM tetraborate at pH 9.0 as running buffer) by directly injecting into the CE the complex mixture of oligosaccharides, enzymes, and incubation buffer, just after a simple drying and centrifugation procedure. The analysis revealed pH-dependent differences in the catalytic behavior of the BTH with respect to its minimum substrate (i.e., the hexasaccharide), and, interestingly enough, the same hexasaccharide was not a substrate of either of the recombinant enzymes. The dependence of both hydrolytic and transglycolytic abilities of the investigated enzymes on substrate concentrations and pH of the medium were also studied. The kinetic measurements were interpreted in terms of subsite affinity toward substrate moieties in the framework of the model proposed by Highsmith et al. [75].

Capillary electrophoresis was the method chosen by Park et al. [76] to study the action of the bacterial type of hyaluronidase from *Streptomyces hyalurolyticus*, which specifically catalyzes hyaluronic acid depolymerization and does not act on the related glycosaminoglycans. Here, the oligosaccharide mixtures prepared by controlled depolymerization of hyaluronan were fluorescently labeled at the reducing end with ANDS (7-amino 1,3-naphthalene disulfonic acid), and CE analysis was used to follow the kinetics of the enzyme-catalyzed hydrolysis. Analysis was performed in reverse polarity mode (18 kV) using a 20-mM phosphoric acid running buffer adjusted to pH 2.5 with 1 M dibasic sodium phosphate and a fluorescence detection (λ_{ex} 250 nm, λ_{em} 420 nm). The CE results associated with computer simulation confirmed that this lyase acts on hyaluronic acid with random endolytic activity. A final tetrasaccharide-to-hexasaccharide ratio of 2 was also predicted by the modeled action, compared with the value of ~1 that was found experimentally. This discrepancy led to the suggestion that a resistant site repeating unit, corresponding to 10–12 saccharide residues, was present.

More recently the performances of CE as an analytical tool shown studying the action of hyaluronate lyase from *S. hyalurolyticus* on hyaluronic acid (HA) have been compared with that expressed by HPLC [77]. Oligosaccharides with a DP ranging from 4 to about 30 obtained from HA depolymerization catalyzed by *S. hyalurolyticus* were successfully separated by high-performance capillary electrophoresis (HPCE) on uncoated fused-silica capillary tubes using a disodium phosphate/sodium tetraborate/SDS buffer at pH 9. Underivatized products were UV detected at 230 nm exploiting their 4,5-unsaturation. Optimized conditions were applied to analyze the tetra-/hexa-saccharide (T/H) ratio produced by depolymerization of HA samples from several sources. Separation of tetra- and hexasaccharide species performed by HPCE showed a sensitivity 20-fold greater than that obtained by strong-anion exchange (SAX)–HPLC. HPCE was also found to be a better approach than HPLC for analyzing *S. hyalurolyticus*-catalyzed depolymerization products from cross-linked HA sample. Interestingly, values of the T/H ratio

close to 2 were found for the sample of HA of fermentative origin and for the sample extracted from the body of the mollusc *M. galloprovincialis*. The findings have important biological implications, supporting the presence of restricted access sites of definite length [76] that likely originated from ordered secondary (or higher) structures.

7.5 Studies of Natural Processes and of Reactions in Single Cells

A significant number of reports address the application of CE to biosynthetic transformation; the aim is to study natural processes or the characterization of cellular enzymatic activities. CE is particularly suitable for this field of analysis because with simple methods it is possible to analyze small amount of precious or nonpurified samples, most of which do not require the always undesirable sample manipulation.

An interesting example is a report on the use of CE-MS for synthetic in vitro glycolysis studies using ten glycolytic enzymes [78]. This approach has the beneficial ability to perform direct and simultaneous determination of the various metabolites; most of the intermediates and nucleotides were selectively separated within 25 min with the use of a cationic polymer (Polybrene)-coated capillary, and ammonium acetate as the electrolyte. The MS detection of the deprotonated ion forms has been ensured by a continuous flow of ammonium acetate in 50% v/v methanol/water as the sheath liquid. The use of this method yields important information about the regulation of sequential enzyme-catalyzed reactions.

In 2007, Wilson and coworkers [79] described the use of CE-UV for monitoring the reaction of incorporation of UDP-glucose into glycogen, catalyzed by glycogen synthase; the use of a tetraborate buffer and an uncoated capillary enabled the quantification of UDP, the product of the enzyme activity, and the determination of the kinetic parameters as K_m. This new approach is a good alternative to the more conventional method of spectrophotometric or radiometric assays. With appropriate modification, this procedure could be applicable also to other enzymes having nucleotides as substrates.

In recent years, many efforts have been devoted to setting up more sensitive enzymatic assays; there are reports in the literature that the use of fluorescent synthetic substrates enzymatically converted to fluorescent products and a phosphate-borate-SDS-buffered CE-LIF separation method [80] could represent a good answer to this problem. However, the ultimate challenge is the detection of the products formed in a single cell; the typical low sample consumption of CE is a key factor in the choice of this technique. Shoemaker et al. [81] set up a novel CE-LIF method for assaying enzyme from a single cell or small cell populations. The possibility of performing the multiple sampling enabled the detection of enzyme heterogeneity and reaction monitoring over time; this is a relevant feature in comparison to other methods that provide only a "snapshot" of enzyme activities [82–87].

Specifically, the authors report the characterization of the activity of two glucosidases and a acetylgalactosaminyltransferase in *Spodoptera frugiperda* cells using a synthetic fluorescent substrate αGlc(1→3)αGlc-tetramethylrhodamine, and sodium phosphate–SDS–phenyl boronic acid–sodium tetraborate buffer eventually added 10% dioxane to enhance carbohydrates separation.

The same labeling agent, TMR, was used by another research group [88] to monitor biosynthetic transformations of labeled *N*-acetyllactosamine in crude microsomal extracts operated by enzymes. The proposed CE-LIF method enabled the simultaneous detection of various compounds enzymatically synthesized by fucosyltransferase and fucosidase, or by degradation reactions run by galactosidases and hexosaminidase on LacNAc-*O*-TMR. During the 11-min time course analysis, all six molecules were baseline resolved with the use of a running buffer composed of 10 mM Na_2HPO_4, 10 mM borate, 10 mM phenyl boronic acid, and 10 mM SDS (pH 9). The simultaneous use of borate and phenylboronic acid was necessary for the complete separation of the neutral species.

Among the studies of β-galactosidase is the work of two Canadian groups [89, 90]; in the first study, the authors report a fluorescence-based enzymatic assay by CE-LIF for the determination of few β-galactosidase molecules. The enzyme can be assayed by monitoring the generation of fluorescent products, fluorescein-mono-β-D-galactopyranoside, and fluorescein, when the fluorogenic substrate fluo rescein-di-β-D-galactopyranoside is used. Adopting a low-ionic-strength buffer, Tricine, for both the enzymatic reaction and the CE separation, the authors attained good enzyme activity and an efficient separation. In the latter study, the method was further improved; using a substrate based on the dye resorufin, the limit of detection (LOD) result was 40-fold lower than that obtained with a fluorescein-based substrate, thanks to a greater turnover number of the enzyme. Also in this case, the same buffer (HEPES) was used as the medium and the running buffer; another advantage of this CE-LIF method is the use of a relatively inexpensive He–Ne laser instead of the Ar^+ one.

Capillary electrophoresis was also used to overcome analytical separation issues; for example, CZE-UV (tetraborate buffer) turned out to be a complementary, or even more powerful, technique compared to the chromatographic approach for the separation of the Maillard reaction products of glucosamine with glyceraldehyde, glucose, and fructose [91]. A CE method with postreaction derivatization has been successfully used for the enzymatic assay of α1,3-galactosyltransferase, the enzyme implicated in the biosynthesis of α-Gal epitope involved in the hyperacute rejection in xenotransplantation [92]. The authors applied a fluorophore-assisted capillary electrophoresis (FACE) method using an underivatized acceptor substrate; the reaction mixture was directly analyzed by CE-UV under inverted EOF (phosphoric acid/triethylamine buffer), after reductive amination with ANTS. The use of triethylamine (TEA) as an EOF modifier improves the analysis time without a loss in efficacy, and it resolves isomeric disaccharides. With this approach, a purification step is not required, and the absence of a label on the acceptor during the enzymatic reaction precludes any interference of the label with the enzyme.

7.6 Conclusion

Due to the complexity of carbohydrate-involving processes, several complementary analytical approaches may be needed for their characterization. The large number and variety of applications surveyed in this chapter demonstrate the reliability of capillary electrophoresis, which can be successfully applied for the monitoring of the synthesis of glycosides, oligosaccharides, and glycoconjugates, as well as for the characterization of the mode of action of enzymes having carbohydrates as subtrates.

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Chapter 8
Analysis of Intact Glycoprotein Biopharmaceuticals by Capillary Electrophoresis

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Abstract Many of the biopharmaceuticals currently produced are glycoproteins in which the oligosaccharide chains can impact markedly on bioactivity, antigenicity, pharmacokinetics, solubility, stability, and protease resistance. Glycosylation profile may vary based on the host system employed to produce a recombinant glycoprotein. Downstream and upstream processes employed to produce the finished product may also significantly impact on the composition of the protein in terms of glycosylation. It is therefore mandatory to control glycoproteins by robust methods able to provide sufficient details on minor modifications. Capillary electrophoresis (CE) has proved its usefulness not only for the characterization of glycoprotein pharmaceuticals but also as a quality-control (QC) tool allowing accurate quantitation. CE has been demonstrated to provide rapid, automated, and quantitative results combined to a high resolving power that meets the requirement for a QC method. This chapter focuses on the most recent (as of mid-2009) applications of CE aimed at producing methods to address either QC or characterization issues for intact glycoproteins used as biopharmaceuticals or relevant for clinical or diagnostic purposes. For biopharmaceutical applications, the discussion encompasses process monitoring, stability testing, control of the finished product or the purified glycoprotein, as well as the influence of process conditions and production steps on the bioproduct structure and glycosylation. Also, the implications of the different CE modes (CZE, CIEF, SDS–CGE, and MEKC) are highlighted and discussed.

Keywords Capillary electrophoresis • Glycoproteins • Oligosaccharides • Bio pharmaceuticals • Quality control • Stability tests • Biological fluids • Clinical applications

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Abbreviations

ATIII	Antithrombin III
BGE	Background electrolyte
BHK	Baby hamster kidney cells
CDT	Carbohydrate deficient transferrin
CDG	Congenital disorders of glycosylation
CE	Capillary electrophoresis
CE-MS	Capillary electrophoresis coupled to mass spectrometry
CGMP	Caseinoglycomacropeptide
CIEF	Capillary isoelectric focusing
CHO	Chinese hamster ovary
CZE	Capillary zone electrophoresis
DAB	1,4-Diaminobutane
DAP	1,3-Diaminopropane
EOF	Electro-osmotic flow
hCG	Human chorionic gonadotropin
ICH	International Conference on Harmonization
IEF	Isoelectric focusing
MEKC	Micellar electrokinetic chromatography
NESP	Novel erythropoiesis-stimulating protein
QC	Quality control
rhATIII	Recombinant human antithrombin III
rhDNAse	Recombinant human deoxyribonuclease
rhEPO	Recombinant human erythropoietin
rhFSH	Recombinant human follicle-stimulating hormone
rhFVII	Recombinant human factor VII
rhGCSF	Recombinant human granulocyte colony-stimulating factor
rhIFN-γ	Recombinant human interferon-γ
rhIL-7	Recombinant human interleukin-7
rhtPA	Recombinant human tissue plasminogen activator
SDS–CGE	Sodium dodecyl sulfate gel capillary electrophoresis
SDS–PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

8.1 Introduction

Biopharmaceutical is a term that describes therapeutic proteins produced by biotechnology and specifically via genetic engineering or, in the case of monoclonal antibody, by hybridoma technologies [1].

Recombinant monoclonal antibody pharmaceuticals for human therapeutics are growing considerably in number. It is estimated that 30% of new drugs likely to be licensed during the next decade will be based on antibody products [2]. They are becoming more and more prevalent due to their excellent effectiveness,

particularly for oncology, rheumatic diseases [3, 4], and immunosuppression in cardiac transplantation [5].

Many of the biopharmaceuticals currently produced are glycoproteins. Glycosylation of proteins is one of the most important posttranslational modifications that provide a source of heterogeneity for recombinant glycoproteins produced by eukaryotic systems. Difference in site-occupancy glycosylation may result in macroheterogeneity, while variation in the glycan chain structures that can occur at individual glycosylation sites is called microheterogeneity [6]. In glycoproteins, especially produced by biotechnology, the oligosaccharide chains can impact markedly on bioactivity, antigenicity, pharmacokinetics, solubility, stability, and protease resistance [7–10].

Glycosylation profiles may vary based on the host system employed to produce a recombinant glycoprotein, but also on the cell culture conditions or the purification process employed. Due to difference in the cell machinery, host cells employed for the expression of human protein do not have the same capability to glycosylate proteins that the human body does. Deficiencies in sugar transferases are often the major reason for that dissimilarity. For example, recombinant human factor VII proteins (rhFVII) produced from Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cell cultures do not have the same glycosylation pattern. In fact, GalNAc residues are absent at the antennae termini of rhFVII produced in CHO. As a consequence, the BHK-derived rhFVII has a higher biological activity [11]. Recombinant erythropoietin (rEPO) is a heterogeneous mixture of glycoproteins used as pharmaceutical product for anemia treatment. However, great attention is being paid to EPO due to not only the benefits provided by its clinical use but also its misuse by athletes in competition sports. Zhang and Song [12] found that CHO and BHK cells sialylate rEPO better than the other mammalian cells commonly used, such as COS-7 and NSO cell lines [12]. Downstream and upstream processes employed to produce the finished product may also significantly impact on the composition of the protein in term of glycosylation.

In summary, macro- and microheterogeneities and variations in the proportion of different glycoforms within a heterogeneous population lead to functional consequences of therapeutic glycoprotein. Because of the reasons mentioned above, glycoform analysis is a fundamental requirement in the QC of recombinant glycoproteins. It is also mandatory for the characterization of all posttranslational modifications required by the regulatory authorities.

It is therefore indispensable to control these structures by a robust method able to provide sufficient details on minor modifications. In addition, for biotechnology-derived pharmaceuticals, batch-to-batch consistency should be demonstrated to warrant biological activity, stability, and safety. Finally, discrimination between the glycosylated and the nonglycosylated forms or among the different glycoforms of a glycoprotein can be relevant for clinical and diagnostic purposes.

Among the available analytical techniques employed for (glyco)protein analysis (mostly high-performance liquid chromatography [HPLC], electrophoresis, mass spectrometry [MS], and circular dichroism), capillary electrophoresis (CE) has proved its usefulness not only for the characterization of glycoprotein pharmaceuticals

but also as a QC tool. Many reviews have reported the advantages of CE in glycoprotein heterogeneity investigation, including monosaccharides composition [13], glycan screening [14–16], and analysis of intact glycoproteins [17–21]. Other reviews have focused more on quality assessment of recombinant glycoproteins [22] or on glycosylation studies of pharmaceutical glycoproteins [23] or more specifically on antibodies [24].

CE has been demonstrated to provide rapid, automated, and quantitative results combined to a high resolving power that meets the requirement for a QC method. The International Conference on Harmonization (ICH) has recommended this technique in its guidelines on specification for biotechnological and biological products ICH-Q6B [25]. In addition to purity and identity controls, quantitative analyses also have to be performed to provide batch-to-batch consistency and batch release. Finally, stability testing is generally performed to identify parameters (temperature, exposure to light, and pH conditions) that can alter the glycoprotein [26].

European Medicines Agency (EMEA) authorities have approved CE in the monographs of European Pharmacopoeia [27, 28] for the generation of data relating to product identification, assay, or testing for related protein impurities. Moreover, CE is also recognized by the biopharmaceutical industry in various aspects of the development process and for drug product characterization [29].

Indeed, QC can be performed at different stages of protein production (in-process monitoring, on the purified or formulated product, and preclinical testing). In-process tests are performed at critical decision-making steps and at other steps where data serve to confirm the consistency of the process during the production, while the analysis of a formulated product requires analyzing the active glycoprotein among excipients.

This chapter focuses on applications of CE aimed at producing methods to address either QC or characterization issues for intact glycoproteins used as biopharmaceuticals or for clinical or diagnostic purposes. This research area was approached 20 years ago by different pioneering works published as early as 1992. Since then, several reviews have discussed this subject [17–24]. In particular, Girard et al. [21] have extensively reviewed up to 2007 the analysis of glycoproteins by CE, comprising recent innovations to circumvent protein adsorption, improve detection, and provide practical applications of CE to characterize biopharmaceuticals and glycoproteins in the clinical field. Here, CE methods aimed at establishing peptide or glycan mapping are excluded. To avoid redundancies with the Girard review, we emphasize the most recent developments up to mid-2009; each section below discusses a specific target application field.

8.2 General Considerations

Recombinant glycoproteins exhibit a high heterogeneity. The analyst has to differentiate which findings are attributed to the accepted level of heterogeneity of the product and which are attributed to impurities. Process-related impurities encompass

those that are derived from the manufacturing process, such as cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing. Product-related impurities are molecular variants arising during manufacture or storage that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety [25]. These products may appear after chemical (e.g., deamidation, oxidation, fragmentation, isomerization) or physical (aggregation, denaturation) degradation, but also after an enzymatic reaction (exposure to proteases or carbohydrases). As an example, protein aggregation can be encountered at all stages of protein drug development [30].

Glycoprotein analysis can be realized with various CE modes. Capillary zone electrophoresis (CZE) is the most often used method for heterogeneity determination and glycoprotein characterization, depending on the glycoprotein's charge-to-mass ratio. It is very successful for the analysis of glycoforms showing different degrees of glycosylation and that differ, for example, in the number of sialic acid residues. But this method can also resolve proteins having close structures, such as deamidated and oxidized forms of a given protein. However, often, electrostatic or hydrophobic interactions with the inner surface of the capillary result in protein adsorption, which leads to poor reproducibilities of migration times and peak areas and to resolution deterioration. To circumvent this problem and to improve the technique's performance, several strategies are available, which are described comprehensively in several reviews [31, 32]. The most common (and probably most efficient) approach to reduce wall adsorption of proteins relies on permanent coating or dynamic modifications of the capillaries. This latter process consists of altering the background electrolyte (BGE) composition with additives such as zwitterions, amines, polymers, and surfactants. Generally, under a counter-electro-osmotic flow (EOF) mode, the resolution among closely related glycoforms can be improved when electrophoretic mobilities of glycoforms and electro-osmotic mobility (which are in the opposite direction) are similar.

Micellar electrokinetic chromatography (MEKC) can be useful for analyzing glycoproteins, which could exist as noncovalent polymeric forms or which exhibit a limited solubility in aqueous solution. In this mode, surfactants are added to the running buffer at a concentration higher than the critical micellar concentration (CMC). The surfactant interacts with the proteins by forming complexes that generally carry the charge of the surfactant itself.

Capillary isoelectric focusing (CIEF) separates glycoproteins/glycoforms based on their isoelectric point (p*I*) and additionally can be employed for the estimation of the p*I* of the bioproduct of interest as an identity control. It is probably the mode of CE for glycoproteins that has the best resolution.

The sodium dodecyl sulfate gel capillary electrophoresis (SDS–CGE) mode is carried out in capillaries containing gel that are often replaceable polymers in solution and that provide a size-based separation. SDS is a surfactant often employed to confer the same charge density (negative) on all the proteins and thereby the same electrophoretic mobility. Separation, in this case, is based only on the size of the proteins. This mode is particularly useful for estimating the molecular weight of proteins or for separating glycoforms having a high variation

in their glycosylation (macroheterogeneity). However, with glycoproteins, things may be quite different, as SDS is less prone to form complexes with glycoproteins due to their hydrophilicity, and molecular weight estimation may become inaccurate with these molecules. SDS–CGE is routinely employed to analyze monoclonal antibodies [33–35].

8.3 Capillary Electrophoresis and Glycoprotein Pharmaceuticals

8.3.1 Impact of the Process

8.3.1.1 Host Cell or Expression System

Since the different cell lines do not have the same enzymatic machinery, the recombinant glycoproteins derived from different expression systems are generally differently glycosylated. *Escherichia coli* results in aglycosylated protein often being recovered in inclusion bodies, while yeast expression system add sugar chains rich in mannose residues that can be longer than traditional high mannose oligosaccharides, often called manannes [36]. Plants, which glycosylate proteins to which xylose and fucose have been added, are reported to be immunogenic in humans [37]. Insect cell lines glycosylate proteins differently from the mammalian cell patterns and cannot attach complex-type oligosaccharides to the proteins. In contrast, mammalian cells are able to add complex glycan structures that are quite similar to those found in human proteins but without the possibility of adding the bisecting *N*-acetylglucosamine. Variations in glycosylation pattern between cell species are important and go beyond the simple differences in the linkage and branching patterns of the glycans, which enable them to be easily detected by CE.

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine that regulates the proliferation and differentiation of neutrophils. It is commercially available in two forms: glycosylated recombinant human (rh)G-CSF and nonglycosylated rh-met-CSF expressed in CHO cells and the *E. coli* expression system, respectively. Glycosylated G-CSF has an *O*-linked carbohydrate chain attached to threonine-133. Glycosylated and nonglycosylated forms of rhG-CSF cannot be distinguished by traditional biological assays; however, they can be successfully separated by CE under acidic conditions and using a polymer (hydroxypropylmethylcellulose, HPMC) in the BGE [38]. This method enables distinguishing clearly the two products since the glycosylated rhG-CSF showed two distinct peaks, while nonglycosylated rhG-CSF migrated slightly earlier due to the absence of neuraminic acid. In other CZE methods, rhG-CSF expressed in a baculovirus vector system and analyzed by CZE under alkaline conditions without HPMC exhibited a different

glycoform pattern that was attributed to the processing of glycosylated proteins in insect cells that differ from the CHO cells pathways.

Very recently, Thakur et al. [39] developed a CE-MS method to analyze the α subunit of recombinant human chorionic gonadotropin (rh-αhCG) produced in a murine cell line. This glycoprotein consist of two subunits, both of them have two *N*-glycosylation sites, whereas subunit-β carries four *O*-glycosylated serine residues. hCG is considered a challenging biopharmaceutical due to its high heterogeneity [39]. A polyvinylalcohol (PVA)-coated capillary was used to decrease the adsorption, while acetic acid, which is compatible with MS detection, was used as the BGE. This CE-MS method was able to compare the glycoform profile of this glycoprotein produced from mouse or CHO cell lines. The authors found that the intact recombinant αHCG produced in CHO was less complex than that produced in mouse cells. Glycoforms bearing from one to five sialic acid residues were identified from the CHO-derived product, in contrast with glycoforms from the mouse cell line, which contained up to nine sialic acids. This emphasizes the importance of selection of the cell line so as to have the desired glycosylation pattern.

The vascular endothelial growth factor 165 ($VEGF_{165}$) is the predominant form and the most efficient angiogenic factor in the VEGF family (a complex family of proteins that regulate angiogenesis). Besides its angiogenic effect, its implication in neuroprotection has raised significant expectations in the therapeutic field. Recombinant human $VEGF_{165}$ produced by two different systems – *SF*-21 insect cell and *E. coli* – have been studied by De Frutos's group [40] using two CE methods to distinguish glycosylation heterogeneity. Two conditions were compared in order to suppress protein adsorption: a dynamic coating based on the addition of spermine in an SDS-containing buffer, and the use of a permanent polycationic-coated capillary. The best result was obtained with the Poly-LA 313-coated capillary, which allowed a slight separation of seven peaks for $VEGF_{165}$ glycoforms (Fig. 8.1).

The comparison of the electropherograms of $VEGF_{165}$ expressed in insect cells by both CE methods with those obtained for human $VEGF_{165}$ expressed in a nonglycosylating organism (*E. coli*) showed that there are more than one nonglycosylated form of $VEGF_{165}$ and several glycosylated forms. At the same time, the authors adapted the CZE method, which employed the poly-LA 313-coated capillary for the coupling to electrospray ion– time of flight –mass spectrometry (ESI-TOF-MS) [41]. This technique provided structural information about the different forms of $VEGF_{165}$, including glycosylation and loss of N- and C-terminus amino acids. Moreover, methionine oxidation in *E. coli*-derived VEGF could be detected.

The same group developed a two-step CIEF method to achieve the separation of the different glycoforms of $VEGF_{165}$ derived from insect cells and to compare the obtained profile to that of VEGF produced in *E. coli.* A neutral-coated capillary based on polyacrylamide was used in combination with the addition of 7 M of urea to the BGE to suppress protein aggregation [42]. This method was able to differentiate the glycosylated from the nonglycosylated recombinant $VEGF_{165}$ with up to seven different glycoforms distinguished in the case of the insect cell-derived product.

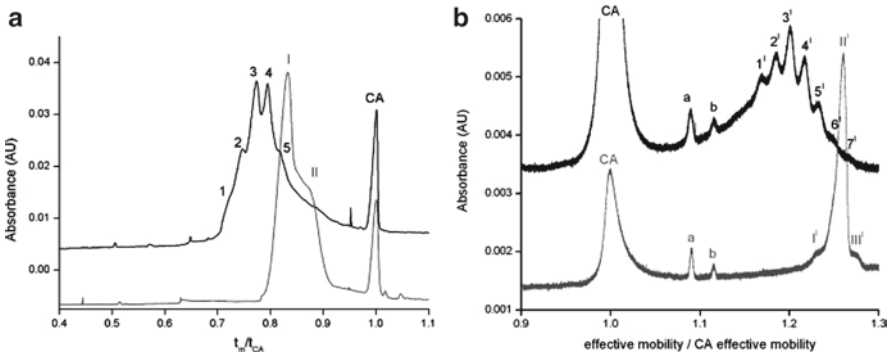

Fig. 8.1 Analysis of human $VEGF_{165}$ expressed in insect cells (*black line*) and human $VEGF_{165}$ expressed in *Escherichia coli* (*gray line*) by capillary electrophoresis (CE) under two different conditions. (**a**) Dynamically coated silica capillary: 50 μm inner diameter, 60 cm effective length, 67 cm total length; applied voltage 20 kV; background electrolyte (BGE), 40 mM sodium phosphate, 10 mM sodium tetraborate, 25 mM sodium dodecyl sulfate (SDS), 6 mm spermine, pH 7.2. Peak identification: carbonic anhydrase (CA), peaks 1–5 corresponding to human $VEGF_{165}$ expressed in insect cells, peaks I and II of human $VEGF_{165}$ expressed in *E. coli.* (**b**) Poly-LA 313-coated silica capillary: 50 μm inner diameter, 50 cm effective length, 58.5 cm total length; applied negative CE voltage −12 kV; BGE, 40 mM sodium phosphate, 1.6 mM sodium tetraborate, pH 3.0; Peak identification: peaks 1–7 corresponding to human $VEGF_{165}$ expressed in insect cells, peaks I–III corresponding to human nonglycosylated $VEGF_{165}$ expressed in *E. coli* cells (reprinted from ref. [40], with permission)

8.3.1.2 Cell Culture Conditions

Cell culture conditions (nutriments, presence of serum, pH of the medium, density of cells, type of cell feeding, and scale of the production) can have a significant impact on the glycosylation of secreted recombinant glycoproteins and on the stability of the products. Degradation, desialylation, and fragmentation of proteins can occur quite easily under certain cell culture conditions.

Rustandi et al. [43] reported the application of SDS–CGE for monoclonal antibody (mAb) development. Antibody-type pharmaceuticals of immunoglobulin G (IgG) have commonly attached to their heavy chains (HCs) *N*-glycosylated carbohydrate chains that play important roles in the effectiveness of antibodies. The glycan moieties are attached at a common site on the Fc region of the HC through asparagine 297. The high molecular weight of IgG represents a major challenge when analyzing the intact molecule. Therefore, the analysis of reduced IgG fragments, such as the Fc and Fab parts as well as light and heavy chains, is preferred to reduce the complexity and provides more information [44]. SDS–CGE was employed, in particular, for purity monitoring, but also to demonstrate the impact of cell-culture media and the cultivation process on the attachment of *N*-glycans to the HC of the antibody (Fig. 8.2). Condition B in Fig. 8.2 was found optimal with a very small amount of nonglycosylated heavy chain (NGHC), while the additional peak obtained under cell culture condition C (corresponding to an increase of mass of +2 kDa) indicated the appearance of an unusual glycoform carrying two sites of N-linked glycan in HC (HC +2 kDa).

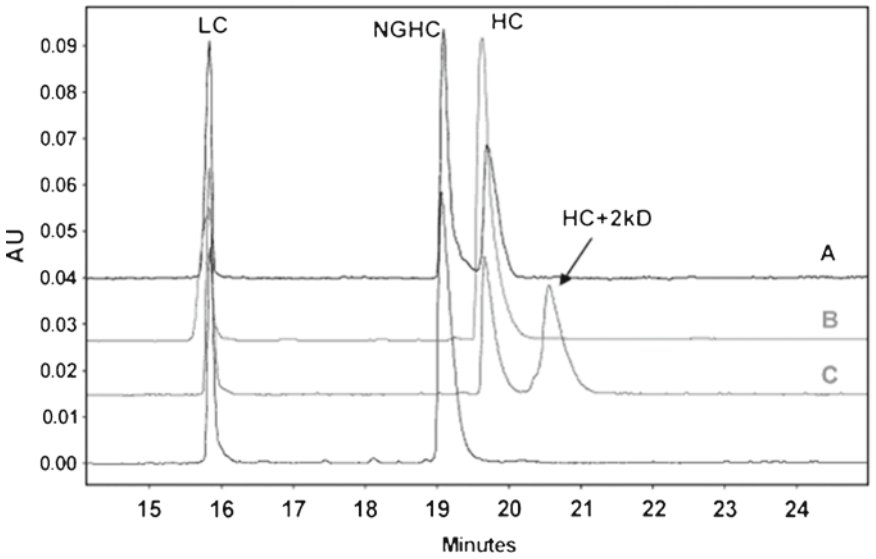

Fig. 8.2 Electropherograms of reduced monoclonal antibody (mAb) produced from various cell cultures. *Cell culture A* shows clearly high nonglycosylated heavy chain (NGHC), which is not wanted for mAb development. *Cell culture B* is the intended product with very low NGHC (0.5%). *Cell culture C* is mAb with unexpected extra peak called HC +2kDa. The trace at the *bottom* is peptide-*N*-glycosidase F (PNGase F)-treated mAb produced in cell culture C indicating that the HC +2 kDa peak contains additional N-linked glycan (reprinted from ref. [43], with permission)

Berkowitz et al. [45] have shown qualitatively and quantitatively, by their CZE method conducted in extreme acidic condition, the impact of using different CHO cells strains or different growth media conditions on the microheterogeneity of a produced therapeutic glycoprotein. In this method, triethanolamine was employed to prepare the phosphate buffer and generated an anodic electro-osmotic flow, while the glycoprotein that was positively charged migrated in the opposite direction. This feature enabled a high resolution among different glycoforms. The results showed qualitative and quantitative differences between the glycoprotein obtained in the two different culture media.

Schmelzer and Miller [46] have investigated the impact of culture conditions on the glycosylation of IgG_{2a} produced by hybridoma cells. They first employed MEKC method to detect changes in macroheterogeneity of IgG_{2a}. Then, a CIEF method was used to determine the p*I* distribution of the different glycoforms. The influence of the level of galactose incorporated into the cell culture media as well as that of osmolality and CO_2 partial pressure (pCO_2) in growth media were investigated. The MEKC method was conducted on fused silica capillary using borate buffer containing 0.5 M SDS. Borate buffer is well known to promote glycoform separation through its interaction with sugars. CIEF was carried out on polyacrylamide-coated capillary with the addition of methylcellulose. The results showed that the changes in p*I* were significant under hyperosmotic stress.

8.3.1.3 Purification Process

The relative proportion of different glycoforms of a therapeutic glycoprotein may vary based on the purification process employed. In addition, performing glycoform profiling after different purification protocols enables making a selection of the best downstream process to be used in order to yield not only the highest concentration of desired glycosylated glycoprotein but also the composition of glycoform population that will give the best efficacy of the biopharmaceutical.

Recombinant human interleukin-7 (rhIL-7) produced from CHO is currently under clinical development. rhIL-7 is a heterogeneous glycoprotein that bears at least three *N*-glycosylation sites. Very recently, Alahmad et al. [47] developed a CZE method using an acidic buffer composed of citrate–triethanolamine to achieve the separation of the different glycoforms of rhIL-7. Different batches were analyzed to evaluate the batch-to-batch consistency during the scale-up of the production and to compare also different purification procedures (Fig. 8.3). The CZE profiles obtained clearly demonstrated that the replacement of the anion-exchange chromatographic media by a novel and more capacitive one in the purification protocol altered quantitatively the proportion of the distribution of the different glycoforms. Indeed, the batch obtained with the improved purification has more highly sialylated glycoforms than does the batch obtained with the previous protocol. This method was shown to yield information on subtle modifications of rhIL-7 glycosylation between batches

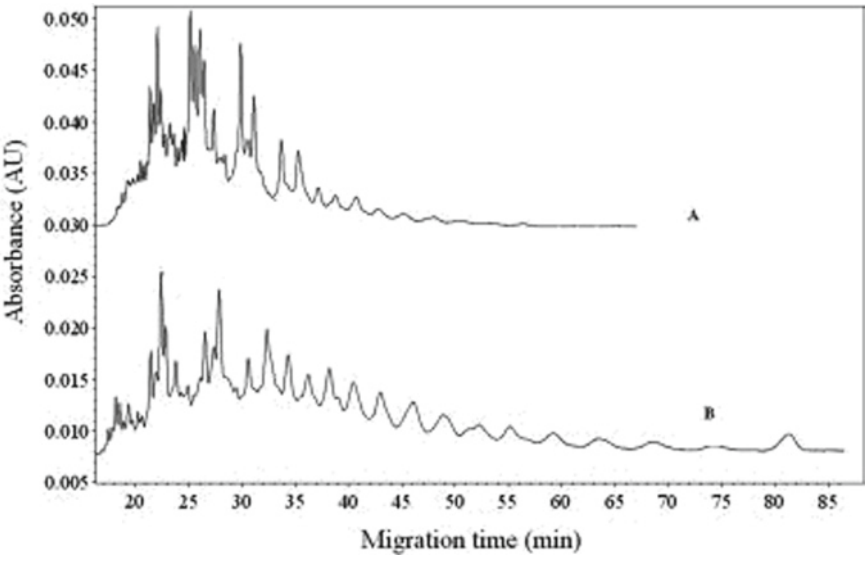

Fig. 8.3 Capillary zone electrophoresis (CZE) analysis of different batches of recombinant human interleukin-7 (rhIL-7) obtained from different production scales and purification processes. Batches A and B are produced at the same scale (200 L Bioreactor), but purified using different downstream processes. Conditions: 25 mM citrate/triethanolamine (TEOOA) at pH 2.6; applied voltage, 15 kV; detection, 214 nm; fused silica capillary (50 cm × 75 μm) (adapted from ref. [47], with permission)

purified differently, and was considered by the authors as a supportive analytical tool during process optimization. The same authors point out the same kind of differences for batches produces on a different scale (bioreactor of 12 L vs. 200 L) [47].

Later, these same authors further improved the separation of rhIL-7 glycoforms and fully validated a CZE method in alkaline conditions for this glycoprotein under current good manufacturing practices [48].

Tran et al. [49] used a two-step CIEF method to monitor the glycosylation differences between two batches of caseinoglycomacropeptide (CGMP). The two batches had different preparation procedures. To avoid the adsorption, the analysis was carried out on a polyacrylamide-coated capillary. The results showed that the glycoforms of the two CGMP batches had almost the same p*I* range, although the profiles of the two batches differed in terms of the number of peaks, suggesting a higher O-glycosylation heterogeneity for one of the two batches.

Buchacher et al. [50] used CGE and CIEF methods to compare the profile of several ATIII preparations purified using different commercially available affinity gels with immobilized heparin. ATIII is a single-chain plasma glycoprotein indicated to be an anticoagulant of high therapeutic value. The pattern of the electropherograms was influenced by the type of gel used for isolation. The protein has four *N*-glycan moieties attached to Asn96, Asn135, Asn155, and Asn192, while no O-glycosylation or other posttranslational modifications are reported. Two major groups of isoforms of AT were found: the group of α-ATIII isoforms, which carry glycans on all four above-mentioned Asn-residues; and the group of β-ATIII isoforms, which carry glycans on only three Asn-residues, lacking that on the N135. β-ATIII shows a higher affinity to heparin than α-ATIII, leading to different α-ATIII/β-ATIII ratios, based on the type of affinity gel used.

In the examples mentioned above, CE is a useful tool for illustrating the impact of different production processes on the glycosylation pattern. Thus, this technique can be particularly helpful in making a decision on the cell line, cell culture conditions, and purification protocol that are most suitable for producing the biopharmaceutical. It can provide qualitative and quantitative information, and, based on the CE modes employed, can lead to a global comparison or to a more detailed examination of subtle variations in glycosylation patterns.

8.3.2 Quality Control

8.3.2.1 In-Process Monitoring

Quality control (QC) of recombinant glycoproteins is often ascertained during bioprocessing steps due to the multiple steps involved in a production and purification process and the cost of the whole production. Not all the separation modes of CE are compatible with in-process monitoring. Indeed, the in-process monitoring entails analyzing a nonpurified compound within a protein mixture that may be present in a complex medium. As an example, CIEF, which is particularly sensitive

to the presence of salts, surfactants, and ionizable molecules, is not adapted to this purpose. The other modes have been seldom employed for in-process controls.

Freitag et al. [51] used an HPLC-CZE method to analyze cell-free culture supernatants. The sample was first separated on a strong anion exchanger (membrane adsorber) using a linear salt gradient. In this dimension the rhATIII was separated from the immunoglobulins and bovine transferrin as well as from the majority of the other protein contaminants. The rhATIII-containing fraction, which still contains considerable amounts of bovine serum albumin (BSA) and other proteins, was then analyzed by CZE. Despite the complicated setup, the ATIII analysis remained highly reproducible. In addition, the authors emphasized the superiority of the HPLC-CZE method over the enzyme-linked immunosorbent assay (ELISA) tests in terms of time, cost, and reproducibility.

Goldman et al. [52] used the MEKC method to monitor the *N*-glycosylation degree of recombinant human interferon-γ (rhIFN-γ) expressed in CHO cells. rhIFN-γ has N-linked glycans at Asn25 and a variably occupied site (Asn97). The intact glycoprotein was resolved rapidly into three variants (depending on the site occupancy (2N, 1N, and nonglycosylated). The CIEF method was also used to quantify the high sialylated glycoforms among the 11 separated glycoforms. These methods enabled good control of the bioreactor operation to avoid undersialylation of the product, which may occur during the stationary and death growth phase of the batch culture.

Wang et al. [53] proved by a MEKC method that continuous culture of CHO cells in a fluidized-bed bioreactor is better than the other continuous cultures for EPO production. Clear differences in both peak shapes and migration times of the rhEPO were observed when compared with unglycosylated EPO, suggesting that adequate glycosylation was obtained.

8.3.2.2 Purified Products

Assessment of purified product is an inevitable step during the recombinant glycoprotein production. Purified product represents the product of interest with the same level of quality and purity as that used for clinical trials but without excipients. This product should contain impurities at a level below the acceptance criteria given in monographs of the European Pharmacopoeia. This step demands a validated method that is able to check not only the product consistency but also the presence of impurities, since the biological processes are complex and many factors can influence the quality of the resulting product. The routine analysis of a purified product can be done using CZE as well as MEKC or CIEF, since the samples are relatively clean and devoid of any other materials that may interfere with the electrophoresis process. Generally, this kind of study concerns profiling of glycoforms or the determination of degradation products.

Erythropoietin has been subjected to extensive development over the last decade by several groups to achieve the glycoform profiling of this heterogeneous glycoprotein that carries more than 40% of sugars. The European Pharmacopeia has

published a monograph for CZE separation of rhEPO glycoforms in which the cooperative effect of 1,4-diaminobutane (DAB) and urea was exploited to obtain a well-resolved separation of rhEPO glycoforms [28]. DAB was used as additive because of its effectiveness in reducing the EOF and urea as chaotropic agents providing disruption of hydrophobic and noncovalent interactions, resulting in reduced solute adsorption [54]. However, Kinoshita et al. [55] found that this method was not reproducible, since repetitive analyses deteriorated the resolution. Later Diez-Masa's group [56] proposed three other CZE methods by modifying the nature and additives of BGE to separate rEPO glycoforms. They also compared their method's compatibility with that of other more sensitive detection systems (laser-induced fluorescence [LIF] or MS) [56]. The European Pharmacopoeia method was then improved by strongly preconditioning the capillary with voltage applied for 12 h.

The separation of the reference erythropoietin BPR (biological product reference) into eight peaks occurred in a predictable manner in order of increasing number of sialic acids contained in the glycoforms. This method allowed differentiating EPO of recombinant and urinary origin [57]. However, the separation buffer containing DAB and 7 M urea was not compatible with online ESI-MS detection. That is why, later, ionene polymers similar in the structure to polybrene have been proposed as a dynamic coating for the separation of rhEPO glycoforms in 15 min. These polymers have the advantage of being compatible with MS detection. The 6,6-ionene-coated capillary and the online CE-ESI-MS have been applied to the differentiation of endogenous from recombinant EPO [58]. Simultaneously, Sanz-Nebot et al. [59] investigated experimental conditions employing volatile electrolyte systems to achieve the separation and characterization of rhEPO glycoforms using CE and ESI-MS methodologies. The influence of the coating, the composition, the concentration, and the pH of the separation electrolyte have been studied. A polybrene-coated capillary combined with a buffer containing 400 mM acetic acid–ammonium acetate at pH 4.75 provided a reproducible separation of EPO glycoforms. CE-ESI-TOF-MS using polybrene or UltraTrol low normal (LN)-coated capillary with a volatile BGE such as ammonium acetate has been developed by another group for the analysis of the glycoforms of rhEPO [60]. More recently, a carboxymethyl chitosan-coated capillary was successfully applied to separate rhEPO using an optimized and volatile BGE containing acetic acid–ammonium acetate for its further application to CE-MS [61]. The results demonstrated that rhEPO glycoforms could be well separated within 8 min with good reproducibility and resolution.

Other separation modes, such as the CIEF method, have also been developed for rhEPO-BPR analysis [62, 63]. The use of a neutral capillary, a mixture of broad and narrow pH-range carrier ampholytes, combined with the optimization of focusing voltage and focusing time, allowed the separation and quantitation of seven peaks of rEPO in 12 min, with apparent p*I* ranging from 3.78 to 4.69 [62]. This method was further improved by optimizing electrolytes, using internal standardization and a complete depletion of excipients (Tween 20, trehalose, arginine, NaCl, and phosphate) contained in the rhEPO-BPR. This led to a more precise

CIEF method (less than 6 min) with an intra-assay RSD (relative standard deviation) of less than 0.5% for index time and less than 1.5% for index area.

Other biopharmaceuticals also have been extensively investigated to find a routine control based on CE. This is the case with recombinant tissue plasminogen activator rtPA, which displays a macroheterogeneity (the number of site occupancies is different between the two variants) and a strong tendency to aggregate to the capillary wall. A CZE method was first developed, and then a one-step CIEF was proposed [64, 65]. Finally, Thorne et al. [66] compared three different CE modes – CZE, CIEF and SDS-CE – for rtPA characterization.

Recombinant human IFN-γ is one example of a successful glycoform profiling achieved by MEKC [67]. But this mode has been seldom employed.

Felten et al. [68] developed a CZE method to profile recombinant human deoxyribonuclease (rhDNAse). This glycoprotein is an enzyme manufactured in CHO cells and has been approved for the alleviation of the debilitating effects of cystic fibrosis. The authors used a PVA-coated capillary under acidic or alkaline conditions. They reported the beneficial effect of the addition of calcium ion on the resolution.

Kremser et al. [69] proposed three electrophoretic methods for separating glycoforms of ATIII from human plasma. CZE, using PVA-coated capillary, was shown to be more accurate in quantifying the relative proportion of the two groups of isoforms ATIII-α and ATIII-β. The resolution of the isoforms was significantly improved when adding 4 M urea in the BGE containing 1 M acetic acid at pH 2.5 and in the presence of 0.1 M NaCl in the sample solution. CIEF of ATIII was performed with 1% ampholyte pH 4–6 containing 4 M urea and 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) using PVA-coated capillary. The p*I* values of the main isoforms of ATIII-α and ATIII-β found with CIEF, using internal calibration, were in agreement with those determined by two-dimensional gel electrophoresis (2-DE). The CZE method was then coupled online to ESI-quadrupole ion trap-MS by Demelbauer et al. [70] to enable high accuracy of the molecular mass determination. Therefore, the carbohydrate composition could be calculated, assuming there are no variations in the protein moiety.

Berkowitz et al. [45] developed a simple and rapid CZE method for the QC of a therapeutic glycoprotein. The authors employed different kinds of digestion – neuraminidase and peptide-*N*-glycosidase F (PNGase F), prefractionation of the glycoprotein by anion-exchange, and deamidation of the glycoprotein – to monitor the extensive microheterogeneity of the therapeutic recombinant glycoprotein and to get more information on the glycoform profile in terms of oligosaccharide structures. These peak assignments were used to calculate parameters that provided quantitative information on the level of sialylation, glycosylation, and deamidation of the glycoprotein. This method afforded a high and reproducible resolution fingerprint of the heterogeneity of the glycoprotein, which was attributed either to deamidation or to glycosylation microheterogeneity of the protein.

Triple- and double-layered rotavirus-like particles (RLPs) are candidate vaccines against rotavirus infection, and are composed of the main rotavirus structural proteins VP2, VP6, and VP7. Rotavirus VP7 is a glycoprotein that forms the viral capsid outer layer and is essential for the correct assembly of triple-layered RLPs.

Mellado et al. [71] developed for the first time an SDS–CGE method applied to the analysis of recombinant VP7 produced in an insect cell infected by baculovirus. This method was applied to the characterization of a purified VP7, obtained with a novel purification strategy, designed to obtain highly pure monomeric VP7 required for the RLPs' in vitro assembly. SDS–CGE was also employed for the analysis of RLPs that could be resolved in four peaks attributed to the viral proteins VP2, VP6, and VP7 or isoforms of them based on their apparent molecular mass (MW_{app}) [72]. The proposed method clearly, quickly, and reproducibly discriminated glycosylated from nonglycosylated VP7 and enabled the quantitative and qualitative characterization of RLPs in terms of the relative proportion of these proteins for QC purposes. This method was shown comparable to both sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization (MALDI)-TOF-MS in terms of sensitivity.

8.3.2.3 Finished Product

The main challenge in analyzing a finished product is to obtain sufficient separation between the glycoprotein of interest and the excipients present in the formulated product. A finished product of most recombinant glycoproteins, commercialized for therapeutical use, are, at present, either a lyophilisated powder or an injection solution. The excipients are generally added to the finished product to stabilize it, to enhance its solubility, to prevent its aggregation, or to prevent the adsorption of the protein into the container. To this purpose, the finished product may contain surfactants, sugars, polyhydroxylated compounds, human serum albumin, amino acids, polypeptides, and salts mixed with the active ingredient. When excipients are proteins, sufficient selectivity is expected from the CE method to separate the active ingredient from interfering species.

The great number of applications reported in the literature have proven that CE is able to characterize finished products even when they are complex and heterogeneous glycoproteins, to compare the formulated product obtained from different manufacturers, and to check batch-to-batch consistencies.

In 1997, Bietlot and Girard [73] developed a CZE method for the analysis of rhEPO in formulated preparations that contained a large quantity of human serum albumin (HSA). The method was conducted on an amine-coated capillary at a high concentration of phosphate buffer and pH 4. The addition of $NiCl_2$ in the running buffer was very useful in modifying the electrophoretic mobility of has, enabling the resolution between the two proteins. Since then, many authors have proposed new buffers or new capillary coatings to achieve reproducible and high-resolution analyses of rhEPO, and a monograph describing the CZE separation of EPO glycoforms was published by the European Pharmacopoeia in 2002 [28] and updated in 2005 [74]. This evolution of the methodology applied to the characterization of rEPO glycoforms was discussed in Section 8.3.2.2.

Novel erythropoiesis-stimulating protein (NESP) is a recently approved hyperglycosylated analog of EPO that has a lower p*I* than rhEPO. Indeed, NESP differs

from human EPO at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr), allowing the additional attachment of two *N*-linked oligosaccharides, each containing up to four terminal sialic acids. As a consequence, this analog has a longer circulation life time and can be administrated less frequently. This formulated analog can be separated into seven well-resolved peaks by using a buffer at pH 4.5 instead of at pH 5.5, as recommended for rhEPO [75] by the European Pharmacopoeia [28].

Human serum albumin is added to some pharmaceutical preparations as an excipient, as is the case with several commercial preparations of EPO. It has been observed that HSA interfered with or precluded the separation of rhEPO bands by CZE, probably due its strong interaction with the capillary wall, modifying the zeta-potential and thus EOF. An immuno-chromatographic method to remove HSA from rhEPO pharmaceutical formulations and a procedure to concentrate the sample prior to the CZE analysis have been developed [76]. The CZE method was similar to that proposed by Watson and Yao [54]. A computer program that was developed for the study of commercial samples of α- and β-epoetin and darbepoetin-α [77] enabled the accurate assignment of the different forms of rEPO even when HSA was present at less than 2 mg/mL [76].

To further improve the CZE method recommended by the European Pharmacopoeia, Benavente et al. [78] proposed a multivariate calibration method using partial least-squares (PLS) to characterize the binary mixture of two types of rhEPO (epoietin-α and -β formulated as Epopen® and NeoRecormon®, respectively). The characteristic electrophoretic profiles used together with the PLS-1 multivariate regression model enabled the determination of epoietin-α and -β contents in the rhEPO provided by the European Pharmacopoeia as a biological reference product (BRP) (Fig. 8.4).

Polysorbate 80, which is contained in some EPO formulations, interferes with the CZE analysis proposed by the European Pharmacopeia for EPO [74]. Unfortunately, the desalting procedure based on the use of molecular mass cutoff filters, as described in this monograph, is not efficient to remove this excipient. Zhang et al. [79] very recently proposed an improvement in this CZE method by modifying the preconditioning procedure of the capillary. The capillary was flushed with a combination of NaOH and HCl solution, water, and buffer over a long period, rendering the method more reproducible. The second improvement was achieved using a nonionic detergent (NID) trap cartridge to remove polysorbate. This method was applied to characterize 2K IU/mL and 40K IU/mL EPO samples that are epoietin-α formulations (Raritan®, Johnson & Johnson Ortho Biotech, Raritan, NJ). The relative areas of each isoform for the two concentrations of formulated EPO were considered to be equivalent to that of the epoetin-α standard. Four sets of analyses were performed to assess the repeatability (RSD of relative area <0.7%) and intermediate precision (RSD of relative area <6.8%). The results showed that repeatability and intermediate precision have been improved.

The biophysical properties of two commercially available rhEPO-α products, Epogen® and Eprex®, both of which are produced under similar conditions but by different manufacturers (Amgen, Thousand Oaks, CA and Ortho Biotech Products,

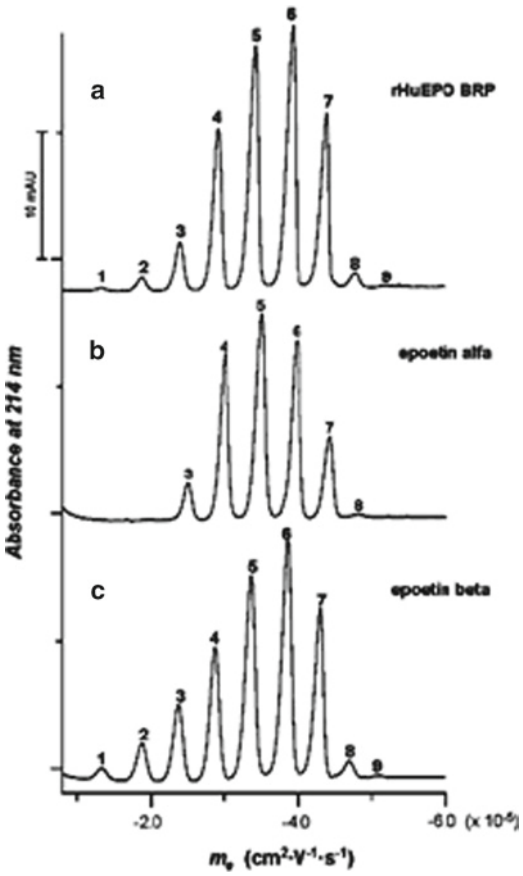

Fig. 8.4 CZE separation of rhuEPO glycoforms using the European Pharmacopoeia method: (**a**) rhEPO BRP, (**b**) epoetin-α (Epopen), (**c**) epoetin-β (NeoRecormon) (reprinted from ref. [78], with permission)

L.P., EastBridgewater, NJ) were studied by other authors [80]. In this study, CZE was one of the techniques used to compare the EPO products. Using amine-coated capillary and a high concentration of phosphate buffer at pH 4.2 containing 8 M urea, the authors showed that isoform type and distribution for all samples were similar. They concluded that the sialic acid content was the same.

This group recently conducted a biochemical assessment of EPO products from Asia versus those from the United States, specifically the epoietin-α manufactured by Amgen (Thousand Oaks, CA) [81]. A similar CZE method to the one described above was utilized, among a set of techniques, to compare the isoform distribution of rhEPO samples, and found that the purported copies of rhEPO from Korea, India, and China contained either more glycoforms or different glycoform distribution or other impurities compared to the Amgen-derived product Epogen®. This finding resulted either from different expression systems or different manufacturing processes used by the different companies.

Recently, Dou et al. [82] developed a CIEF method with whole-column imaging detection and Polyvinylpyrrolidone (PVP)-coated capillary to compare EPO-α and -β marketed by Roche (Recormon) and by Kirin (Espo). This method combined the use of urea and short-range pH ampholytes to provide a rapid and high-resolution profiling of rhEPO able to distinguish EPO-α from EPO-β (Fig. 8.5). By correction with the standard p*I* markers, the reproducibility for the peak position of each glycoform was excellent, with RSD values of 0.3 and 0.9% for run-to-run and day-to-day reproducibility, respectively. The authors concluded that the technique combined with a preconcentration step by ultrafiltration could be a good alternative for the detection of rhEPO misuse by athletes.

In two previous papers, rhEPO was analyzed by CZE-ESI-TOF-MS using a polybrene-coated capillary. A large number of glycoforms were detected with high

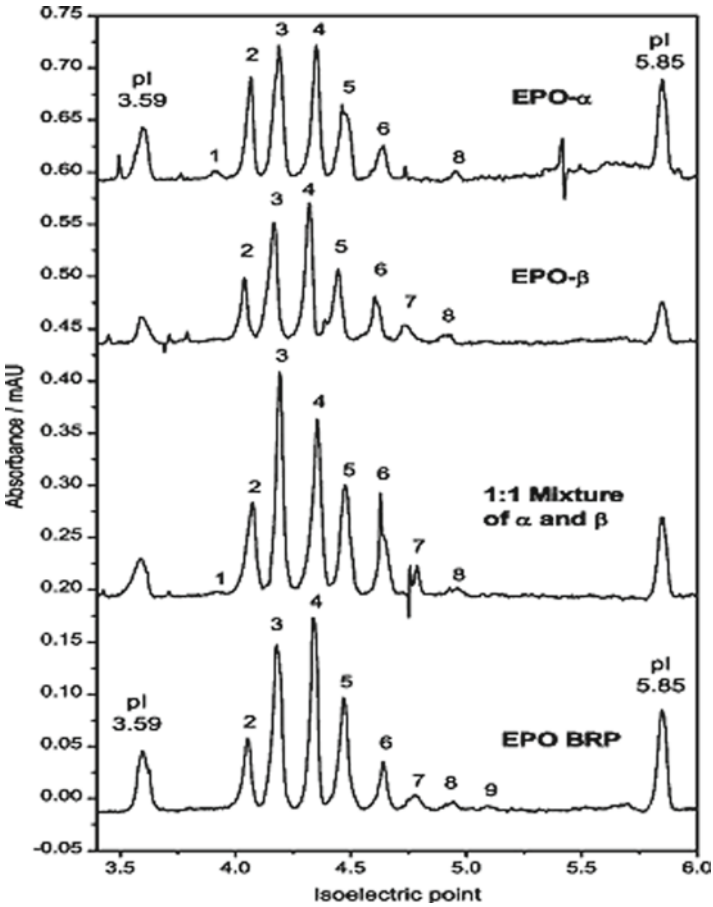

Fig. 8.5 Comparison of different EPO samples by capillary isoelectric focusing (CIEF) combined with the whole-column imaging detection. Samples: 1.0 mg/mL EPO of containing 4 M urea, 1% PVP, and 2% Fluka ampholytes pH 3–10 (reprinted from ref. [82], with permission)

mass accuracy [60], which demonstrated the suitability of the method for distinguishing between two types of commercial rhEPO (α and β from Eprex® and NeoRecormon®) [83]. A well-documented characterization of rhEPO combining glycan and glycoform analyses by the method previously described was performed by the same group [84]. In this latter work, a new commercial coating – UltraTrol Dynamic Pre-Coats low normal (LN), a polyacrylamide-based physically adsorbed coating – was tested and was found to provide a better resolution of rhEPO glycoforms compared with the polybrene (PB)-coated capillary. The LN coating generated a lower EOF than that obtained with the PB-coated system, resulting in electrophoretic mobilities of rhEPO glycoforms that are closer to those of EOF (in absolute values), a situation more favorable in terms of resolution. However, the cost of this latest generation of mass spectrometers (orthogonal acceleration, TOF) remains high. Therefore, Sanz-Nebot's group [85] explored the use of a more conventional analyzer ion trap (IT). Although an IT is not the ideal mass analyzer for high-molecular-weight glycosylated compounds, the CE-ESI-IT-MS using a neutral LN coating found that it was possible to carry out a rapid identification of three main glycoforms of rhEPO. Reproducibility studies confirmed the lack of hydrolytic stability and blending of the LN coating at extremely acidic BGE, causing problems with MS detection. In contrast, the cationic UltraTrol high reverse (HR) coating provided enhanced hydrolytic stability, making it more suitable for online MS coupling but at the expense of a poorer glycoform resolution.

An international collaborative study was carried out by Storring et al. [86] to assess whether isoelectric focusing (IEF) and CZE methods for predicting the in vivo biological potency of recombinant follicle-stimulating hormone (rFSH) were capable of ranking rFSH preparations based on their in vivo biological potencies. The isoform distribution of four preparations of highly purified follitropin beta (Organon) that differ from their isoform compositions and biological potencies has been compared quantitatively. The CZE method used with a fused silica capillary and a borate buffer containing diaminopropane (DAP) as a dynamic coating was found to be sufficiently accurate, precise, and robust for this purpose, although the interlaboratory geometric coefficient of variation was less favorable than that obtained with the IEF method.

Zhou et al. [87] reported a CZE method to characterize nonglycosylated rhG-CSF preparation (GRAN300® injection) and glycosylated rhG-CSF preparation (GRANOCYTE® injection), which contained a large amount of HSA as a stabilizer. In this case, the adsorption problem was more pronounced with HSA than with the biopharmaceutical rhG-CSF itself. Although it was difficult to separate the glycosylated rhG-CSF from the nonglycosylated counterpart in the Tricine buffer under alkaline conditions, the separation could be greatly improved by the addition of NaCl and by the introduction of DAB onto the BGE, which decreased the charges on the capillary surface and hence amplified the differences in the electrophoretic mobilities between the different forms of rhG-CSF. The glycosylated rhG-CSF was separated into two glycoforms, while the nonglycosylated rhG-CSF migrated as a single and sharp peak. The large peak in the CE profile of the GRANOCYTE® injection was caused by the carrier protein has, which did not affect the characterization

of rhG-CSF in the commercial dosage forms. CIEF was also used to determine the identity and purity of the nonglycosylated rhG-CSF. The nonglycosylated rhG-CSF bulk sample was found to contain three ingredients with p*I*s of 6.75, 6.02, and 5.09, while GRAN300® presented only one species with a different p*I*. Thus, CIEF afforded the detection of composition differences in the rhG-CSF that was not achieved by CZE.

Urinary trypsin inhibitor (ulinastatin) is a characteristic protein pharmaceutical that contains both glycosaminoglycans and N-linked glycans and has been used for the treatment of acute pancreatitis. Matsuno et al. [88] presented a comparative study of ulinastatin preparations from different lots or different companies using several analytical methods. MEKC, one of these methods, was used to compare the electrophoretic profiles obtained. Using a 50-mM borate buffer (pH 9.3) containing 100 mM SDS, a single broad peak was observed. The migration time and peak shape of this single peak enabled demonstrating the comparability of two commercial products.

Taga et al. [89] validated a CZE method using a newly developed, carboxylated capillary to determine the content of nonglycosylated heavy chains (NGHC) in an IgG-type pharmaceutical. As a model, tocilizumab (brand name Actemra®) was reduced prior to CZE analysis to yield a well-resolved separation between the heavy and light chains (HC and LC, respectively). Indeed, the analysis of the native tocilizumab and its nonglycosylated counterpart showed only one single sharp peak. SDS at 0.1% was added to the weak acidic phosphate buffer to discriminate NGHC from HC. The method was reproducible (RSD of migration times <0.6% and RSD of peak areas <3.9%) and quite fast, 6 min, thanks to the carboxyl groups ensuring a constant EOF and preventing protein adsorption onto the capillary wall. The advantage of this method is illustrated in Fig. 8.6 by a comparison of the profiles obtained using a noncoated silica and the carboxylated coated capillary. The carboxylated capillary gives three well-resolved peaks with excellent repeatability, contrasting with the two broad peaks (corresponding to HC and LC) observed with the silica capillary. In addition, on this latter capillary, the analysis time increased upon repeated analyses due to EOF becoming slower. Excellent durability of the carboxylated capillary was demonstrated since migration times did not change over 200 runs.

rhIL-7 in its formulation form has been recently analyzed by a validated CZE method based on the guidelines of ICH-QB6 [48]. The specificity of the method was demonstrated by analyzing the storage buffer of rhIL-7 containing sodium acetate, sodium chloride, and other stabilizer and antioxidant ingredients. The method developed was specific for rhIL-7 glycoforms, since it was able to discriminate them from the component matrix present in the buffer formulation. The separation buffer was a 25-mM sodium borate at pH 10 containing 12 mM diaminobutane (DAB) used as a dynamic coating agent of the capillary. This method enabled the separation of seven peaks ranging from low to high sialylated glycoforms. Moreover, this method could ascertain the batch-to-batch consistency upon the analysis of two batches with strict application of the current good manufacturing practices to one of them.

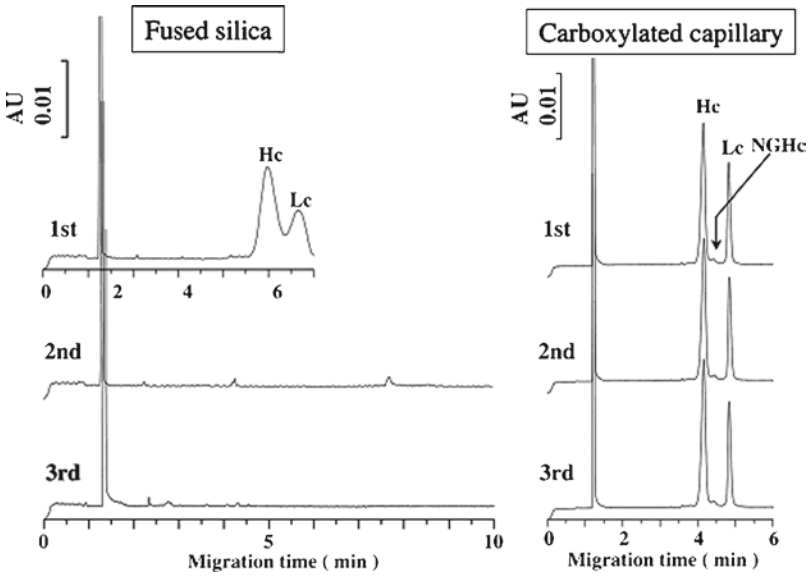

Fig. 8.6 Comparison of capillary electrophoretic separation of tocilizumab in a carboxylated capillary and a bare fused silica capillary over three successive runs. 50 μm inner diameter, effective length 40 cm, running buffer, 25 mM phosphate buffer (pH 5.8) containing 0.1% SDS; applied voltage, 28 kV; temperature, 25°C; hydrodynamic introduction (1 psi = 6.8947 kPa, 5 s); UV detection at 214 nm. *Hc* heavy chain; *Lc* light chain; *NGHc* nonglycosylated heavy chain (reprinted from ref. [89], with permission)

Thus, as we have demonstrated, biological processes are complex, and many factors can influence the quality of the resulting product. Monitoring of glycosylation at all stages of a bioprocess has became a necessity to ensure product quality and batch-to-batch consistency, but the methods to be developed cannot be equivalent for a purified or a finished product control. The in-process analysis with CE remains less explored, due probably to the complexity of the culture media and supernatants, making it quite challenging to maintain a high resolving power by CE. In this regard, the CIEF mode is not suitable for in-process control due to a potential high salt concentration that affects the pH gradient formation and stabilization, and CZE remains the method most employed at all stages.

8.3.3 Stability Testing

Up- and downstream processes in the manufacturing of recombinant products can generate impurities, which can be either process- or product-related. QC of therapeutic glycoprotein pharmaceuticals thus includes also impurity testing. Moreover, formulation and storage conditions may promote potential forms of related proteins. CE has proved to be a suitable technique for evaluating glycoprotein stability.

To this purpose, biopharmaceutical glycoproteins are subjected to different stress conditions to identify critical parameters for their stability as well as the most probable degradation pathways. CE has been largely exploited to evaluate stability of recombinant human monoclonal antibody (rhmAB) [34, 90]. Hunt et al. [91] characterized the rhmABHER2 by CIEF and SDS–CGE methods, and studied its stability at two storage temperatures, 5 and 37°C for 27 days. The analyses have been carried out on nonreduced samples. The CIEF method detected a slight decrease in peak area percentage of several isoforms at 37°C, indicating a probable deamidation of the glycoprotein. Simultaneously, the SDS–CGE method showed that some fragmentation of the mAb occurred during the storage of the glycoprotein under elevated temperatures.

Berkowitz et al. [45] monitored the stability of a 24-kDa glycoprotein as a function of temperature and pH over time. They compared CZE results from those obtained via HPLC methods, which were capable of monitoring deamidation and desialylation on the glycoprotein. The trends in the level of deamidation and desialylation obtained by the two separation techniques were found to be similar.

Recently, Rustandi et al. [43] evaluated by an SDS–CGE method under reducing conditions the stability of mAb by investigating the impact of the formulation buffers, elevation of temperature, light exposure, and high pH stress. As an example of their study, the effect of UV light on the mAb is illustrated in Fig. 8.7. In fact, UV light exposure over 309 h at 4°C affected the mAb by forming aggregates

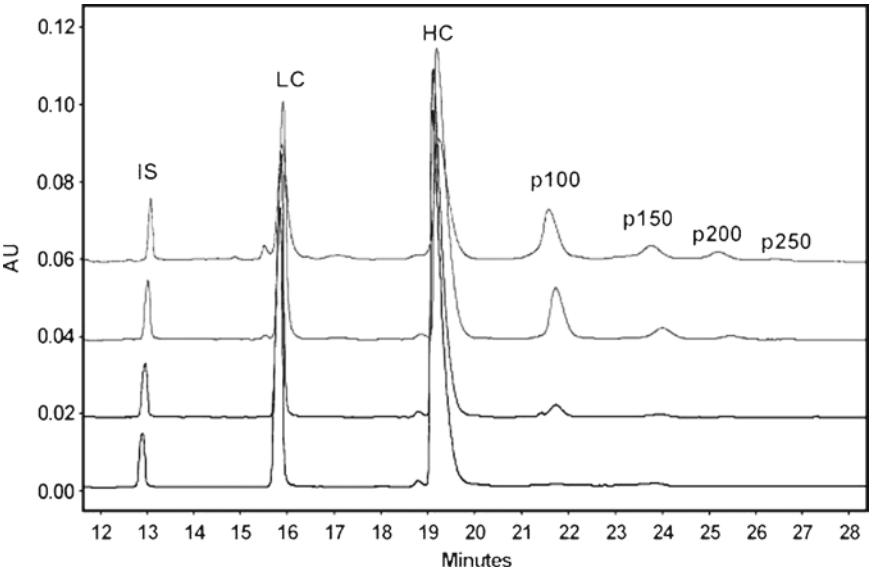

Fig. 8.7 Electropherograms of reduced mAb to evaluate the photostability under ultraviolet (UV) light, traces from *bottom* to *top*: 0, 20, 98, and 309 h. The apparent MW of aggregates p100, p150, p200, and p250 were approximate based on the MW markers. The electropherograms were normalized to the internal standard (IS) peak (reprinted from ref. [43], with permission)

(p100, p150, p200, and p250). The increase of peak intensity of p100 and p150 is concomitant with the decrease of the HC one, indicating that these covalent aggregates were mainly formed from the HC. Accelerated temperature stability was also investigated at 4, 37, and 45°C for 4 weeks. The results showed that the majority of clipping occurs in HC, and most clipping provoked by high temperatures occurs in the hinge region of the antibody. Finally, high pH stress on product stability was studied on samples held at a pH between 6.5 and 9.3 for 1 week and at 45°C. The clipping pattern, at high pHs, was similar to that obtained for temperature stress. One peak was identified as likely to be a result of a beta-elimination reaction at high pH. The data indicated that severe change happened after storage of the bioproduct at pH 7.9, and that most of the clipping occurred in the HC part of the antibody.

Glycoprotein stability can be evaluated by CE to assess its quality as well as its sensitivity to various external parameters. SDS–CGE is widely employed for the stability testing of mAb. These examples highlight the power of CE in detecting changes in protein structure subjected to various stresses, such as slight structure modifications (deamidation) or major ones (aggregation, fragmentation) that affect its biological function.

8.4 CE in Biological Fluids for Clinical Application

Biological fluids contain important information on the physiopathological organism state. Proteins and glycoproteins can serve as biomarkers for the diagnosis of certain diseases, or to evaluate the efficacy of therapeutic treatments. Diagnosis does not rely only on quantification of a given biomarker. For instance, some diseases can affect the glycosylation profile of plasma glycoproteins without altering their expression level. Since CE is a separation technique that can give reproducible results with good accuracy in a short time, it can be a very useful tool for doing clinical studies and for discovering biomarkers [92, 93]. However, glycoform analysis in biological fluids is often hampered by problems in detection sensitivity, since the concentration of glycoforms may be very low in a complex mixture of proteins in the sample. Therefore, CE analysis in biological fluids is often associated with a sample preparation step to preconcentrate the protein of interest. The coupling of CE with MS is particularly useful in this regard [94, 95].

In 1994, Morbeck et al. [96] reported a CZE method to analyze the microheterogeneity of hCG using a borate buffer and DAP as the EOF modifier. Seven to eight glycoforms could be baseline resolved with these conditions. The profile obtained from an hCG hormone purified from the urine of a patient with metastatic choriocarcinoma was compared to that obtained with other hCG preparations from healthy persons. The authors concluded that the relative percentage of each glycoform was different between the preparations.

Hiraoka et al. [97] developed a CIEF method to analyze the β-trace protein (β-TP, lipocaline-type prostaglandin D synthetase) that is a sialic acid-containing

glycoprotein. A one-step CIEF method, which consists of performing the mobilization by EOF along with a focalization step, was carried out on a neutral capillary and using the N,N,N',N'-Tetramethylethylenediamine (TEMED) as a gradient extender. The concentration of this biomarker was known to increase in the cerebrospinal fluid (CSF) of patients recovering from brain damage and those with pathological brain atrophy. This study evidenced a variation of the charge microheterogeneity of this β-TP associated with various neurological disorders (neurodegenerative disorders with brain atrophy, multiple sclerosis).

One-step CIEF was also reported by our group to resolve the glycoforms of the heterogeneous recombinant human immunodeficiency virus (HIV) envelope glycoprotein (gp160) [98]. Analysis was conducted using a PVA-coated capillary and a mixture of three ampholytes from the acidic to alkaline pH range containing saccharose and 3-(cyclohexylamino)-1-propanesulfonic acid to avoid precipitation, since this phenomenon can occurred easily at a pH close to the p*I* of proteins. The rapidity and precision of the one-step CIEF approach were demonstrated. This method was valuable for determining the difference in the glycosylation heterogeneity of the gp160 from two subpopulations of the virus HIV-1 originating from two continents, considering that five different clades of this virus exist all over the world.

Transferrin was extensively analyzed by CE as a biomarker of alcohol abuse or carbohydrate-deficient transferrin (CDT) [99] and also as a model of the congenital disorders of glycosylation (CDG) diagnosis. One important section of Girard et al.'s review [21] is devoted to this topic, so we will only briefly describe this clinical application of CE. There are two categories of CZE methods reported in the literature to achieve the resolution of transferrin glycoforms from serum. The first entails the use of alkylamines in the BGE as dynamic coating reagents [100], while the second is based on the formation of double ionic layers at the silica surface [101, 102] with an available commercial kit (Ceofix-CDT Analis, Suarlée, Belgium). Both strategies aim at avoiding protein adsorption to the capillary wall and at ensuring reproducible analyses; the problem of adsorption is not a concern regarding transferrin itself, but it is for the other serum proteins present in the samples. Sanz-Nebot et al. [103] developed a CZE-MS method to characterize transferrin glycoforms using volatile buffer of ammonium acetate and a double-layer coating.

In 2007, the detection of CDT was proposed using a fully automated method developed in the Capillarys™ CDT kit (Sebia, Evry, France), which relied on a multicapillary electrophoresis system. This system provided the advantages of high productivity and total automation. The analytical features of this method have been evaluated compared to those of single capillary electrophoresis [104, 105], and this system's good analytical performance was demonstrated. Recently, Parente et al. [106] evaluated this kit's ability to screen CDG [106].

Salivary proteins present a variety of posttranslational modifications (PTMs) such as glycosylation and phosphorylation. The protein component of saliva has great diagnostic importance, since many diseases affect the salivary glands and oral health [107], and because saliva is a readily accessible body fluid. Guo et al. [108] identified 1,381 small proteins and bacterial proteins from saliva by a CIEF-based multidimensional separation platform coupled with ESI-MS.

Alpha acid glycoprotein (α-AGP) is one of the major acute-phase proteins in serum. It is a single chain of 183 amino acids with very low p*I* of 2.8–3.8 because of the presence of numerous terminal sialic acid residues and a very high carbohydrate content of 45% [109]. Kakehi et al. [110] analyzed α-AGP glycoforms in slightly acidic conditions (pH 4.5) on a dimethyl-polysiloxane-coated capillary. They analyzed serum samples from patients suffering from methicillin-resistant *Staphylococcus aureus* (MRSA) that was acquired during hospitalization. Although they attempted to find some characteristics in the patients' sera, a conclusive relationship was not observed between the relative abundance of each glycoform and the clinical data.

Later, Lacunza et al. [111] compared different α-AGP samples purified from cancer patient sera and healthy volunteers by the CZE method using a computer program to assign accurately the α-AGP peaks. They obtained a baseline resolution of 11 peaks. This method was found to be promising, since a difference in the peak area percentage of α-AGP glycoforms between sick and healthy individuals could be observed. The same group developed a CIEF method to compare α-AGP isoform patterns from healthy individuals and from patients suffering from ovarian cancer and lymphoma (Fig. 8.8) [112, 113]. The four samples were analyzed and presented no qualitative difference, since the same six main peaks were observed in both the healthy samples and the patient samples. However, quantitative differences in the

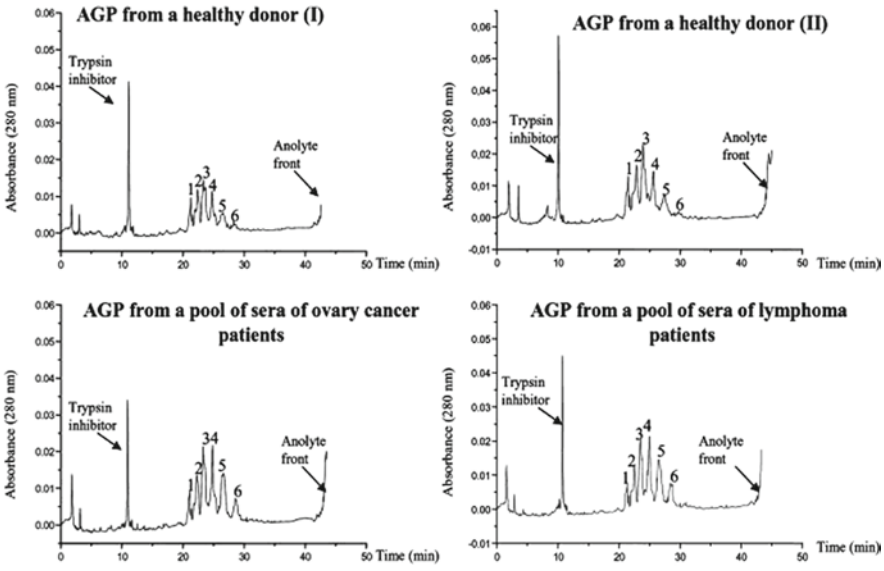

Fig. 8.8 CIEF profiles of human alpha acid glycoprotein (α-AGP) samples. Analytical conditions: PVA-coated capillary, total length: 27 cm (effective length: 20 cm). Catholyte: 20 mM NaOH titrated with H_3PO_4 to pH 11.85. Anolyte: 91 mM H_3PO_4 in CIEF gel. Sample mixture in CIEF gel: 1.5 mg/mL AGP, 0.25 mg/mL trypsin inhibitor, 5.6 M urea, 20 mM NaCl, and 6.3% v/v ampholytes 2–4, 2.5–5, 3–5, 3–10 in the ratio (3:1:1:1) v/v. Injection of sample mixture 1.5 min at 20 psi. Focusing time: 10 min at 20 kV. Hydrodynamic mobilization step: 20 kV and 0.5 psi N2 pressure. Detection: 280 nm. Temperature: 20°C (reprinted from ref. [113], with permission)

profile were observed between AGP from the healthy donors and the two pools of α-AGP from cancer patients. The authors checked that the differences were not due to the inherent variability between runs. Samples from cancer patients showed a higher proportion of the most acidic glycoforms (peaks 3–6) in accordance with the hypersialylation of glycoconjugates that is often reported in cancer patients.

The numerous examples described here emphasize the emerging interest in glycoprotein analysis in the clinical field and for diagnosis, and the usefulness of CZE and CIEF methods to achieve this goal. These studies showed not only that the evaluation of the concentration of one given biomarker in body fluids is a valuable diagnostic tool, but also that the more detailed study of the alteration of its glycosylation heterogeneity is also valuable. This opens the door to new generations of glycoprotein-based diagnosis, not through measuring the simple variation in the expression level of the protein but rather by detecting subtle modifications of the glycosylation state associated with diseases.

8.5 Conclusion

Considering the number of CE methods already developed so far for the analysis of glycoprotein pharmaceuticals, CE is clearly an emerging technique in the context of quality control of these bioproducts and for allowing accurate quantitation. The high resolution power, automation, rapidity, and convenient manipulation make CE and particularly CZE, CIEF, and SDS–CGE such interesting methods that they could substitute for the conventional gel electrophoresis or be used as alternative to capillary HPLC methods. These advantages meet the requirement for QC methods that are generally performed at different stages of biopharmaceutical manufacturing processes. Applicability of CE to the biopharmaceutical analytical field entails detailed information on the glycosylation microheterogeneity of glycoprotein, quantitation of the product, purity assessment, and stability studies, all contributing to the development of more effective and safe pharmaceuticals. This explains why the European Pharmacopoeia recommends CE methods in monographs about glycoproteins.

As a general trend, CZE and CIEF are used to profile and quantify glycoforms, except in the case of mAb, for which SDS–CGE is generally used under reduced conditions. To avoid protein adsorption to the capillary wall, methods relying on covalent or permanent modification of capillaries are increasing, with new capillaries appearing that bear charges, since a neutral polymeric coating can sometimes present nonspecific interaction with proteins and decrease the efficiency by the suppression of the EOF. However, dynamic coating remains the preferred strategy, probably due to issues linked to long-term stability of the permanently coated capillaries. Once a method has been developed and optimized for the characterization of a given glycoprotein, it should be adapted to the stage of production process at which the glycoprotein will be analyzed (in process, and for the finished product). In addition, problems related to protein adsorption may not be evidenced initially, and successive improvements of separation conditions are often

necessary. This is exemplified by the considerable number of reports on the CE of EPO, since the first developed method that allowed a well-resolved separation of a high number of glycoforms of EPO. Since 1993, this method has been continuously optimized and improved up to very recently, either to obtain a higher reproducibility or to remove impurities that interfere in the separation.

Structural analysis is not possible by CE methods, which is why coupling CE to MS is attracting interest. This strategy requires, however, separation buffers that are sufficiently volatile and compatible with MS.

One limitation inherent to CZE is the low concentration sensitivity. This is not an issue when finished or purified products have to be analyzed, but this can become a serious limitation for clinical and diagnostic applications. This can be addressed by combining CZE with on-line pre-enrichment techniques, such as immunoaffinity- or lectin-based systems to preconcentrate glycoproteins. This integration of sample preparation with the separation step can be particularly easy, if electrokinetic separations are performed not within a capillary but inside microchip possessing separation channels connected to reactors or preconcentration chambers. We anticipate that the clinical and diagnostic fields, relying on the analysis of glycoforms as biomarkers, will be one of the important area of research in the future.

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Chapter 9
Capillary Electrophoresis and Capillary Electrophoresis–Mass Spectrometry for Structural Analysis of *N*-Glycans Derived from Glycoproteins

Miyako Nakano, Kazuaki Kakehi, Naoyuki Taniguchi, and Akihiro Kondo

Abstract This chapter highlights recent developments in the analysis of proteins glycosylated of the amino groups (*N*) of asparagine residues (i.e., *N*-glycans) by capillary electrophoresis (CE) and capillary electrophoresis–mass spectrometry (CE-MS). First, the analysis of intact glycoproteins by CE and CE-MS is reviewed. In glycoform analysis of a heterogeneous protein due to different glycosylation, multiforms are observed on CE. Second, to perform detailed analysis of *N*-glycans in glycoproteins, *N*-glycans released from glycoproteins through chemical or enzymatic reactions are analyzed by CE. A number of reagents for labeling *N*-glycans to improve the sensitivity and several different separation modes of CE have been developed. Finally, to obtain detailed structural information, the advantages and limitations of various methodological approaches and techniques as well as mass spectrometric instrumentation are discussed, particularly in the context of glycan analysis.

Keywords Capillary electrophoresis • *N*-glycans • Mass spectrometry • Capillary zone electrophoresis • Capillary gel electrophoresis • Capillary isoelectric focusing • Micellar electrokinetic chromatography • Capillary electrochromatography • Capillary affinity electrophoresis

Abbreviations

2-AA	2-aminobenzoic acid
2-ABA	2-aminobenzamide
2-AMAC	2-aminoacridone
2-AP	2-aminopyridine
3-AA	3-aminobenzoic acid

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3-ABA	3-aminobenzamide
4-AA	4-aminobenzoic acid
4-ABN	4-aminobenzonitrile
6-AQ	6-aminoquinoline
7-ANDS	7-aminonaphatalene-1,3-disulfonic acid
AA-Ac	3-acetamido-6-aminoacridone
AFP	α-fetoprotein
ANS	2-aminonaphthalene-1-sulfonate
ANTS	8-amino-naphatalene-1,3,6-trisulfonicacid
APTS	7-aminopyrene-1,3,6-trisulfonate
BA	*N*-quaternized benzyl-amine
BAP	2-amino-6-amidobiotinyl-pyridine
BPE	peak electropherogram
CAE	capillary affinity electrophoresis
CBQCA	3-(4-carbobenzoyl)-2-quinolinecarboxaldeyde
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CHO	Chinese hamster ovary
CIEF	capillary isoelectrofocusing
CZE	capillary zone electrophoresis
DMSO	dimethylsulfoxide
Dol-P	dolichol phosphate
Dol-P-P-GlcNAc	dolichol pyrophosphate GlcNAc
EIEs	extracted ion electropherograms
EOF	electro-osmotic flow
ER	endoplasmic reticulum
Fmoc-Cl	9-fluorenylmethyl chloroformate
GlcNAc-P	GlcNAc-phosphate
HCC	hepatocellular carcinomas
He-Cd	helium-cadmium
huEPO	human erythropoietin
IEF	isoelectric focusing
LC	liquid chromatography
LCA	*Lens culinaris* agglutinin
LIF	laser-induced fluorescence
MEKC	micellar electrokinetic chromatography
NBD-F	7-nitro-2,1,3-benzoxadiazole-4-fluoride
NP	normal phase
PEG	polyethyleneglycol
PEO	poly(ethylene oxide)
pI	isoelectric point
PMP	1-phenyl-3-methyl-pyrazolone
PMPMP	1(4-methoxy)phenyl-3-methyl-5-pyrazolone
PVP	polyvinylpyrrolidone
rhuEPO	recombinant human erythropoietin
RP	reverse phase

SA	sialic acid
SDS	sodium dodecyl sulfate
SiA	sialic acids
TOF	time-of-flight
WGA	wheat germ agglutinin
WIDC	capillary imaging detection

9.1 Introduction

Glycosylation is a crucial event that dominates post- or cotranslational modifications, which occur in more than 50% of eukaryotic proteins [1]. For example, most proteins present in human sera, expect for albumin and some other proteins, are glycoproteins.

Glycoproteins are modified with glycans that are covalently attached to the Asn residue in the Asn-X-Ser/Thr sequence (*N*-glycans), where X is any amino acid other than proline [2, 3]. In contrast, *O*-glycans are attached to Ser/Thr residues of proteins (Fig. 9.1a). This chapter describes the analysis of *N*-glycans. Figure 9.1b shows representative *N*-glycans. All *N*-glycans have a common core structure, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-, and are classified into three types: (1) oligomannose (high-mannose) type, in which only Man residues are attached to both of the Manα1–6 and Manα1–3 arms of the core structure; (2) hybrid type, in which Man residues are attached to only the Manα1–6 arm, an "antenna" structure produced through the action of *N*-acetylglucosaminyltransferases being attached to the Manα1–3 arm; and (3) complex type, in which the "antenna" structure is attached to both the Manα1–6 and Manα1–3 arms.

The biosynthesis of *N*-glycans in eukaryote is briefly shown in Fig. 9.1c. The biosynthesis begins on the cytoplasmic face of the endoplasmic reticulum (ER) membrane with the transfer of GlcNAc-phosphate (GlcNAc-P) from uridine diphosphate GlcNAc (UDP-GlcNAc) to the lipid-like precursor dolichol phosphate (Dol-P) to generate dolichol pyrophosphate GlcNAc (Dol-P-P-GlcNAc). Glycans are not primary gene products but rather secondary ones, as the result of biosynthesis by various combinations of glycosidases and glycosyltransferases. The synthesis of glycans is also controlled by the level of nucleotide donors and nucleotide transporters, and substrate availability. It should be noted that these glycosidases/glycosyltransferases present in the ER and Golgi are specific enzymes catalyzing the release/addition of monosaccharides at specific positions with specific configuration of the glycan backbone [4]. These enzymes are exquisitely sensitive to the physiological state of the cell in which the glycoprotein is expressed. Thus, the populations of glycan attached to the Asn residue in a mature glycoprotein depends on the cell type in which the glycoprotein is expressed and on the physiological status of the cell. The status is regulated during development and differentiation, and varies under pathophysiological conditions such as cancer and inflammation [5–7].

Glycans have been reported to participate in many biological processes, such as cell growth, migration, embryonic development, differentiation, inflammation,

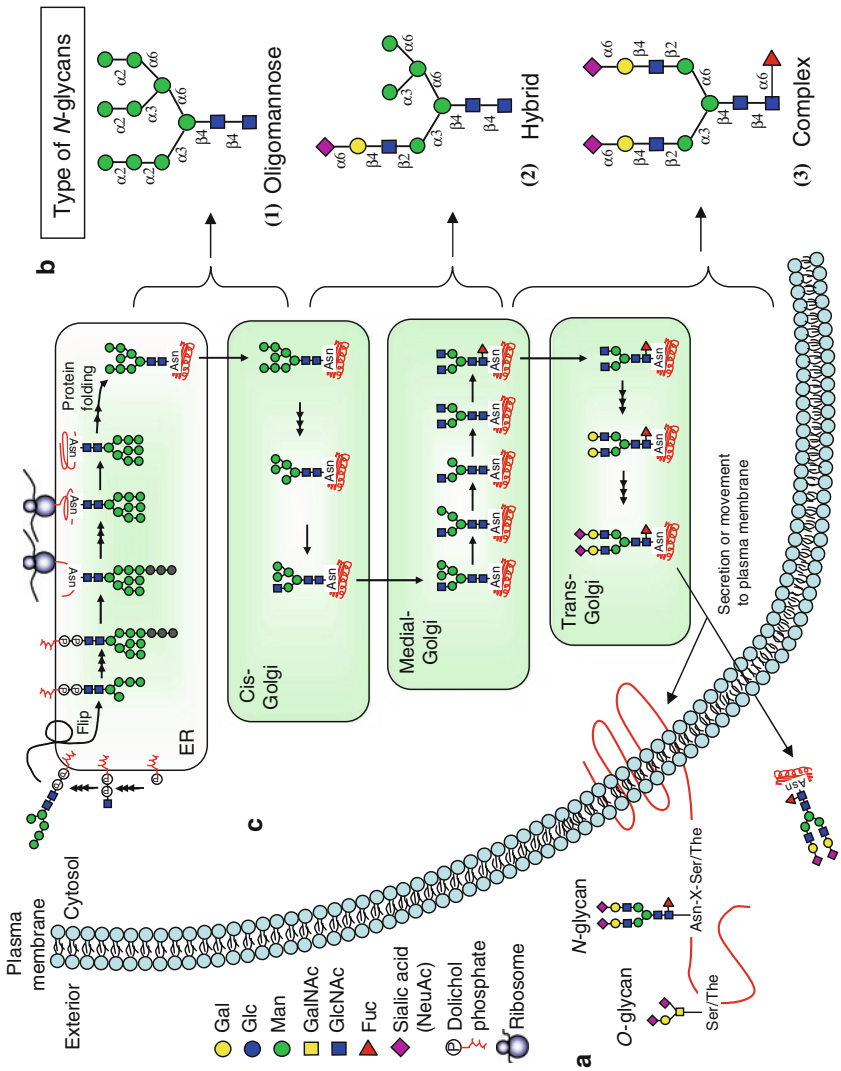

Fig. 9.1 Features of glycans on glycoproteins. (**a**) *N*-glycans and *O*-glycans in common amino acid(s) sequences of proteins. (**b**) Classification of *N*-glycan types. (**c**) Biosynthesis of *N*-glycans on proteins (From Varki et al. [15], with permission)

tumor invasion, fertilization, immune defense, molecular recognition, inter- and intracellular signaling, host–pathogen interactions, cell trafficking, transmembrane signaling, cell adhesion, division processes, viral replication, and parasitic infections. Furthermore, glycan chains can significantly alter the conformation of a protein and may consequently modulate the functional activity of a protein as well as protein–protein interactions. Hence, glycoprotein-glycans represent a valuable tool for the fine-tuning and modulation of protein structure and function, "intervening in the social life of cells" [8–16]. Therefore, the analysis of *N*-glycans is very important for investigating their mechanisms or their functions in detail.

Capillary electrophoresis (CE) was introduced as a novel method for separation techniques in the 1980s [17], and commercial use began in 1988. Since the 1990s, it has been demonstrated that CE can be used as a powerful tool for the analysis *N*-glycans derived from glycoproteins. It offers inherently high efficiencies and some selectivity due to its different modes [18–20].

9.2 Isolation and Direct Analysis of Intact Glycoproteins

Potential *N*- or *O*-glycosylation sites on a glycoprotein are usually occupied to various degrees. Moreover, a wide range of structurally different glycans is present at a particular glycosylation site. Hence, all glycoproteins must be considered as heterogeneous mixtures of closely related glycosylation variants, so-called glycoforms, consisting of a number of different glycans attached to an individual glycosylation site with various degrees of site occupancy.

Human erythropoietin (huEPO) is a glycoprotein hormone produced primarily by the kidneys that stimulates red blood cell production. Recombinant human erythropoietin (rhuEPO), generally produced in Chinese hamster ovary (CHO) cells, is used as a therapeutic protein. In addition, the misuse of EPO by athletes is an important problem. Therefore, the profiling of EPO glycoforms is a critical means of quality control in the pharmaceutical industry and anti-doping analysis in sports. rhuEPO has three *N*-linked and one *O*-linked site, and the glycans have various numbers of sialic acid residues [21, 22]. Isoelectric focusing (IEF) in the slab gel format has been widely used for glycoform analysis of rhuEPO based on differences in sialic acid content [23]. An example of IEF–gel electrophoresis patterns is shown in Fig. 9.2a. Two standard samples of rhuEPO derived from CHO cell lines were examined. One of the preparations (S-1) yielded five distinct bands, while the other (S-2) yielded only four bands. Although both samples yielded five bands during the staining procedure, the destaining procedure abolished the band with the highest isoelectric point (pI) value for S-2. This means that the whole procedure requires skillful techniques to obtain good and reproducible results. In addition, the procedure is time-consuming in gel preparation, staining-destaining procedures, and scanning for quantitative determination of each material.

Several separation modes of capillary electrophoresis have been developed to overcome the problems of slab gel IEF. Capillary zone electrophoresis (CZE) and

capillary isoelectric focusing (CIEF) are the most powerful modes of CE for glycoform analysis. Excellent resolution has been reported for glycoform analysis of ribonuclease B, ovalbumin, α_1-acid glycoprotein, horseradish peroxidase, and rhuEPO [24–27]. Mechref and Novotny [28] listed various applications in the area, summarizing the types of analyzed glycoproteins along with the employed modes of CE. Also, analytical parameters such as buffer type, pH of the background electrolyte, and buffer additives (organic cations, detergents, and water-soluble polymers) are also included. The 2002 European Pharmacopeia describes methodology for rhuEPO glycoforms involving CE [29]. The separation is performed using bare fused silica and separation buffer containing 1,4-diaminobutane and urea. Although excellent resolution of seven glycoforms is observed in the first analysis, the glycoform resolution becomes irreversibly worse on several repeated analyses [30–32]. Kinoshita and coworkers [26] successfully separated rhuEPO glycoforms using the CZE mode with good reproducibility, as shown in Fig. 9.2b. The separation can be performed using a commercially available surface-modified capillary for capillary gas chromatography with a running buffer containing hydroxypropylmethycellulose (HPMC) within 1 h. Nine peaks are observed between 30 and 60 min, and the run-to-run and day-to-day residual standard deviations (RSDs) (%) are 1.04–1.67 and 1.01–1.99, respectively. This method is thus suitable for routine glycoform analysis.

In contrast, the CIEF mode is a perfect alternative for IEF gel electrophoresis for glycoform analysis [33,34]. Although CIEF provides definite pI values for the resolved bands, the CIEF methods proposed so far are associated with relatively poor resolution. The major reason for this poor resolution is that a mobilization step is required to force the focused bands through the detector after the focusing step. Recently, CIEF with whole-capillary imaging detection (WCID) was developed, which eliminates the mobilization step because of observation of the whole capillary absorption image with a CCD camera. An example of glycoform analysis involving CIEF-WCID is shown in Fig. 9.2c. Dou et al. [35] successfully separated rhuEPO glycoforms using a fluorocarbon-coated fused-silica capillary with 4 M urea, 1% polyvinylpyrrolidone (PVP), and 2% ampholytes (pH 3–10) containing two pI markers (pI 3.59 and 5.85) within 10 min [35]. The excellent resolution and speed of CIEF-WCID make it a powerful tool for routine glycoform analysis of glycoprotein pharmaceuticals.

Glycoform analysis by CZE–mass spectrometry (MS), especially electrospray ion (ESI)-orthogonal accelerating time-of-flight (TOF), has reviewed by Amon et al. [36]. An instance of CZE-MS analysis of glycoforms of rhuEPO is shown in Fig. 9.2d. The CZE separation was achieved based on the number of sialic acid residues, and the sequences of monosaccharides on the protein could be distinguished with a high-resolution ESI-orthogonal accelerating TOF instrument. In Fig. 9.2d, a base peak electropherogram (BPE) and extracted ion electropherograms (EIEs) of glycoforms bearing 8–14 sialic acids (SiAs) are shown. For example, in this figure, EIE of 14 SiA indicates an electropherogram of a glycoform containing 14 SiAs, whose main molecular mass is 30,178.6 m/z (=165 amino acids (backbone protein) + 14 sialic acids + 22 hexoses + 19 *N*-acetylhexosamines + 3 fucoses). EIE of 14 SiAs was obtained from four major ions (1776.2, 1887.2, 2012.9, and 2156.7 m/z) in

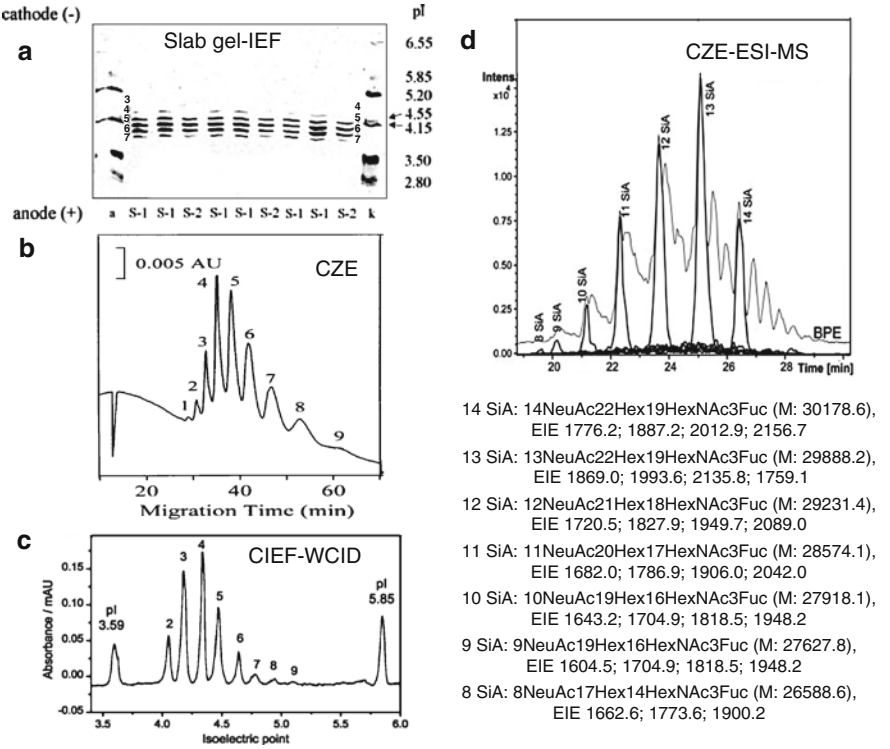

Fig. 9.2 Analysis of rhuEPO glycoforms. (**a**) Isoelectric focusing (IEF) slab gel electrophoresis of rhuEPO. Bands 3–7 observed in lanes S-1 and S-2 correspond to peaks 3–7 in (**b**). Analyzed samples: two standard samples (S-1 and S-2) of rhuEPO produced from Chinese hamster ovary (CHO) cell lines were provided by the European Pharmacopeia as biological reference products (BRPs). Lanes a and k, isoelectric point (pI) markers. (**b**) Capillary zone electrophoresis (CZE) of rhuEPO. Analyzed sample: 1 mg/mL of rhuEPO-BRP. Analytical conditions: capillary, DB-1 capillary (57 cm [effective length, 50 cm], 100 μm inner diameter); running buffer, 10 mM acetate buffer (pH 5.7) containing 0.5% w/v of hydroxypropylmethycellulose (HPMC); applied potential, 12.5 kV; and detection, UV absorption at 200 nm. Peaks 3–7 correspond to bands 3–7 in (**a**), respectively. (**c**) Capillary isoelectric focusing with whole-capillary imaging detection (CIEF-WCID) of rhuEPO. The pI values of peaks 2–9 are 4.04, 4.17, 4.33, 4.45, 4.63, 4.77, 4.93, and 5.08, respectively. Analyzed sample: 1 mg/mL of rhuEPO-BRP. Analytical conditions: capillary, fluorocarbon-coated fused silica capillary (5 cm, 100 μm inner diameter); sample buffer, 4 M urea, 1% PVP, and 2% Fluka ampholytes, pH 3–10, containing two pI markers, pI 3.59 and 5.85; applied potential for focusing, 3 kV; and detection, UV absorption at 280 nm with WCID. Images were taken automatically every 30 or 20 s. The anolyte and catholyte were 100 mM phosphoric acid and 100 mM sodium hydroxide containing 1% (w/v) PVP, respectively. (**d**) Capillary zone electrophoresis–electrospray ion–mass spectrometry (CZE–ESI–MS) of rhuEPO. Based peak electropherogram (BPE) and extracted ion electropherograms (EIEs) of glycoforms with different numbers of sialic acids. EIEs were obtained for the three or four major ions of the respective charge distributions, as indicated together with the deconvoluted mass. Analyzed sample: 2.5 mg/mL of rhuEPO-BRP. Analytical conditions: capillary, LN-coated capillary (100 cm, 50 μm inner diameter); running buffer, 2 M HAc; applied potential, 30 kV; detection, ESI-TOF MS; sheath liquid, 1% HAc in 2-PrOH:water (1:1) at 4 μL/min; and nebulizer gas, 0.2 bar. An electrospray potential of −4.5 kV was applied at the inlet of the MS (ESI positive mode). (**a** and **b** from Kinoshita et al. [26], with permission; **c** from Dou et al. [35], with permission; **d** from Balaguer et al. [37], with permission)

the respective charge distributions, as indicated together with the deconvoluted mass. In this electropherogram, a total number of 44 different glycoforms and at least 135 isoforms, when accounting also for differences in the acetylation of the glycans, can be distinguished [37]. For such extensive analysis of iso- and glycoforms, the EPO concentrations should be in the range of several mg/mL, as well as for analysis by CZE with an ultraviolet (UV) detector or analysis by CIEF-WICD.

For glycoform analysis of low concentrations (μg-pg/mL) of glycoproteins, such as in biological samples, effective pre-concentration of the proteins along with improvement of the sensitivity of the CZE, CIEF-WICD, and CZE-MS methods will be necessary.

9.3 Release of *N*-Glycans from Glycoproteins

Glycoform analysis is useful for evaluation of glycoproteins as whole molecules based on their glycan heterogeneity. However, glycoform analysis is mainly based on the differences in the pIs of molecular species that have different numbers of SiA residues. Therefore, information about glycan structures obtained by glycoform analysis is limited, and we have to analyze glycan profiles. Analysis of the glycans released from a core protein is a broadly applied strategy that has some distinct advantages: (1) It is well suited for establishing an overview of glycans, and many glycan structures have been already confirmed by a number of research projects. (2) Quantitation of glycans becomes more feasible, because the released glycans can be derivatized with chromogenic or fluorogenic reagents and can be detected with specific and sensitive detectors. Moreover, when a mass spectrometer is used as the detector, there are less specific differences in ionization among glycans without peptide substructures, because ionization strongly depends on the sequence of amino acids. (3) Analyses of glycan linkages using exoglycosidases provide more detailed information on the structures. Chemical and enzymatic methods are available for the release of *N*-glycans, such as chemical release and enzymatic release.

9.3.1 Chemical Release

N-glycans are often cleaved from glycoproteins with anhydrous hydrazine [38, 39]. This method is used widely for the comprehensive analysis of *N*-glycans derived from organs or cells as well as from glycoproteins.

Chemical release by hydrazine (i.e., hydrazinolysis), however, has several major disadvantages. First, the acetyl groups of *N*-acetylamino sugars, and the acetyl and glycolyl groups of SiAs, are often hydrolyzed under the reaction conditions. Second, the residual hydrazide or amino groups are often combined with the reducing termini of some glycans. Third, high reaction temperatures often result in the loss

of the reducing end GlcNAc. Finally, it is essential to maintain strictly anhydrous conditions. Therefore, this method is not used so much now.

9.3.2 Enzymatic Release

The first choice for the release of *N*-glycans is an enzymatic reaction involving endoglycosidases such as endoglycosidase H (Enzyme Commission [EC] number: 3.2.1.96; cleaves between the two *N*-acetylglucosamine residues in the core of oligomannose *N*-glycans), endoglycosidase F (EC number: 3.2.1.96; cleaves between the two GlcNAc residues in the core of oligomannose or biantennary *N*-glycans), peptide-*N*-glycosidase F (EC number: 3.5.1.52, referred to as PNGase F or *N*-glycanase; cleaves between the Asn and GlcNAc residues of oligomannose, complex, or hybrid *N*-glycans; requires at least one amino acid at both the amino terminals, and does not work if GlcNAc next to Asn has α1-3-fucosylated), and peptide-*N*-glycosidase A (EC number: 3.5.1.52, referred to as PNGase A; cleaves between the Asn and *N*-acetylglucosamine residues of oligomannose, complex, or hybrid *N*-glycans; requires at least one amino acid at both the amino terminal, and will work even if GlcNAc next to Asn is α1-3-fucosylated). The structure of GlcNAc next to Asn is α1-3-fucosylated, and is commonly found in plants.

For the release of *N*-glycans attached to Asn residues through *N*-glycosidic linkages (structure 1 in Fig. 9.3), digestion with PNGase F is most frequently performed, and the glycans are released as *N*-glycosylamines (structure 2). The *N*-glycosylamines are readily hydrolyzed to free forms (structure 3) under slightly acidic conditions, but are relatively stable under slightly basic conditions. Structures 2 and 3 are analyzed by CE after appropriate modification such derivatization with a chromogenic or fluorogenic reagent (structures 4, 6, 7, 9, 11, and 12), or reduction (structure 13).

9.4 Analysis of *N*-Glycans by Capillary Electrophoresis

9.4.1 Derivatization of Released N-Glycans and the Detection

A mapping database of *N*-glycans without labeling was developed, and structural analysis was performed by simple comparison of migration times [19]. Approximately 80 different sialylated *N*-glycans of known structures were separated and detected at 194 nm. However, this database is available for structural confirmation of sialylated structures, and a large amount of a glycoprotein sample is often required for full assignment. Therefore, labeling of *N*-glycans has been developed for biological samples.

Derivatization of glycans serves two purposes: (1) attachment of a charged group to enable or facilitate CE separation, which is advantageous for the analysis of neutral glycans; and (2) enhancement of the sensitivity of a highly sensitive detector using

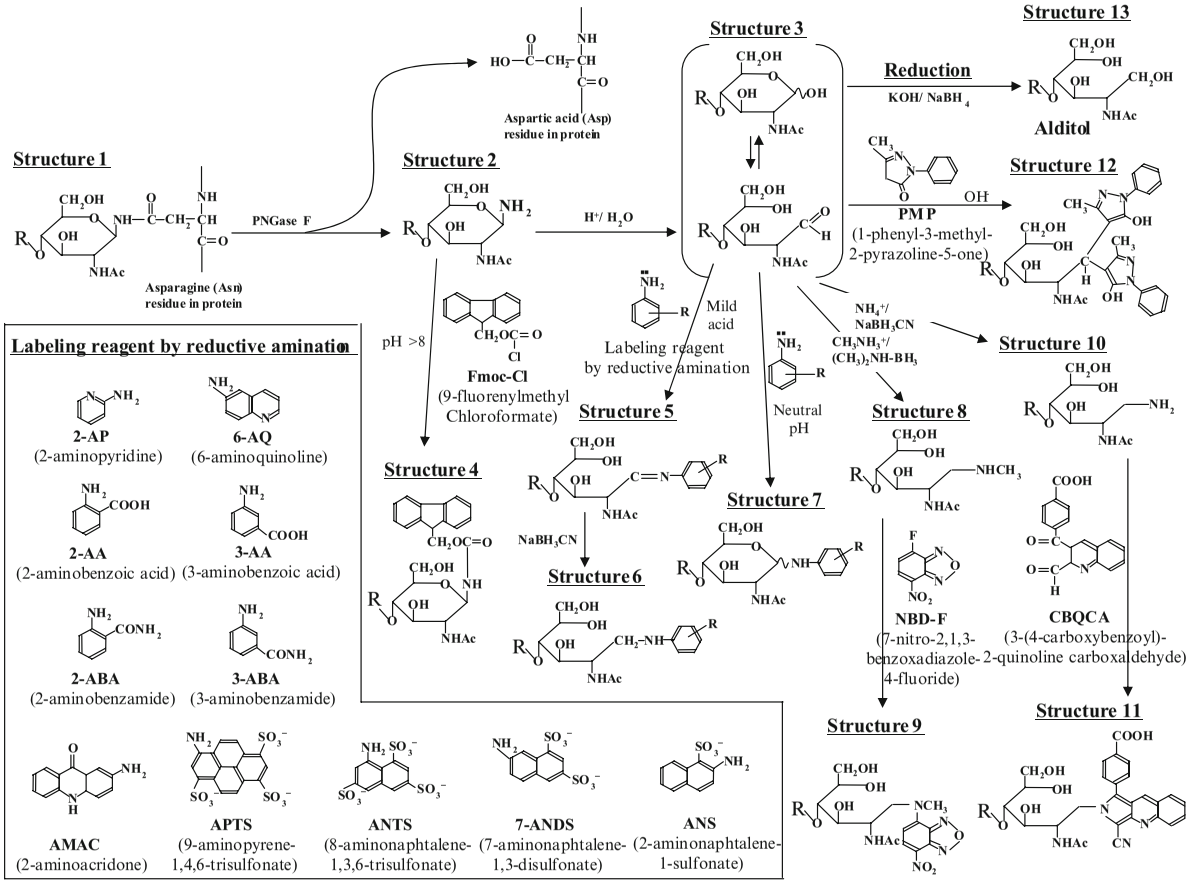

Fig. 9.3 Flowchart for the labeling procedures and the labeling reagents for *N*-glycans after the release of *N*-glycans from proteins

laser-induced fluorescence (LIF) detection. Various labeling reagents have been reported, and typical labeling reagents and labeling procedures are shown in Fig. 9.3.

The labeling method involving reductive amination (structures $1 \rightarrow 2 \rightarrow 3 \rightarrow 5 \rightarrow 6$ in Fig. 9.3) is usually used not only for CE analysis but also for other analytical methods such as high-performance liquid chromatography (HPLC). The chromogenic or fluorogenic reagents used for the labeling are 2-aminopyridine (2-AP) [20, 40–42], 2-amino-6-amidobiotinyl-pyridine (BAP) [43], 6-aminoquinoline (6-AQ) [41, 44], 2-aminobenzoic acid (2-AA) [45–49], 3-aminobenzoic acid (3-AA) [50, 51], 4-aminobenzoic acid (4-AA) [52], 2-aminobenzamide (2-ABA) [50, 53], 3-aminobenzamide (3-ABA) [50], 4-aminobenzonitrile [54], 2-aminoacridone (AMAC) [55–59], 3-acetamido-6-aminoacridine (AA-Ac) [60, 61], 9-aminopyrene-1,4,6-trisulfonate (APTS) [18, 51, 62–73], 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) [40, 67, 74], 7-aminonaphthalene-1,3-disulfonate (7-ANDS) [73], and 2-aminonaphthalene-1-sulfonate (ANS) [73].

The labeling reaction starts with the attack of the lone pair of amino groups of the reagent on the carbonyl groups of reducing sugars, yielding a Schiff base (imine derivative, structure 5 in Fig. 9.3). Mild acidic conditions are necessary to promote the reaction, and the formed Schiff base is reduced with sodium cyanoborohydride to a stable secondary amine (structure 6). Typical reaction conditions are as follows: temperature, 37–90°C; reaction time, 1 h to overnight; acidic conditions, acetic acid or citric acid; reducing reagent, $NaCNBH_3$; and organic solvent for the labeling reagent, methanol or dimethylsulfoxide (DMSO). 2-AP and aminonaphthalene sulfonic acids such as ANTS are detected with a helium–cadmium (He–Cd) ion laser. Aminobenzoic acid isomers such as 2-AA and 2-ABA are also detected with a He–Cd ion laser. APTS derivatives are detected with an argon ion laser. Aminoacridones such as AMAC are also detected with an argon ion laser. Optimization of these parameters is necessary for quantitative determination to minimize sample decomposition.

In many cases, removal of the excess reagents in the labeling reaction is necessary. A cleanup procedure involving extraction with organic solvents is performed when a hydrophobic reagent such as AMAC is available. Alternatively, the labeled glycans are purified by means of a hydrophilic filter in the presence of acetonitrile and subsequent elution with water [53]. Mort et al. [75] reported the use of Sephadex beads carrying aldehyde groups in the reaction mixture (excess reagent reacted with the "scavenger beads") and reported complete removal of the excess reagent. A gel filtration method involving Sephadex G-10 and Biogel P-2 columns or packed 96-well filter plates has also been reported [50, 76, 77].

Many researchers compared the labeling reagents to find the most suitable one for their analysis. Bigge et al. [78] extensively studied the reaction conditions for the derivatization of *N*-glycans with 2-ABA and 2-AA. Kakehi et al. [50] compared the reaction efficiency on reductive amination using maltose as a model with nine monoamino benzene derivatives, and they concluded that 3-ABA showed the highest reactivity. However, 2-ABA was the most intensely fluorescent and 4-ABN was the most effective chromogenic compound. They proposed that 3-ABA was the first choice for the analysis of *N*-glycans, and the weakness in sensitivity was overcome by employing a He–Cd laser-induced fluorometric detector. Kamoda et al. [51]

compared APTS and 3-AA as the labeling reagents for the analysis of *N*-glycans of antibody pharmaceuticals. The APTS derivatization method is widely used due to the rapid analysis time and high sensitivity. They found that the 3-AA derivatization method showed sufficient resolution for characterization of the *N*-glycans in antibody pharmaceuticals, and almost equivalent repeatability and accuracy to those of the APTS derivatization method. Although the 3-AA method needs a longer analysis time than the APTS method, it should be noted that this is not an important disadvantage, because a CE apparatus is generally equipped with an auto-injector. Furthermore, the 3-AA derivatization method has some advantages: high purity reagents are easily commercially available, and 3-AA labeled *N*-glycans can be analyzed by HPLC and are stable at under −20°C for several months.

APTS, ANTS, or analogous negatively charged labels have been widely used for the CZE separation of glycans in connection with (laser-induced) fluorescence detection. 2-AP, 2-ABA, and analogous compounds have positive charges based on amino groups, and thus are available as well as the driving force in CE.

A slight modification of the reductive amination procedure has been reported for the analysis of *N*-glycans. The reaction of an amino-compound with the carbonyl groups of reducing sugars yields glycosylamines (structure 7 in Fig. 9.3), when reducing reagents are not present. Disaccharides labeled with 4-AA via the formation of glycosylamines were analyzed by CE-ESI MS [52]. The authors reported that the glycosylamine approach instead of reductive amination provides more information on linkage and anomeric configuration on negative-ion ESI [52]. Honda et al. [79] reported a novel derivatization procedure for reducing sugars. Reducing sugars (structure 3 in Fig. 9.3) are converted to *N*-methylglycamines (structure 8) in the presence of methylamine and the dimethylamine-borane complex. The resultant *N*-methylglycamines are derivatized with 7-nitro-2,1,3-benzoxadiazole-4-fluoride (NBD-F) (structure 9). The derivatives show strong absorbance at 490 nm, and their fluorometric properties are appropriate for the LIF detector equipped with an argon ion laser. The main advantage of this scheme is that sialic acids are not released from sialylated glycans under the mild conditions, and thus it can be applied to the analysis of trace amounts of sialylated oligosaccharides [79]. 3-(4-Carboxybenzoyl-)-2-quinoline carboxyaldehyde (CBQCA) has been used for the ultrasensitive detection of compounds having amino residues [80]. On *N*-glycan analysis, the reducing ends of glycans are converted to 1-amino-1-deoxyalditols (structure 10 in Fig. 9.3) through reductive amination. The resultant 1-amino-1-deoxyalditols are derivatized with CBQCA (structures 11). Liu et al. [44] analyzed *N*-glycans derived from bovine fetuin using CBQCA by CE-LIF. The isoindole derivatives thus formed exhibited excitation maxima at 442 nm blue line with the He–Cd ion laser and the 456 nm secondary line with the argon laser. This procedure has the advantage that it is not necessary to remove the excess reagent, since the unreacted compound does not exhibit fluorescence.

The UV-absorbing tag, 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP), and its methoxy analog, 1-(*p*-methoxy)-phenyl-3-methyl-2-pyrazolone (PMPMP), have been used to derivatize reducing *N*-glycans under weakly basic conditions (structure 12 in Fig. 9.3) [81, 82]. Various modes of CE for the analysis of

PMP-derivatives have been reported, including borate complexation, micellar electrokinetic chromatography (MEKC) in the presence of sodium dodecyl sulfate (SDS), and complexation with alkaline earth metals [83–85].

Recently Kamoda et al. [86] reported a novel means of derivatization of *N*-glycosylamines (structure 2 in Fig. 9.3) yielded on PNGase F digestion. After the release of *N*-glycans with PNGase F, the resultant *N*-glycosylamine (structure 2) is stable under the relatively basic conditions in which the enzyme reaction with PNGase F is performed, and easily subjected to in situ derivatization of glycosylamine-type glycans with 9-fluorenylmethyl chloroformate (Fmoc-Cl). The structure of Fmoc-labeled *N*-glycans (structure 4) is shown in Fig. 9.3. The Fmoc labeling method has some advantages: (1) it is a simple procedure, as Fmoc reagent is just added to the reaction solution after digestion with PNGase F, and it is easy to remove excess reagent by extraction with chloroform; (2) it is a high-speed analysis that is completed within 4 h, including the enzymatic glycan-releasing reaction and the labeling reaction before analysis; (3) it has high sensitivity – 5 and 30 times higher than that for 2-AA and PA-labeled *N*-glycans, respectively, with a fluorescent detector; and (4) it enables an easy recovery of free-form glycans (structure 3 in Fig. 9.3) by incubation with morpholine in a dimethylformamide solution at room temperature. Nakano et al. [87] analyzed Fmoc-labeled *N*-glycans derived from several glycoproteins such as fetuin, α1-acid glycoprotein, immunoglobulin G (IgG), and transferrin by CE–MS. The detailed results are presented in Section. 9.5. Lamari et al. [88] have done a comprehensive review of derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometric structure analyses.

On CE–MS, *N*-glycans are often reduced to their alditol forms (structure 13 in Fig. 9.3). Reduction of the reducing ends of glycans (structure 3) with a reducing agent such as sodium borohydride under weakly basic conditions affords alditols (structure 13). CE analysis of *N*-glycans as their alditol forms has not been reported, probably due to the low sensitivity. Kakehi et al. [89] reported the analysis of *O*-glycans derived from bovine submaxillary mucin and swallow nest material by CE, with monitoring of the absorbance at 185 nm, since *O*-glycans are released chemically through β-elimination in the presence of sodium borohydride. The study demonstrated only minor differences between phosphate and borate buffers as background electrolytes, suggesting no significant contribution of borate complex formation to the resolution of glycans.

9.4.2 Separation Modes

Several separation modes of capillary electrophoresis have been demonstrated to give excellent resolution of *N*-glycans during the past decade: (1) capillary zone electrophoresis (CZE); (2) capillary gel electrophoresis (CGE); (3) capillary isoelectric focusing (CIEF); (4) micellar electrokinetic chromatography (MEKC); (5) capillary electrochromatography (CEC); and (6) capillary affinity electrophoresis (CAE). An appropriate combination of these separation modes makes it possible to resolve *N*-glycans released from glycoproteins.

9.4.2.1 Capillary Zone Electrophoresis

This is the basic mode for the CE analysis of *N*-glycans. In the CZE mode, the analyte ions resolved based on their individual electrophoretic mobilities pass through the detector as "analyte zones." The inner surface of the silica capillary has a negative charge due to ionization of the silanol groups. The ionized silanol groups on the capillary wall attract cationic species in the buffer. These cationic species are driven toward the cathode. This force is called the electro-osmotic flow (EOF), and the EOF is usually significantly faster than the solute velocity.

On CZE in normal polarity [inlet (+)−outlet (−) of CE capillary], the cations are observed first, because they move with the EOF + and their own mobilities. Neutral solutes are eluted next, but are unresolved because they do not have charges and move only with the EOF. Anions such as sialylated or phosphorylated *N*-glycans are eluted last because they migrate based on their negative charges in the opposite direction to the EOF, but the EOF is the very fast, and so they are gradually pulled back to the anode. Therefore, CZE is useful for the analysis of *N*-glycans with different charges.

We can modulate the EOF by modifying the inner surface of the capillary with some neutral polymers. Capillaries modified with polymers are commercially available under names such as DB-1 (dimethylpolysiloxane), DB-17 (50% phenyl methylpolysiloxane), PVA (polyvinyl alcohol), and PAA (polyacrylamide). These coatings are used to suppress the EOF. In contrast, cationic polymers such as Polybrene (PB) are used to generate a strong anodic EOF.

The CZE mode has been studied for the analysis of the following glycoprotein samples: ovalbumin [20, 40, 50, 74], ribonuclease B [41, 50, 63, 73], fetuin [44, 50, 63, 74], thyroglobulin [50], α1-acid glycoprotein [25, 66], and IgGs of antibody pharmaceuticals [45, 51, 69, 90, 91]. Figure 9.4 shows examples of the analysis of *N*-glycans derived from antibody pharmaceuticals with the CZE mode. In Fig. 9.4a–c, *N*-glycans derived from rituximab were derivatized with APTS after PNGase F digestion and were detected at 520 nm, with excitement with an argon laser with a 488 nm filter [69]. In Fig. 9.4d and e, *N*-glycans derived from trastuzumab were derivatized with 3-AA after PNGase F digestion, and analyzed at 405 nm and with excitement with a He–Cd laser with a 325 nm filter [90]. Complex-type biantennary *N*-glycans with zero, one, or two galactose residues (G0, G1, or G2) are abundantly present in IgG molecules. G1′ is a positional isomer of G1. In Fig. 9.4a, these glycans are well resolved within 4 min. The structures of G1 and G1′ were confirmed by comparing CE profiles obtained on sequential enzymatic digestion. *N*-glycans released by digestion with PNGase F were further digested with β-*N*-acetylglucosaminidase to remove the terminal GlcNAc residues on G0, G1, and G1′, and then injected to CE after derivatization with APTS. One GlcNAc residue is released from G1 and G1′ (Fig. 9.4b). Then, digestion with α1-2,3 mannosidase, which specifically cleaves the terminal Man residue linked through an α1-3 linkage, released Man residues only from G1, indicating that G1 has a galactose residue on the Manα1-6 arm.

In Fig. 9.4d and e, the mixtures of the *N*-glycans (G0, G1, G1′, and G2) labeled with 3-AA showed similar profiles between 23 and 27 min. Because 3-AA derivatives

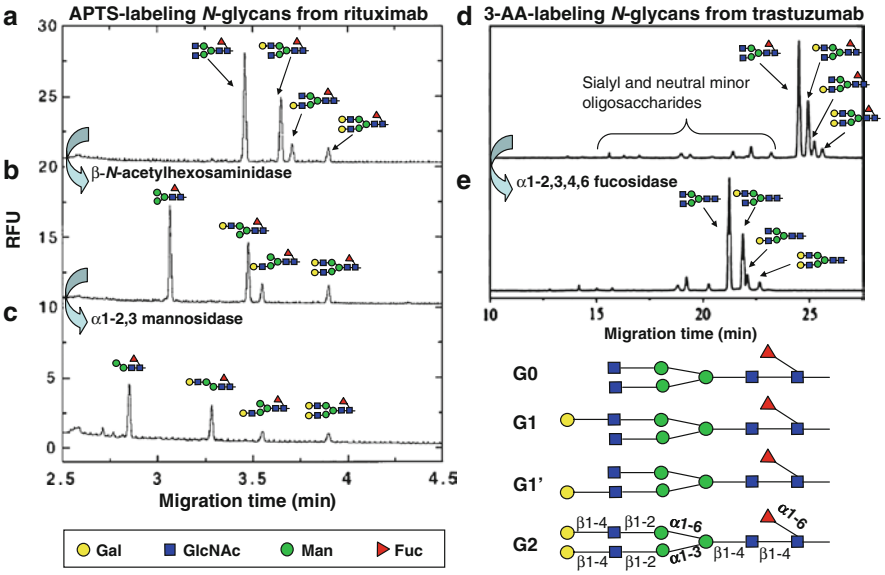

Fig. 9.4 CZE analysis of *N*-glycans derived from antibody pharmaceuticals with different modes of derivatization. (**a**–**c**) Electropherograms of *N*-glycans obtained from rituximab after sequential enzymatic digestion steps: (**a**) PNGase F; (**b**) β-*N*-acetylhexosaminidase; and (**c**) α1-2,3 mannosidase. Samples were derivatized with APTS before capillary electrophoresis (CE) analysis after each endoglycosidase digestion. Analytical conditions: capillary, neutral hydrophilic polymeric coated capillary (27 cm [effective length, 20 cm], 50 μm inner diameter); applied potential, 700 V/cm; detection, fluorescent detection at 488 nm with excitation with an argon laser with a 520 nm filter. The released *N*-glycans were dissolved in 100 mM sodium citrate phosphate (pH 5.0, 40 μL), followed by the addition of β-*N*-acetylhexosaminidase (15 U, 10 μL) and incubation at 37°C for 18 h. Enzyme α1-2,3 mannosidase (135 U) was buffer-exchanged into 50 mM sodium citrate/5 mM $CaCl_2$, pH 6.0, supplemented with 100 mg/mL of BSA; a total volume of 10 μL was added to the sample, followed by incubation at 37°C for 60 h. (**d** and **e**) Electropherograms of the *N*-glycans obtained from trastuzumab. Samples were derivatized with 3-AA after digestion with PNGase F (**d**) and then the 3-AA labeled *N*-glycans were digested with α1-2,3,4,6 fucosidase (from bovine kidney; Glyko, Novato, CA) (**e**). Analytical conditions: capillary, DB-1 capillary (50 cm [effective length, 40 cm], 50 μm inner diameter); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 7.5% w/v PEG70000; applied potential, 30 kV; and detection, fluorescent detection at 405 nm with excitation with a helium–cadmium laser with a 325-nm filter. The 3-AA-labeled *N*-glycan mixture was dissolved in 20 mM sodium citrate/phosphate buffer (pH 6.0, 25 μL), followed by the addition of fucosidase (25 mU, 5 μL) and incubation at 37°C overnight (**a**–**c** from Kamoda and Kakehi [90], with permission; **d**, **e** from Ma and Nashabeh [69], with permission)

have less negative charges than APTS derivatives, the analysis requires a longer time, and the observed number of theoretical plates is lower than that observed with the APTS method. However, we can observe both oligomannose type *N*-glycans and minor sialylated *N*-glycans, as indicated in Fig. 9.4d. Digestion of these glycans with α1-2,3,4,6 fucosidase, which specifically cleaves the Fuc residue linked at α1-6 at the reducing end, showed that the peaks between 21 and 23 min

represented *N*-glycans corresponding to G0–G2, which do not have a fucose residue at the reducing end, as shown in Fig. 9.4e.

9.4.2.2 Capillary Gel Electrophoresis

This mode is used for the size-based separation of biological macromolecules. The separation of glycans is achieved by the sieving of glycans, when they migrate through gel matrix in the capillary. The analysis is performed by filling the capillary with a sieve-like matrix, for example, of linear polymer solutions such as polyethyleneglycol (PEG) and polyethyleneoxide (PEO). This molecular sieving effect is based on the hydrodynamic volume differences between glycans in solution. Due to this mechanism of separation, distinct resolution of certain structural isomers has been demonstrated using CGE [18, 66, 92]. Kakehi et al. [92] were able to distinctly separate a mixture of hyaluronic acid polymers from dp8 to dp190 within 1 h using PEG as the matrix. Guttman et al. [66] separated positional isomers of oligomannose type *N*-glycans derived from ribonuclease B using PEO. Difference in the electrophoretic mobilities of the oligomannose type *N*-glycans was compared with those of the linear molecules of malto-oligosaccharides (glucose oligomers) at different PEO concentrations in the running buffer system. The authors observed that an increase in PEG concentrations causes increases in the relative mobilities of the oligomannose type *N*-glycans compared to those of the linear glucose oligomers. Analysis of the data indicated that this increase in relative migration time was not due to sieving, but seemed to be related to the mannose content and the hydrodynamic volume of the branched glycans as well as to the viscosity of the separation medium.

9.4.2.3 Capillary Isoelectric Focusing

This mode is used to separate biological molecules, mainly proteins, based on differences in their pI. CIEF is performed by filling the capillary with a mixture of ampholytes and the sample, and then forming a pH gradient. By applying an electric field across the capillary with a basic solution at the cathode and an acidic solution at the anode, the ampholytes and solutes migrate until they reach a region where their overall charge is neutral (pH = pI). The ampholyte and the solute zones remain extremely narrow because diffusion to a zone of different pH results in the generation of charge and subsequent migration back to the proper zone. For the CIEF mode, isolation and direct analysis of intact glycoproteins is described above (see Sect. 9.2).

9.4.2.4 Micellar Electrokinetic Chromatography

This mode can separate neutral molecules as well as charged ones using a running buffer containing ionic surfactants such as SDS and taurodeoxycholic acid. The surfactant aggregates the molecules to yield micelles. Micelles have a three-dimensional structure with the hydrophobic moieties of the surfactant in the interior

and its charged moieties at the exterior. The separation is based on the hydrophobic interaction with the micelles. When an *N*-glycan interacts with micelles of SDS, the *N*-glycan migrates slower under the conditions that generate EOF in normal polarity. The 3-AA-labeled *N*-glycans derived from bovine fetuin were separated through MEKC using SDS and detected as direct UV absorbance at 200 nm [50]. The AMAC-labeled *N*-glycans derived from bovine fetuin and RNase B were separated using taurodeoxycholic acid and detected with an argon ion laser [55, 56]. The NBD-labeled *N*-glycans derived from bovine fetuin were separated using SDS and detected by the argon ion laser [79]. The *N*-glycans derived from recombinant tissue plasminogen activator were separated using SDS and detected with monitoring direct UV absorbance at 200 nm [93]. The best separation capacity was observed upon the addition of a divalent ion (Mg^{2+}) to the SDS electrolyte.

9.4.2.5 Capillary Electrochromatography

This mode is a fusion of liquid chromatography (LC) and CE. The capillary is packed with a stationary phase similar to that used for LC. The EOF moves the mobile phase when an electric field is applied to the packed column. The migration of the analytes through the stationary phase is prompted by EOF endured by the mobile phase. Over the last 15 years, monolithic stationary phases have been extensively investigated for HPLC and CEC separation, and the field has been recently reviewed [94–96].

Monolithic columns are mainly divided into two types, organic polymer-based monoliths [97, 98] and silica-based monoliths [99, 100], based on the composition of the monomer used for the polymerization reaction. Organic polymer-based monoliths have undergone more development and applications than the counterpart silica-based monoliths due to the relative ease of their preparation and the wide range of their chemistry. Organic polymer-based monoliths are being increasingly employed for LC and CEC separation by reverse-phase (RP) chromatography, normal-phase (NP) chromatography, hydrophilic interaction chromatography, affinity chromatography, and ion exchange chromatography. Mixtures of the 2-ABA-labeled *N*-glycans derived from ovalbumin and α1-acid glycoprotein were analyzed on a glyceryl monomethacrylate–ethylene dimethacrylate (GMM–EDMA) monolithic capillary column by NP-CEC [101]. Several structurally similar components of glycans, in addition to certain structural isomers, were analyzed using porous polyacrylamide monoliths with suitable ligands, such as 2-cyanoethy acrylate (hydrophilic separation factor) and ionic ethenesulfonic acid (needed for EOF generation) [102, 103]. However, the separation efficiency and resolution of glycans observed on the CGE and CZE modes are better than those observed on the CEC mode [104, 105].

9.4.2.6 Capillary Affinity Electrophoresis

This mode is an analytical approach by which the migration of carbohydrate molecules is observed in the presence of a lectin. Lectins are glycoproteins that recognize and bind with carbohydrate residues of specific sugar structures.

Therefore, the different migration times of different structures of *N*-glycans can be observed. Uegaki et al. [106] reported a model study involving determination of the interaction between a mixture of a simple glycan and a lectin. Kinoshita and Kakehi [107] and Nakajima et al. [108–110] developed a technique for glycomics studies involving profiling of complex mixtures of glycans derived from glycoproteins. Recently, Kakehi and Kinoshita [111] reviewed the details of CAE procedures for glycan analysis. CAE enables the characterization of glycans even at the 10^{-18} mol level, at which structural information cannot be obtained with the present MS technology, and thus will be a powerful tool for the analysis of minute amounts of glycan mixtures [110].

Figure 9.5a and b illustrates the principle of CAE for the classification of glycans. In the initial step, a mixture of fluorescently labeled glycans is analyzed by capillary electrophoresis in an electrolyte that does not contain a lectin (Fig. 9.5a). In the subsequent step, the sample is analyzed in the presence of a lectin whose specificity toward carbohydrates is well established. When the lectin recognizes carbohydrates A, B, and C (peaks A, B and C, respectively) with different specificities, the order of migration of the carbohydrate chains changes, as shown in Fig. 9.5b. By repeating similar procedures using a set of lectins whose specificities are also well known, it will be possible to categorize all carbohydrate chains without prior isolation of the glycans.

A typical example of CAE for obtaining structural information on *N*-glycans derived from bovine α1-acid glycoprotein (Fig. 9.5d) is shown in Fig. 9.5e–g. We found that bovine α1-acid glycoprotein contains biantennary carbohydrate chains that contain one, two, three, or four SiA residues, as shown in Fig. 9.5e. The asialoglycans that were previously digested with sialidase were resolved into two peaks (Fig. 9.5f). These two peaks were further resolved into four peaks in the presence of wheat germ agglutinin (WGA), which discriminates between Galβ1–4GlcNAc and Galβ1–3GlcNAc branches at the outer parts of the glycans (Fig. 9.5g). By analogy with structure information on triantennary glycans derived from bovine fetuin, which contains both Galβ1-4GlcNAc and Galβ1–3GlcNAc branches, it can be concluded that biantennary glycans contain both branches. In addition, the linkage positions of *N*-acetyl/glycolyl-neuraminic acids were confirmed by specific sialidase digestion (Fig. 9.5d) [112].

9.5 Analysis of *N*-Glycans by Capillary Electrophoresis–Mass Spectrometry

Recently, glycan analysis by CE–MS has become very popular, because MS provides so much information, such as the detailed structures and mass numbers of glycans. This means CE assumes a separation function and MS assumes a detector instead of or in addition to that of the LIF detector. But on-line CE–MS of glycans is still at a developmental stage, since in CE the interface is always a compromise between optimal mass source performance and elevated separation efficiency.

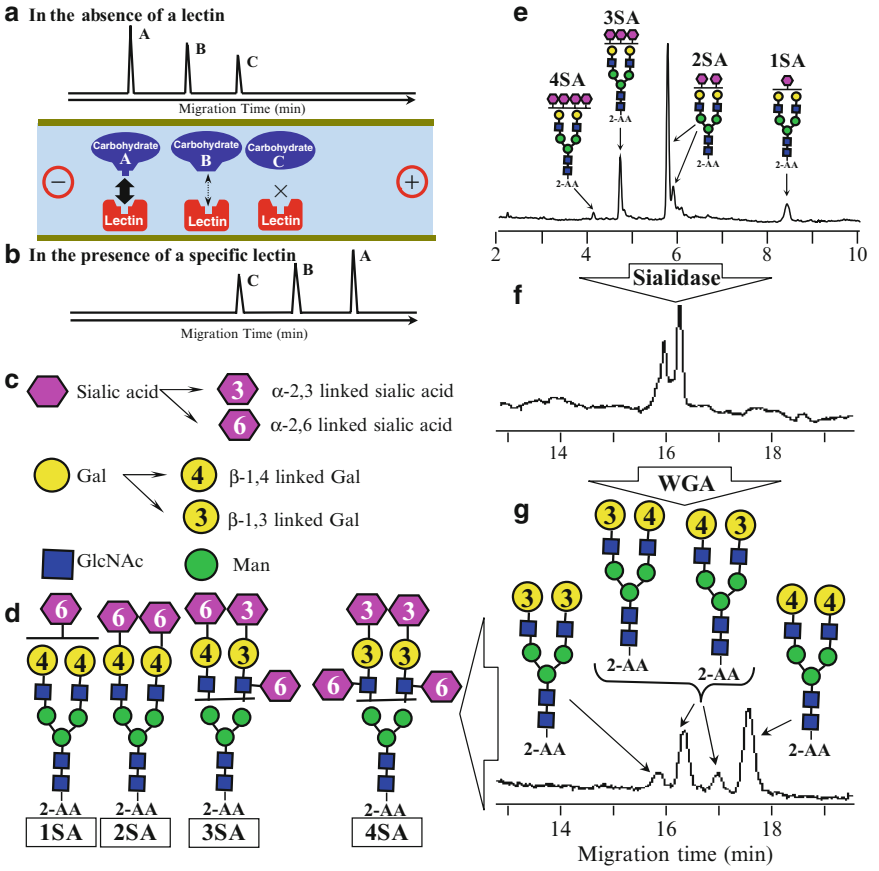

Fig. 9.5 Capillary affinity electrophoresis (CAE) for the structural confirmation of *N*-glycans. Principle of CAE (**a** and **b**). (**a**) Analysis of fluorescently labeled glycans without a lectin. (**b**) Analysis of fluorescently labeled glycans in the presence of a lectin in the electrolyte. When the lectin recognizes glycan A (peak a), the peak is observed later. In contrast, glycan C (peak c) does not show affinity for the lectin, and is observed at the same migration time as that in the absence of the lectin. A typical example of CAE for obtaining structural information on *N*-glycans derived from bovine α1-acid glycoprotein (**d**) is shown in **f** and **g**. (**e**) Analysis of *N*-glycans labeled with 2-AA derived from bovine α1-acid glycoprotein. (**f**) Analysis of *N*-glycans labeled with 2-AA after sialidase digestion (asialo-*N*-glycans). (**g**) Analysis of asialo-*N*-glycans in the presence of wheat germ agglutinin (WGA), which discriminates between Galβ1–4GlcNAc and Galβ1–3GlcNAc branches. We conclude that bovine α1-acid glycoprotein has biantennary glycans containing both Galβ1–4GlcNAc and Galβ1–3GlcNAc branches. In addition, we were also able to confirm the linkage positions of *N*-acetyl/glycolyl-neuraminic acids by means of specific sialidase digestion (**d**). Analytical conditions: running buffer, 100 mM Tris-acetate buffer (pH 7.4) containing 0.5% poly(ethylene glycol) (PEG70000) and lectins; capillary, eCAP N-CHO coated capillary (30 cm length (effective length, 10 cm) 50 μm inner diameter); applied potential, 20 kV; injection: pressure method (0.5 psi for 5 s); fluorescence detection at 400 nm with excitation with a helium–cadmium laser (320 nm). SA, sialic acid (From Nakano et al. [112], with permission)

Indeed, the buffers normally used for the optimal CE separation of glycans contain nonvolatile salts, such as borate ions, or surfactants, such as SDS, which noticeably improve the separation selectivity for underivatized and derivatized glycans [113]. These buffer additives impair reliable mass detection, although some attempts for glycan analyses using borate at low concentrations (i.e., 10 mM) have been reported [52]. Therefore, the CZE and CEC modes with volatile buffers are mainly used for CE–MS analysis. CE–MS analyses of glycans were reviewed by several researchers [28, 36, 114–117].

9.5.1 Interfacing with Mass Spectrometry

For on-line CE–MS, only ESI is an available source now, and an ion trap is mainly used as the analyzer followed by TOF. For CE–ESI MS, a number of interfaces have been described, as reviewed by several authors [43, 118, 119]. The most commercially available interface is a triple-tube sprayer, of which the innermost tube is for the sample with the running buffer for CE, the middle tube for the sheath liquid, and the outermost tube for the nebulized gas. The sheath liquid is essential for obtaining a current bridge, delivering a suitable amount of liquid for the generation of a stable spray, and adjusting the solvent composition for attaining good ionization yields in the chosen ion mode. Sheath-liquid interfaces usually allow one to attain good reproducibility. The flow rate of the sheath liquid with a standard CE sprayer is commonly 2–4 μL/min. The sheath liquid mainly comprises an alcohol (methanol, 2-propanol, mostly near 1:1 v/v) in aqueous solution. For analysis in the positive ion mode, the aqueous solution usually contains small amounts of acidic components such as formic or acetic acid, or a volatile buffer such as ammonium acetate [28, 36, 114–117].

Sheathless interfaces are also used to increase the sensitivity, because the analyte is diluted with the sheath liquid at the ionized source for MS. In the sheathless interfaces the analyte infused directly from the CE microspray tip into the MS. The most effective sheathless configurations are based on coating of the sprayer tip with a conductive layer to provide the electrical contact needed for both CE and ESI. Gold [120], copper [121], and carbon [122] have been used as materials for the conductive layer. On-line sheathless CE–MS with the normal polarity using an alkaline buffer, which is assisted by the EOF, has been developed for the analysis of negative charged glycans, such as sialylated glycopeptides and glycosaminoglycans [123, 124]. On the other hand, on-line sheathless CE–MS with the reverse polarity [inlet (–)–outlet (+) of CE capillary] with an acidic buffer, which is assisted by pressure or by coating of the capillary walls to sustain the electrospray, has also been reported for the analysis of derivatized and underivatized *N*-glycans [125].

Regarding interfacing to matrix-assisted laser desorption ionization (MALDI) MS, it is possible to spot an analyte onto a MALDI target using an interface. The MALDI-matrix can be added dissolved in the sheath liquid [126, 127].

9.5.2 *Derivatization of N-Glycans for Capillary Electrophoresis–Mass Spectrometry Analysis*

Derivatization of *N*-glycans has some distinct advantages for CE–MS analysis: (1) high-resolution separation and high sensitivity in detection due to attachment of the charged group of a labeling reagent, as described in Sect. 9.4.1; (2) enhancement of the ionization yield on MS ionization; and (3) achievement of detailed MS/MS fragmentation for investigation of the linkage patterns of *N*-glycans. Therefore, various labeling reagents have been employed for this purpose, as shown in Fig. 9.3. Although the same labeling reagents are used for CE–MS analysis as those for CE analysis, unlabeled *N*-glycans (referred to as reducing *N*-glycans or free *N*-glycans, structure 3 in Fig. 9.3) are also analyzed as well as labeled *N*-glycans in CE–MS [114].

N-glycans with free reducing ends are obtained by digestion by PNGase F, and the released *N*-glycans are usually separated from the deglycosylated proteins by some cleanup procedures such as solid-phase extraction chromatography, lectin-affinity chromatography, and hydrophilic-interaction LC. The *N*-glycans released from RNase B, bovine fetuin, AGP, and rhuEPO were analyzed by CE-ESI-quadrupole ion trap (QIT) (CE: reverse polarity with a PVA-coated capillary; MS: negative mode) or CE-ESI-TOF (CE: normal polarity with a non-coated capillary; MS: negative mode) [37, 125, 128]. Negative-charged *N*-glycans such as sialylated *N*-glycans were clearly observed with the negative mode of MS. However, the fragmentation ions of *N*-glycans that have lost sialic acids on MS/MS analysis are hard to detect in the negative mode.

APTS and ANTS have strong fluorescence, and also strong anionic characteristics, enhancing the resolution of their glycan derivatives in CE analysis [114]. APTS-labeled *N*-glycans derived from cellobiohydrolase I and hemocyanin were analyzed by CE-ESI-IT (MS: negative mode) [129–131]. ANTS-labeled *N*-glycans derived from RNase B, fetuin, and antibody pharmaceuticals were analyzed by CE-ESI-IT (MS: negative mode) [125, 131]. *N*-quaternized benzyl-amine (BA) was investigated as a new labeling reagent for MS or CE–MS analysis [126]. The BA-labeled *N*-glycan significantly improved CE separation and enhanced the MS detection sensitivity with 10-fold higher sensitivity than that for underivatized *N*-glycans.

A novel alternative labeling method for glycans involving 9-fluorenylmethyl chloroformate (Fmoc-Cl) instead of the conventional reductive amination procedure was reported (structure 4 in Fig. 9.3) [86]. Originally the Fmoc-labeling method was used for HPLC analysis with a fluorescence detector. As described in Sect. 9.4.1, the Fmoc-labeling method is simple and allows high-speed analysis, which can be performed within 4 h, including the enzymatic glycan-releasing reaction and the labeling reaction. Therefore, the Fmoc-labeling method was applied to high throughput and sensitive analysis of *N*-glycans [87]. The detailed results are shown in the next section.

9.5.3 Capillary Zone Electrophoresis–Mass Spectrometry

Both underivatized and derivatized *N*-glycans have been extensively analyzed by CZE-MS [36] for glycoscreening in the biomedical field [117]. In terms of CZE analysis without MS, the separation of *N*-glycans has frequently been performed using nonvolatile buffers such as phosphate, citrate, and borate buffers in order to achieve good separation. Especially with borate buffers, linkage-specific separation of glycans can be achieved due to the complexation by borate. However, nonvolatile buffers are not suitable for CZE-MS analysis because of interference with ionization on MS. Therefore, volatile buffers such as formic acid [129], ammonium acetate [130, 131], ammonium formate [87, 130], and aminocaproic acid [132] buffer are used as running buffers for CZE. In the case of ESI-MS, underivatized *N*-glycans are mainly separated with a noncoated capillary in the normal polarity, and are mainly detected in the negative mode on MS [37, 125, 128]. *N*-glycans labeled with negatively charged reagents such as ANTS and APTS are separated either in a noncoated capillary in the normal polarity [131] or in a coated capillary in the reverse polarity [125, 129], and are detected in the negative mode on MS. In terms of CZE-MALDI-MS, BA-labeled *N*-glycans in off-line combination using fraction collection were reported [126]. Also, when permethylated *N*-glycans were analyzed by CZE-MALDI-MS, permethylation significantly enhanced the ionization and allowed detailed MS/MS fragmentation for investigating the linkage patterns of *N*-glycans [133].

Typical results of *N*-glycan analysis using CZE-ESI MS are shown in Fig. 9.6. We analyzed *N*-glycans of α-fetoprotein (AFP) derived from normal human placenta and human hepatoma cells in order to reveal any differences in *N*-glycan structures. AFP is an oncofetal glycoprotein that contains a single glycosylation site at asparagine 232, and is a well-known tumor marker for hepatocellular carcinomas (HCCs). However, the AFP concentration is also elevated in other liver diseases, such as chronic hepatitis and cirrhosis. Recently, the *Lens culinaris* agglutinin (LCA)-reactive fraction of AFP (AFP-L3) has been measured as a more specific marker for HCC. AFP-L3 reflects HCC-specific changes in the glycans of AFP. LCA is a lectin which exhibits affinity to biantennary glycans with α1-6 Fuc [134]. Using LCA, AFP can be divided into three types (L1, L2, and L3), as shown in Fig. 9.6c. AFP-L1 and L3 are the major components of AFP in the serum of HCC patients. Addition of a fucose residue is mainly observed when there is a structural change in *N*-glycans caused by HCC, and AFP-L3 is highly specific to HCC. Purified AFP from both normal placenta and hepatoma cells was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 9.6a) followed by western blotting with anti-AFP antibodies (Fig. 9.6b). Although there was no difference between AFP derived from normal placenta and hepatoma cells on both SDS–PAGE analysis and western blotting, there was big difference on electrophoresis with LCA (Fig. 9.6c). AFP derived from normal placenta yielded one band at L1 position, and AFP derived from hepatoma cells yielded two bands at L1 and L3 positions.

We next tried to analyze *N*-glycans in these AFPs by the CZE–ESI–MS technique. *N*-glycans released from AFPs were derivatized with Fmoc before analysis

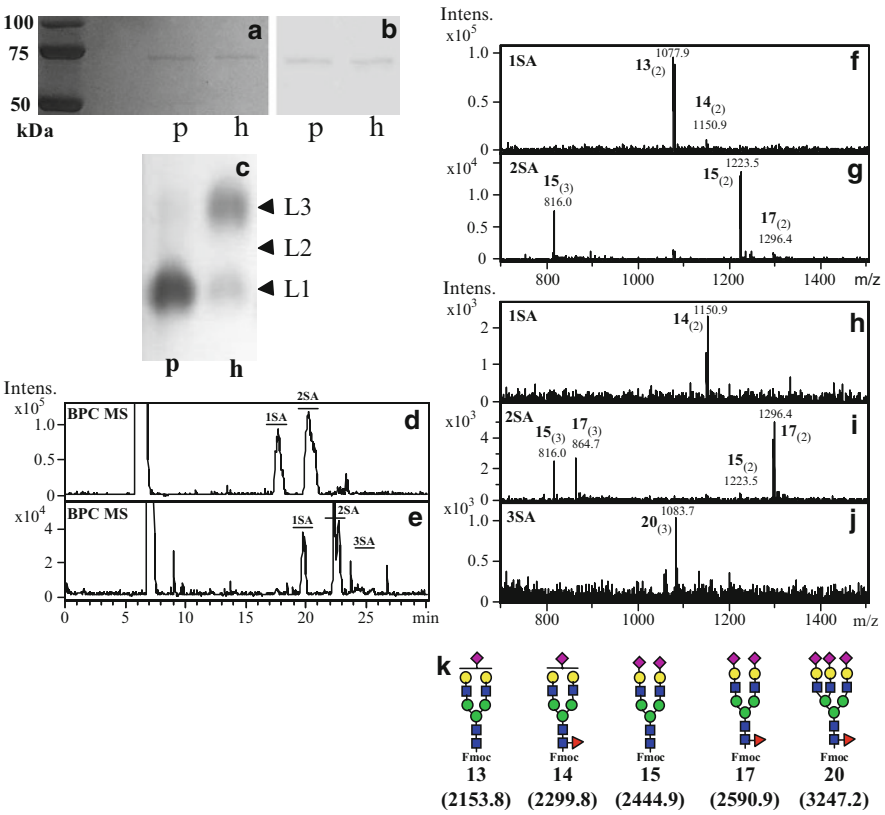

Fig. 9.6 CZE–ESI–MS analysis of Fmoc-labeled *N*-glycans derived from qqqα-fetoprotein (AFP)AFP. (**a**) SDS-PAGE (12%) analysis of AFP derived from human normal placenta (100 ng, lane p) and hepatoma cells (100 ng, lane h), followed by CBB staining. (**b**) Western blot analysis using anti-AFP antibodies for AFP derived from human normal placenta (1 ng, lane p) and hepatoma cells (1 ng, lane h). (**c**) Lectin (LCA)-affinity electrophoresis for AFP derived from human normal placenta (300 ng, lane p) and hepatoma cells (100 ng, lane h). (**d**) BPC MS of Fmoc-labeled *N*-glycans in AFP derived from human normal placenta. (**e**) BPC MS of Fmoc-labeled *N*-glycans in AFP derived from hepatoma cells. (**f** and **g**) Averaged mass spectra of the 1SA and 2SA sections in d. (**h**–**j**) Averaged mass spectra of the 1SA, 2SA, and 3SA sections in E. (**k**) List of *N*-glycan structures in this figure. The numbers in parentheses are the mass numbers represented as [M+H]+ of Fmoc-labeled *N*-glycans. CE, normal polarity with a fused silica capillary (100 cm × 50 mm inner diameter); CE buffer, 50 mM ammonium acetate; applied potential, 30 kV; sheath liquid, 50/49.9/0.1 (v/v/v) MeOH/water/formic acid (2 mL/ min); MS, positive mode; nebulizing gas, N2, 8 psi; voltage of the capillary outlet, –4 kV; at temperature, 300°C; and flow rate of nitrogen gas for drying, 4 L/min (from Nakano et al. [87], with permission)

by CZE–ESI–MS. *N*-glycans were observed in the order of the attached number of sialic acid residues (Fig. 9.6d and e). *N*-glycans of 1SA and 2SA released from AFP derived from normal placenta were monosialo-biantennary ones (13 in Fig. 9.6f) and disialo-biantennary ones (15 in Fig. 9.6g), respectively. We could detect α1-6 fucosylated monosialo- and disialo-biantennary glycans (14 in Fig. 9.6f and 17 in

Fig. 9.6g) with trace amount. On the other hand, the *N*-glycans with 1SA, 2SA, and 3SA released from AFP derived from hepatoma cells were α1-6 fucosylated monosialo-biantennary ones (14 in Fig. 9.6h), α1-6 fucosylated disialo-biantennary ones (17 in Fig. 9.6I), and α1-6 fucosylated trisialo-triantennary ones (20 in Fig. 9.6j), respectively. Disialo-biantennary glycans without Fuc (15 in Fig. 9.6i) were also detected as a minor component. Although the electrophoresis with LCA (Fig. 9.6c) revealed differences in the patterns of *N*-glycans in AFP derived from normal placenta and hepatoma cells, the CZE–ESI–MS technique could reveal the differences based on the detailed *N*-glycan structures.

9.5.4 Capillary Electrochromatography–Mass Spectrometry

Over the last 15 years, monolithic columns have been investigated for HPLC and CEC analysis (see Sect. 9.4.2). CEC principally offers a wide range of retention selectivity together with separation conditions that appear compatible with MS operation. On CEC-ESI-MS analysis, unlabeled oligomannose type *N*-glycans derived from RNase B were separated on a polar monolithic column (in-house amino capillary column) and continuously introduced into the ESI-MS [135]. MS/MS analysis was also performed to obtain sequence, branching, and linkage information on the *N*-glycan structures. The running buffer used for CEC was a volatile buffer, that is, acetonitrile/water/ammonium formate buffer (55:44:1 v/v/v, pH 3.0). Several structural isomers of oligomannose type *N*-glycans were separated on the CEC column, and they showed different fragment patterns on MS/MS analysis. On CEC-MALDI-MS analysis, a mixture of unlabeled *N*-glycans and *O*-glycans derived from bile salt-stimulated lipase was separated on a polar monolithic column (in-house cyano capillary column), and a micro-deposition device was used for the MALDI-MS analysis [136]. The running buffer used for CEC was a volatile buffer with the same composition as above. Several structural isomers of *N*-glycans were separated on the CEC column, and were seen in the 3D electrochromatogram (time, *m*/*z*, and MS intensity). About 50 distinct peaks of *N*-glycans were observed.

9.6 Conclusion

This chapter discussed *N*-glycan analysis by CE and CE–MS using various labeling methods and various CE modes. CE analysis exhibits high-speed analysis with high resolution, and makes it possible to analyze small amounts of *N*-glycan samples. It is well known that most protein therapeutics, such as monoclonal antibody pharmaceuticals and other biopharmaceuticals and cancer biomarkers, are glycoproteins. *N*-glycosylation in most biopharmaceuticals affects their drug efficacy, safety, and stability, and the *N*-glycosylation is dependent on the manufacturing process and the expression system. Therefore, the evaluation of the *N*-glycan in detail is quite

important for quality control in the development of biopharmaceuticals. In the case of cancer biomarkers, the variation of glycosylation is closely related to oncogenic transformation. Therefore, *N*-glycan characterization of the cancer biomarker glycoproteins enables a distinction to be made between normal and tumor origins, and suggests a valuable biochemical tool for diagnosis. Therefore, CE analysis, especially CZE, is suitable for routine analysis such as for quality control of protein therapeutics. The CZE for glycan analysis offers a low running cost, short separation time, and a high-resolution technique that requires only a small amount of analyte. Fully automated procedures including enzymatic digestion, derivatization, purification of labeled *N*-glycans, CE separation, and MS analysis are expected to become available for routine analysis in the near future.

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Chapter 10
Monosaccharide Compositional Analysis of Glycoproteins and Glycolipids: Utility in the Diagnosis/Prognosis of Diseases

Yehia Mechref

Abstract Different analytical methods have been developed to effectively and accurately determine monosaccharide composition of glycoproteins and glycolipids in biological and biomedical samples. These methods included high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD), high-performance liquid chromatography with fluorescence detection (HPLC-FL), and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). This chapter reviews the development of these methods, and evaluates their potential application in the diagnosis or prognosis of diseases.

Keywords Capillary electrophoresis • Glycoproteins • Oligosaccharides • Monosaccharides • Glycolipids • Diagnosis • Prognosis • HPAEC-PAD • HPLC-FL • HPLC-MS • Laser-induced fluorescence

Abbreviations

ABEE	p-aminobenzoic acid ethyl ester
AMAC	8-aminoacridone
ANDS	7-aminonaphthalene-1,3-disulfonic acid
ANS	2-aminonaphthalene-1-sulfonic acid
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
APTS	1-aminopyrene-3,6,8-trisulfonic acid
CE	capillary electrophoresis
CEC	capillary electrochromatography
CE-LIF	capillary electrophoresis with laser-induced fluorescence detection
CGE	capillary gel electrophoresis

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CZE	capillary zone electrophoresis
DMB	1,2-diamino-4,5-methylenedioxybenzene
EOF	electro-osmotic flow
Gal	galactose
GalN	galactosamine
GalNAc	*N*-acetylgalactosamine
GCC	graphitized carbon column
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GlcN	glucosamine
GlcNc	*N*-acetylglucosamine
HCl	hydrochloric acid
HPAEC-PAD	high-performance anion-exchange chromatography with pulse amperometric detection
HPLC-FL	high-performance liquid chromatography with fluorescence detection
HPLC-MS	high-performance liquid chromatography with mass spectrometry
Man	mannose
ManN	mannosamine
MECK	micellar electrokinetic chromatography
MRM	multiple reaction monitoring
NeuAc	*N*-acetylneuraminic acid
NeuGc	*N*-glycolylneuraminic acid
OPD	*O*-phenyllenediamine 2 HCl
PAD	pulsed amperometric detector
TFA	trifluoroacetic acid.

10.1 Introduction

The fact that no more than 30,000–50,000 proteins are encoded by the human genome indicates the importance of posttranslational modifications in modulating the activities and functions of proteins in health and disease states [1]. Glycosylation is the most prevalent posttranslational modification of proteins in mammalian cells, since on average 50% of all proteins are glycosylated. Moreover, many proteins act through oligosaccharide recognition, while cell–cell interactions involve sugar–sugar, or sugar–protein specific recognition. Consequently, aberrant glycosylation has now been recognized as the attribute of many mammalian diseases, including osteoarthritis [2–4], cystic fibrosis [5–8], and cancer [9–11].

Aberrant glycosylation has been implicated in different types of cancer, with numerous glycosyl epitopes known to constitute tumor-associated antigens [12–14]. Also, it has been shown that molecular changes in glycosylation could be associated with the signaling pathways for the malignant transformation of cells [15]. Since the correlation between certain structures of glycans and a clinical prognosis in cancer were first suggested over a decade ago [16, 17], the interest in structural studies of

glycans and other related molecules on cellular surfaces has increased substantially. Moreover, cancerous cells with altered glycosylation of their surface proteins eventually shed such proteins or their fragments into the circulating fluids. Monosaccharide analysis of such fluids could reveal the altered glycosylation, thus leading to the possible discovery of many biomarker candidates for disease-related glycan alterations.

Accurate and reliable analysis of monosaccharide residues attached to glycoconjugates requires quantitative cleavage of glycosidic, high yield derivatization (if needed), and efficient separation of monosaccharide residues or derivatives (chromatographic or electrophoretic techniques). However, fundamental difficulties originate from the molecular complexity of the glycoconjugates. Generally, glycoproteins and glycolipids possess multiple oligosaccharide structures existing at different abundances and different monosaccharide compositions. Moreover, the same glycoprotein originating from different sources (same polypeptide sequence) possesses different glycans with different ratios of monosaccharide residues. Monosaccharides that are commonly encountered on glycoproteins are neutral, basic, and acidic residues. Some of these monosaccharide residues are structural isomers (Fig. 10.1). For example, glucose (Glc) and mannose (Man) are epimers with respect to C(2), while Glc and galactose (Gal) are epimers with respect to C(4). Others are very different, on the other hand, such as Glc, an aldose, and *N*-acetylneuraminic acid (NeuAc), a 9-carbon carboxylated keto-monosaccharide that is derived from *N*-acetylmannosamine and pyruvic acid. *N*-Acetylneuraminic acid and its derivatives are often referred to as sialic acids (Fig. 10.2). The diversity and labile chemical aspects of this group of monosaccharides complicate the repertoire of methods needed for complete monosaccharide analysis.

Monosaccharide analysis of glycoconjugates involves three steps: monosaccharide release, derivatization (if required), and quantitative separation with proper detection techniques. Currently, the last step is easily accomplished through high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-performance liquid chromatography with fluorescence (HPLC-FL), or mass spectrometry (HPLC-MS), and capillary electrophoresis with laser-induced fluorescence (CE-LIF). This chapter discusses the optimum conditions for efficient release of monosaccharides, highlights the advantages and disadvantages of the different techniques utilized to separate and quantify monosaccharides, and describes the use of some of these techniques to monitor the change in glycan compositions as a consequence of disease progression.

10.2 Chemical and Enzymatic Release for Monosaccharide Compositional Analysis

Acid or enzymatic hydrolysis of glycoconjugates has been routinely used to release intact monosaccharides by cleavage of glycosidic bonds. Each class of monosaccharide is cleaved differently, depending on the employed acid-hydrolysis conditions. For most glycoconjugates, quantitative release of amino monosaccharides is achieved using 4–6 M hydrochloric acid (HCl) and 3–6 h of incubations at 100°C [18].

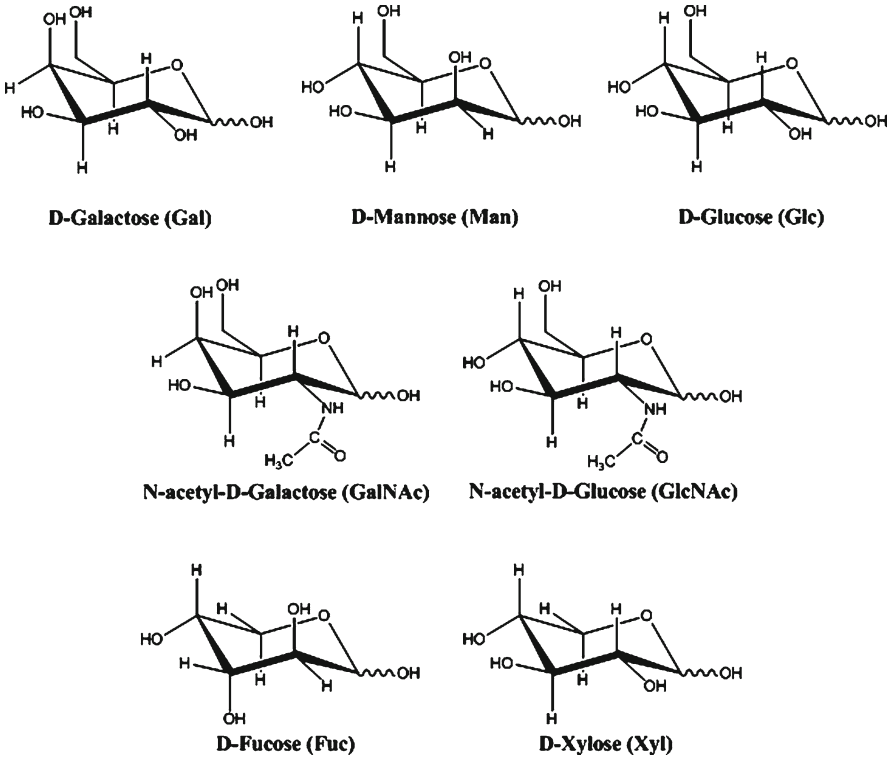


Fig. 10.1 Structures of neutral and amino monosaccharides associated with glycoproteins and glycolipids

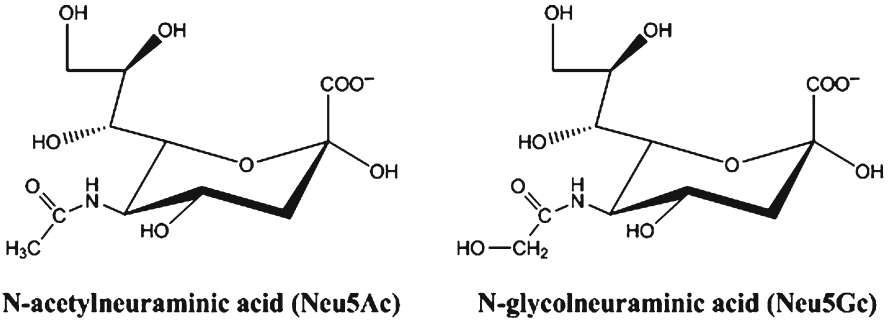


Fig. 10.2 Structures of sialic acids

Amino monosaccharides are stable under these conditions for at least 12 h [19, 20]. Gal and Man significantly degrade under those conditions; however, it has been reported that they are efficiently released using 2 M HCl or trifluoroacetic acid (TFA) [20]. A 50% decrease in the efficiency of release of Gal and Man was reported when 2 M HCl was used for hydrolysis [21]. It appears that the discrepancy

between the two reports is due to the inclusion of anion-exchange resin, which reduces Gal and Man losses by 20–30%. Currently, neutral and amino monosaccharides are efficiently released using 2 M TFA and 4–6 N HCl, respectively.

Complete de-*N*-acetylation of *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) is achieved upon incubation with 2 M TFA for more than 3 h [22]. However, a quantitative re-*N*-acetylation of the resulting glucosamine and galactosamine is attained by resuspending dried samples in a saturated sodium bicarbonate solution prior to the addition of acetic anhydride.

Mild-acid hydrolysis is routinely employed to release sialic acids. However, conditions commonly used for the release of sialic acid (0.1 M HCl at 80°C for 30–90 min) result in the release of *O*-acetyl groups [23]. A milder acid hydrolysis condition involving the use of 2 M acetic acid for 1 h at 80°C was reported to eliminate the release of *O*-acetyl groups [18]; however, incomplete release of sialic acid results under these conditions [24, 25]. Moreover, these conditions result in the selective release of the nonacetylated sialic acids, since *O*-acetylated species are relatively more resistant to release [25–27]. Accordingly, enzymatic release of sialic acids is more desirable.

Thus far, enzymatic release of monosaccharides is only employed for sialic acids, since a neuraminidase with broad specificity is commercially available [23]. Although a mixture of *exo*glycosidases that could release all monosaccharides from glycoconjugates would eliminate many of the shortfalls discussed above, this is not currently attainable since *exo*glycosidases capable of efficient and quantitative release of the different monosaccharides associated with glycoconjugates are not available. Therefore, acid-hydrolysis is still more routinely employed for the release of glycoconjugate monosaccharides.

10.3 Chromatographic and Electrophoretic Methods Developed for Monosaccharide Compositional Analysis of Glycoproteins and Glycolipids

10.3.1 High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

High-performance anion-exchange chromatography (HPAEC), coupled to pulsed amperometric detector (PAD), is a simple and convenient method for carbohydrate analysis that is advantageous because of several assets, including analysis speed; adequate separation of anomeric, structural, linkage, and branch isomers; and high sensitivity without pre- or post-column solute derivatization. The separation in HPAEC is based on ionic interactions between the negatively charged oxyanions of carbohydrates, which result from the use of strongly alkaline mobile phase (pH > 12), and the anion-exchange resins. The strongly alkaline mobile-phase is also needed to facilitate the electro-oxidation reaction at the working gold electrode

of PAD. However, the use of an alkaline mobile phase also bears disadvantages, as it induces side reactions. At pH 12, the equilibrium of epimerization of GlcNAc to ManNAc is virtually attained within an hour. Therefore, large oligosaccharides containing GlcNAc or GalNAc as their reducing terminus can suffer epimerization or degradation. This has prompted the reduction of alkali-sensitive oligosaccharides prior to HPAEC-PAD analyses to eliminate such possibilities.

Another disadvantage of HPAEC-PAD is the high-salt content commonly used with this methodology, thus eliminating direct coupling to MS. However, several desalting approaches have been employed to reduce salt content, thus subsequently facilitating on- or off-line MS analysis. When the salt content is not too high, the on-line membrane desalting device may also be satisfactory [28–30]. The commonly practiced methods for desalting the HPAEC fractions containing high levels of salts are dialysis, ion-exchange, and gel filtration. However, the risk of losing the sample as a result of this excessive sample handling is high, thus making it impossible to desalt small quantities of sample.

A different desalting device for the on-line coupling of HPAEC-PAD to electrospray ion–mass spectrometry (ESI–MS) was described in two separate reports [31, 32], involving a design different from previously published work [28–30]. The new desalting device is based on an on-line microdialysis utilizing a cation-exchange membrane unit, which permits the exchange of sodium ions with hydronium ions (for sodium ion concentrations up to 600 mM). An effective on-line desalting of the effluent before its introduction into the mass spectrometer was achieved through electrolysis of water with 500 mA current. The unit coupled to ESI eliminated the need for a booster pump (usually used with the previous device), while the use of water instead of sulfuric acid improved sensitivity.

Despite some of these difficulties, HPAEC-PAD has been shown to be effective for the analysis of N- and O-linked glycans and their monosaccharide composition when released from sufficient amounts of glycoproteins [for reviews, see 33–36]. Hardy and Townsend were the first to explore systematically the separation power of HPAEC-PAD in the analysis of carbohydrates [31]. They succeeded in separating neutral oligosaccharides according to their molecular weight, sugar composition, and linkage of the monosaccharides residues. The method allowed resolution of 1-3, 1-4, and 1-6 positional isomers of neutral oligosaccharides, which have the same number, sequence type, and anomeric configuration of monosaccharides, but feature differences in linkage position of a single sugar. Moreover, the study also demonstrated high retention of reducing oligosaccharides relative to their reduced counterparts, and a substantial decrease in retention time as a result of the presence of Fuc(α1–3) linkage to GlcNAc. The authors also reported the effectiveness of this methodology in resolving N-linked glycans derived from glycoproteins and glycopeptides. However, the separation of only two triantennary oligosaccharides derived from fetuin was demonstrated.

When used in conjunction with other analytical techniques, such as specific *endo*glycosidase digestions, other HPLC techniques, fast atom bombardment (FAB)/MS, and ^{1}H-nuclear magnetic resonance (NMR) spectrometry, HPAEC-PAD was utilized to determine the carbohydrate structures of recombinant human tissue

plasminogen activator expressed in Chinese hamster ovary cells. In this study, HPAEC-PAD permitted determination of the distribution of oligosaccharide structures at individual glycosylation sites. The oligosaccharides released from each glycosylation site were analyzed. This technique has been routinely used for the profiling of N- and O-linked glycans derived from glycoproteins such as mucin [37–39], human immunoglobulin G (IgG) [40, 41] human α_1-antitrypsin [40], human transferring [40] fetuin [19], α_1-acid glycoprotein, [42, 43], recombinant erythropoietin [44], gastric H^+, K^+-adenosine triphosphatase (ATPase) [45], recombinant plasminogen activator [35, 45], and bile-salt-stimulated lipase from human milk [46, 47].

Lee's group [22] was the first to examine anion exchange chromatography for the analysis of common neutral and amino monosaccharide residues associated with glycoconjugates and released through acid hydrolysis. The group demonstrated the ability to quantify monosaccharide composition in biological samples at subnanomole levels using pulse amperometric detection with a gold working electrode. The separation of underivatized fucose, galactosamine, glucosamine, galactose, glucose, and mannose was readily attained using AS-6 pellicular anion exchange resin (4.6×250 mm, Dionex Corp., Sunnyvale, CA) and 22 mM sodium hydroxide as the mobile phase. Rapid and sensitive monosaccharide composition analysis of several glycoproteins was demonstrated, including bovine fetuin, asialo-fetuin, human orosomucoid, and human fibrinogen. Such analysis was performed on 2 M TFA hydrosylate resulting from incubation for 3–6 h at 100°C.

Today, Dionex Corp. (Sunnyvale, CA) is the leader in HPAEC-PAD, providing columns that are stable and reproducible for the separation of both mono- and oligosaccharides. Initially, CarboPac™ PA1 and PA10 were provided to efficiently separate monosaccharides including neutral and amino monosaccharides. Separations of monosaccharides commonly associated with glycoproteins were attained in 40 min using CarboPac PA10 and isocratic 18 mM KOH eluent (Fig. 10.3). The separation involves column regeneration achieved by stepping the eluent to 100 mM KOH for 5 min and equilibrating the column for 15 min. The 5-min wash with 100 mM KOH is needed to flush strongly retained material off column. This step is needed when injecting glycoprotein hydrosylates, since strongly retained hydrolysis products can accumulate on the column and impair its performance. Separation of monosaccharides has also been achieved using CarboPac PA1 through the use of sodium acetate gradient; however, the reproducibility of this column is less desirable relative to CarboPac PA10, which was originally developed for the separation of monosaccharides.

CarboPac PA20 is another Dionex Corp. anion-exchange column allowing faster separation of monosaccharides than the PA10 and PA1 and subsequently increasing sample throughput. Elution of monosaccharides derived from glycoproteins can be completed in less than 12 min using lower hydroxide concentrations and lower flow rates. Amino trap columns are routinely placed in front of CarboPac PA20 to eliminate interference by amino acids that can coelute with some monosaccharides. Column regeneration with 100 mM KOH for 3–5 min following each run is needed when glycoprotein hydrosylates are being analyzed. Figure 10.4 depicts the chromatograms of IgG hydrosylates prepared under two different conditions.

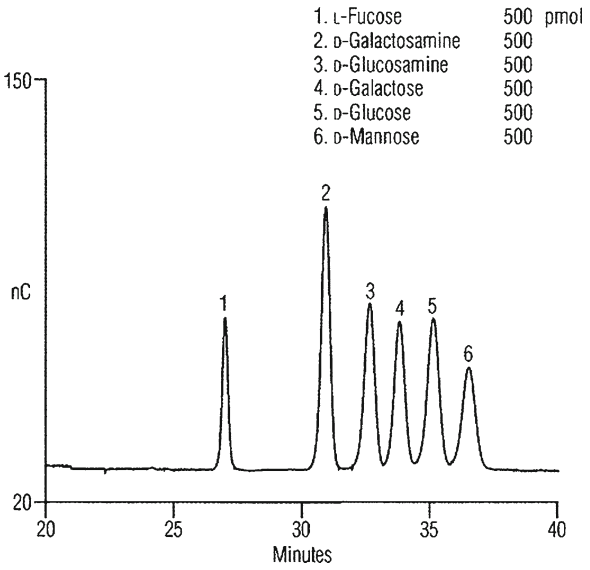


Fig. 10.3 HPAEC-PAD chromatogram of 500 mM monosaccharide standards acquired on CarboPac™ PA10

Monosaccharides associated with IgG were separated over 12 min. The repeatability of peak areas and retention times were better than 5% over 7 days.

The use of HPAEC-PAD for monosaccharide composition analysis was mainly focused on determining the monosaccharide composition of glycoproteins isolated from biological samples or pharmaceutical recombinant glycoproteins. Laferte and Dennis [48] used HPAEC-PAD to determine the monosaccharide composition of two glycoproteins found in the metastatic lymphoreticular tumor cell line known as MADY-D2. The two glycoproteins, migrated at about 110 and 130 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), were immunochemically distinct. They also possessed different proportions of O- and N-linked oligosaccharides. HPAEC-PAD monosaccharide compositional analysis indicated that one of the two glycoproteins contained more than 50% N-linked oligosaccharides structures with sialylated polylactoseamine sequences. Moreover, the carbohydrate content of this glycoprotein was very similar to that of LAMP-1 previously identified in murine fibroblast and macrophage [49]. Monosaccharide compositions of other glycoproteins and recombinant glycoproteins, such as recombinant human tissue plasminogen activator [50, 51], recombinant hepatitis B surface antigen [52], recombinant erythropoietin [44], *Haemophilus influenzae* type b conjugate vaccine [53], glycoprotein variant of human serum albumin [54], and mucin rat small intestine [55], were also rapidly and sensitively determined by HPAEC-PAD.

Although all HPAEC-PAD results that have been reported thus far are comparable to other rapid and sensitive chromatographic approaches, all other approaches require chemical derivatization of monosaccharide to introduce fluorphore/chromaophore (see below) or render the analytes volatile. Derivatization is not favorable

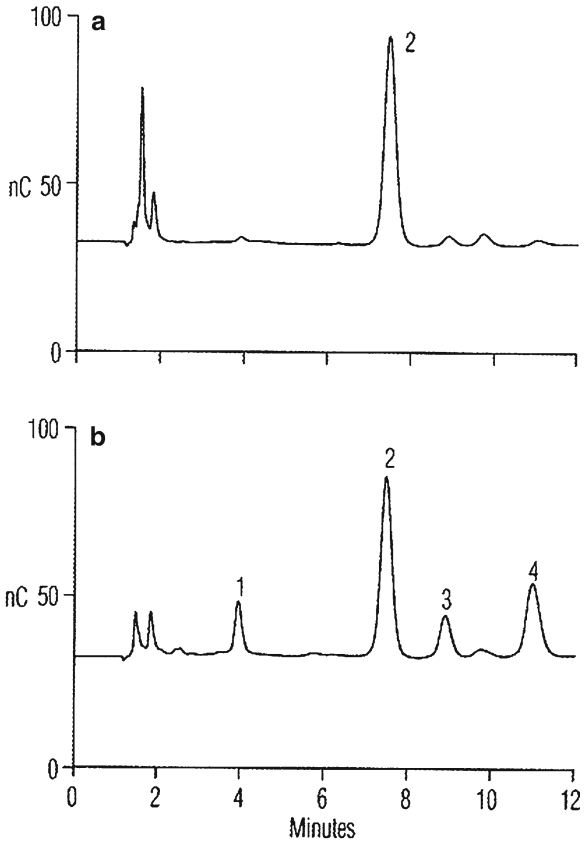


Fig. 10.4 HPAEC-PAD chromatogram of monosaccharides derived from immunoglobulin G (IgG) and acquired on CarboPac™ PA20

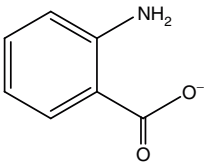
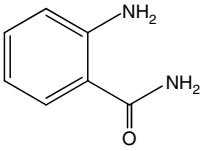
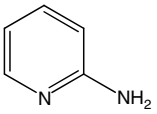
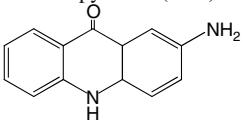
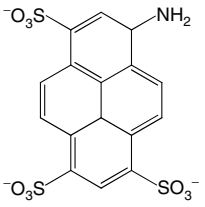
in the case of biological samples because of the complexity of the biological matrix, prompting many side reactions and by-products. In this regard, HPAEC-PAD is superior since no derivatization step is required. However, several components of any biological matrix such as amino acids and peptides are also detectable electrochemically. Therefore, cleanup by a suitable solid-phase extraction method (e.g., cation-exchange resins) may be necessary to separate monosaccharide residues from interfering compounds present in samples. Other caveats have also been associated with HPAEC-PAD monosaccharide analysis [22]. For example, Tris buffer produces a large tailing breakthrough peak, thus interfering with quantification. Another caveat that has been associated with all monosaccharide composition analysis techniques is the ubiquitous nature of Glc contamination in biological samples. This aspect can hinder quantitative analysis at high sensitivity (<100 pmol). Accordingly, HPAEC-PAD analysis of monosaccharide composition is highly rapid and quantitative using 1-nmol quantities of material that is not always possible in the case of biological samples.

10.3.2 High-Performance Liquid Chromatography with Fluorescence Detection

Although the hydrophobic interactions in liquid chromatography (LC), most commonly practiced with octadecyl-substituted siliceous packings, have been the most popular means of HPLC for a long time, this mode of LC has not been effectively employed for the separation of carbohydrates because of the polarity of underivatized oligosaccharides, which prompts only weak interactions with typical reversed-phase packings [56, 57]. Using water as the mobile phase, more polar oligosaccharides elute before the less polar ones [58]. Retention can be generally increased through addition of salts, which modify the surface-penetrating characteristics of the mobile phase, thus enhancing separation. Although reverse-phase (RP)-LC with plain water as the mobile phase found limited application [59], sample derivatization enhances the scope of analysis substantially. While the major reason for derivatization is to improve detection through the attachment of a chromophore or a fluorophore, it also renders the solutes more hydrophobic and subsequently retainable on RP-columns. Carbohydrates with reducing ends are typically pre-column labeled with aromatic amines, thus allowing fluorescence detection at enhanced sensitivity. Derivatization of carbohydrates with a fluorescence tag typically enhances the detection limit by approximately ten times relative to derivatization with an ultraviolet (UV)-absorbing tag. Although the majority of fluorescence tags have been introduced mainly for the labeling of oligosaccharides, the same tags could also be used for the labeling and subsequent fluorescence detection of monosaccharides. Table 10.1 lists the different fluorescence tags that have been thus far utilized for the labeling of monosaccharides for HPLC. Labeling is achieved through reductive amination, hence, the presence of amine groups for all tags listed in Table 10.1. Reaction conditions and the molecular structure dictate fluorescence intensity and the reactivity of the tag toward the analytes. Of course, all of the tags listed in Table 10.1 contain aromatic rings with excitable π-electrons.

Since its introduction in 1978 by Hase et al. [60], reductive pyridylamination has been employed extensively for the RP-LC analysis of glycans cleaved from various glycoproteins. The method is based on the labeling of glycans with 2-aminopyridine (2-AP) by reductive amination prior to the analysis. Initially, this method was demonstrated for characterization of the glycans cleaved from as little as a 10-μg sample of Taka-amylase A [61]. However, a further optimization of the labeling conditions permitted the analysis of pyridylamino derivatives of glycans cleaved from as little as 0.15 nmol of Taka-amylase A or bovine submaxillary mucin [62]. Nevertheless, a comparative survey indicated that 2-AP is less sensitive than the other tags [63]. Currently, 2-amino benzoic acid (2-AA) [64, 65] and 2-aminobenzamide (2-AB) [65] are most widely used because of their high fluorescence properties and reactivity. Many factors typically influence the choice of a suitable tag for the derivatization of carbohydrates, including the purity of tag, reactivity, and foremost compatibility with separation and detection.

Table 10.1 Fluorescence tags used for the labeling of carbohydrates in conjunction with HPLC and CE analyses

Fluorescence tag	Spectroscopic properties (nm)	Analytes	References
 2-Aminobenzoic acid (2-AA)	λ_{ex} 230/360nm λ_{em} 425nm	Mono- and oligosaccharides	[64]
 2-Aminobenzamid (2-AB)	λ_{ex} 330nm λ_{em} 420nm	Oligosaccharides	[65]
 2-aminopyridine (2AP)	λ_{ex} 310/320nm λ_{em} 380/400	Mono- and oligosaccharides	[60–62]
 2-Aminoacridone (AMAC)	λ_{ex} 428nm λ_{em} 525nm	Mono- and oligosaccharides	[115]
 8-Aminonaphthalene-1,3,6-trisulfonic acid (APTS)	λ_{ex} 488nm λ_{em} 520nm	Mono- and oligosaccharides	[111, 113, 114]

Although several fluorescence tags are listed in Table 10.1, HPLC of monosaccharides on a reverse-phase column is currently achieved using 2-AA as a fluorescence tag. It directly derivatizes monosaccharides with reducing ends through reductive amination. Moreover, AA labeling does not require re-*N*-acetylation of amino monosaccharides, and the reaction mixture is injected without the need for cleaning steps. Other fluorescent tags such as 2-AP and 2-AB require re-*N*-acetylation of amino monosaccharides prior to derivatization [66–68]. Figure 10.5 depicts typical chromatograms for monosaccharide standards and monosaccharides derived from recombinant human IgG expressed in Chinese hamster ovarian cells [64, 69]. According to the authors, monosaccharide composition analysis with HPLC and AA derivatization is highly accurate and reproducible with less than 3% relative standard deviation (RSD) [64, 69]. Moreover, the approach offers 83–85% recovery. Although the separation depicted in Fig. 10.5 is achieved in 25 min, a much longer run (about 90 min) is required to obtain a more accurate value for

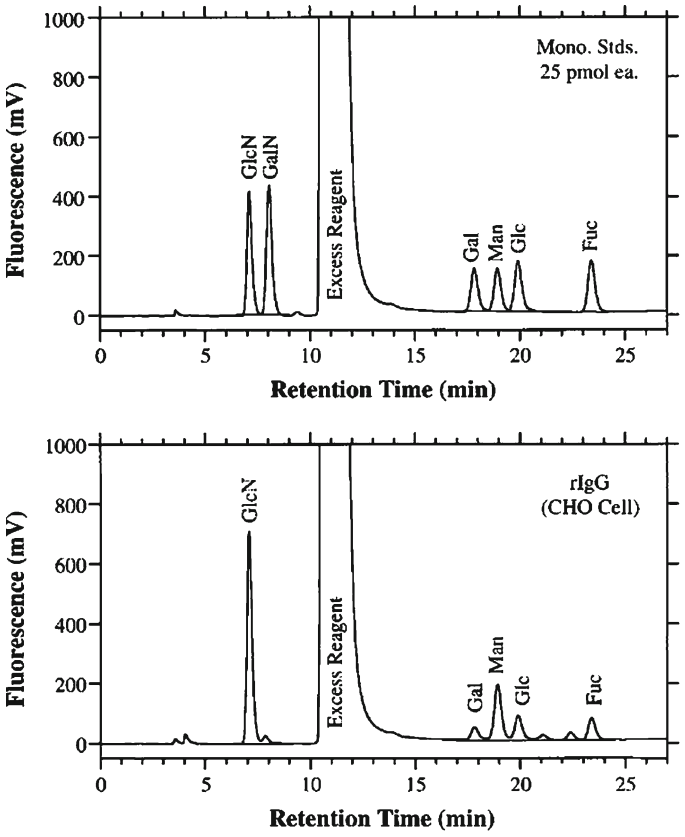


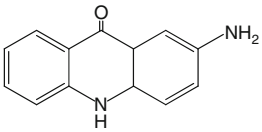
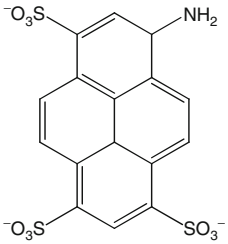
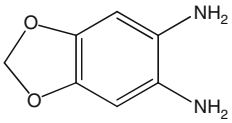
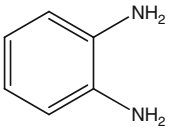
Fig. 10.5 Typical chromatograms obtained with standard monosaccharides (*top*) and ones derived from recombinant IgG expressed in CHO cells (*bottom*) (from Anumula [64] and Anumula and Du [69], with permission)

galactosamine (GalN) that is not separated adequately from mannosamine [ManN, an epimerization product of glucosamine (GlcN)] under the short analysis time [70]. This epimerization is induced by the methanol–acetate–borate reaction medium, resulting in two peaks for every amino monosaccharide [70]. However, it is possible to account accurately for GalN in short runs by subtracting the ManN contribution as a percentage of GlcN, since ManN formed is a constant fraction of GlcN.

The AA method does not allow the determination of sialic acids, since it is a keto-monosaccharide requiring a different tag for derivatization (Table 10.2). Currently, there are three fluorescence tags that are used in conjunction with HPLC to determine monosaccharide composition. The first-is based on the derivatization of sialic acids in dilute sulfuric acid with 1,2-diamino-4, 5-methylenedioxybenzene (DMB) which is a fluorescence tag for α-keto acids [71]. The reaction is performed in the presence of β-mercaptoethanol and sodium sulfite at 60°C for 2.5 h. The highly fluorescent derivatives are separated isocratically in 12 min on a reverse-phase column. This method offers detection limits of 25 and 23 fmol for *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid, respectively [71]. This method was effective in determining the concentration of both *N*-acetylneuraminic and *N*-glycolylneuraminic acids in different glycoproteins and glycolipids, including bovine submaxillary mucin (type I), bovine submaxillary mucin (type I-S), porcine stomach mucin (type II), human α1-acid glycoprotein, bovine brain gangliosides (type III), and bovine brain gangliosides GD_{1A} [71]. Although the utility of this method to determine both *N*-acetylneuraminic and *N*-glycolylneuraminic acids in 5 μL of human and porcine serum or 0.25–2.5 μg of glycoproteins and glycolipids, the sensitivity of the methods permits the simultaneous determination of both *N*-acetylneuraminic and *N*-glycolylneuraminic acids in as little as 0.1 μL of serum or 25–250 ng of glycoproteins and glycolipids. However, the fluorescence of DMB derivatives increases with time, exhibits a curvilinear relationship with DMB concentration, and has a linear range of 3.5–28 pmol [72]. These issues are easily addressed through the addition of an internal standard for quantitative determination using the DMB method [72].

Another fluorescence tag suitable for the derivatization of sialic acid for subsequent HPLC analysis is *o*-phenyllenediamine (OPD) 2 HCl [73]. The derivatization with this fluorescence tag, in contrast to the DMB derivatization, is performed under simple reaction conditions allowing the derivatization to be completed in 40 min. The reaction is completed in one vessel without any need for the purification of the derivatives. Moreover, derivatization with OPD does not require the presence of high concentration of β-mercaptoethanol and sodium sulfite. OPD and DMB sialic acid derivatives are fluorescent substituted quinoxalines that are similar in hydrophobicity, allowing the use of similar separation conditions. However, the linear dynamic range of OPD method extends over two orders of magnitudes (2–450 pmol). OPD derivatization prompts the elution of sialic acid derivatives in order to increase their acetyl content, thus allowing the rapid identification of various sialic acids (Fig. 10.6). This ability is not observed in the case of DMB derivatized sialic acids [67]. The OPD method was found to be highly accurate and reproducible (<3% RSD) for the determination of the sialic acid contents of recombinant IgG

Table 10.2 Fluorescence tags used for the labeling of sialic acid in conjunction with HPLC and CE analyses

Fluorescence tag	Spectroscopic properties (nm)	Method	References
	λ_{ex} 428nm λ_{em} 525nm	CE-LIF	[115]
2-Aminoacridone (AMAC)			
	λ_{ex} 488nm λ_{em} 520nm	CE-LIF	[112–114–115]
8-Aminonaphthalene-1,3,6-trisulfonic acid (APTS)			
	λ_{ex} 373nm λ_{em} 448nm	HPLC	[71]
1,2-Diamino-4,5-methylenedioxy-benzene (DMB)			
	λ_{ex} 232/340nm λ_{em} 420nm	HPLC	[67, 73]
o-Phenylenediamine (OPD)			

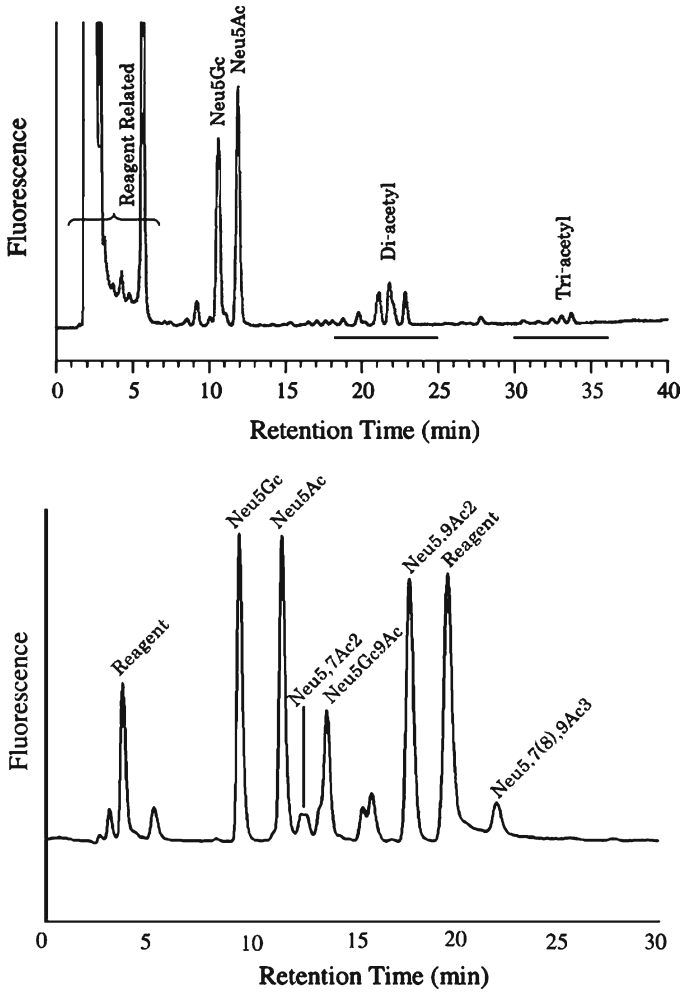


Fig. 10.6 Reverse-phase HPLC separation of sialic acid derivatives: OPD sialic acids from bovine submaxillary mucin (*top*) and DMB derivatives of commercial sialic acids (*bottom*) (from Anumula [67], with permission)

expressed in Chinese hamster ovary cells [74]. This recombinant glycoprotein possesses low levels of sialic acid, presenting a major problem with other techniques. This method also offers high recovery (95–105%).

The third fluorescence tag that has been used for the determination of sialic acid composition is *p*-aminobenzoic acid ethyl ester (ABEE) [75]. This tag has been utilized for determining monosaccharide composition in conjunction with HPLC [76]. The method is based on converting free sialic acids, which otherwise do not react with ABEE, to their corresponding *N*-acylmannosamines using *N*-acetylneuraminic acid aldolase. This conversion allows ABEE labeling of both

sialic acid and other neutral and amino monosaccharides that need to be re-*N*-acetylated to enhance sensitivity. ABEE-derivatized monosaccharides are subsequently determined by reverse-phase HPLC with fluorescence detection. Although the method appears to be effective and allows the simultaneous determination of all monosaccharide composition, including acidic, neutral, and acetylated, the method is less sensitive than OPD and does not allow the distinction between the different types of sialic acid, unless only mild acid hydrolysis is used, but then the ability to simultaneously analyze all monosaccharides is not possible. Accordingly, it appears that thus far the OPD method is the most reliable HPLC-based method for the determination of sialic acids in biological samples.

10.3.3 High-Performance Liquid Chromatography with Mass Spectrometry

Analysis of monosaccharides using LC–MS methods without reverting to derivatization would be an ideal alternative to the methods discussed above; however, its utility has been hindered by the ionization efficiency of neutral monosaccharides using electrospray. Recently, several mass spectrometric studies on ionization of carbohydrates employing adduct formation between the uncharged sugar and various positive and negative ions have been reported [77–79]. Different sugars have been quantified by the LC-multiple reaction monitoring (MRM) mode using Na^+, Cs^+, and NH_4^+ in a positive ESI mode [80–82], and I^- and Cl^- in a negative ion mode [83–86]. Most of these adduct formation methods used post-column addition of the ion and did not provide the required sensitivity needed for the analysis of carbohydrates that are commonly present at low abundance [78, 81].

Chloride attachment by the addition of dichloromethane to a mobile phase consisting of 50% methanol provided excellent sensitivity for sorbitol with a detection limit of 5 pg, but the method still suffered from major disadvantages [85]. The high organic mobile phase content used is not very compatible with the hydrophilic sugars, and a switching valve to clean up the source after each run was required. In addition, the method is complicated by the presence of the two chlorine isotopes, thus rendering it incompatible with the direct quantification of M^{+2} isotopic labeled carbohydrates [84]. Cs^+ attachment to sugars and detection in the positive LC/MRM mode showed sensitivities comparable to those of the chloride attachment method and did not suffer from the isotopic or post-column addition problems that are associated with the chloride adduct [81]. However, the method indirectly monitored the carbohydrate, since the MRM transitions were those of the sugar cesium adduct fragmenting to form Cs^+ product ions. Also, the method was only applied to a few sugars and might not be sensitive to acidic sugars such as the sialic acids, which are expected to ionize better in the negative ion mode.

None of the LC–MS quantification methods, using the ion attachment on monosaccharides, has addressed the problematic simultaneous separation and quantification of all diastereomeric monosaccharides commonly associated with glycoproteins.

This includes glycan structures consisting of the diastereomers glucose, galactose, and mannose; the diastereomers *N*-acetylglucosamine (GlucNc) and *N*-acetylgalactosamine (GalNc); fucose; xylose; and the sialic acids *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc).

Recently, a simple, sensitive, and rapid LC-MRM method for the quantification of all the common monosaccharides was introduced by Mechref's group [87]. Neutral sugar molecules were detected as their alditol acetate anion adducts using electrospray ionization in negative ion MRM mode. The method is robust, rapid, and highly sensitive. It does not require derivatization or post-column addition of reagents. Sialic acids and reduced forms of neutral monosaccharides are efficiently separated using porous graphitized carbon column. As mentioned above, chromatographic separation of monosaccharides and glycans has always been very challenging, due mainly to their highly hydrophilic nature. Carbohydrate retention on a porous graphitized carbon column (GCC) appears primarily due to adsorption. The unique selectivity of GCC and their unmatched ability to resolve isomeric and closely related compounds are brought about by the homogeneous adsorptive nature of this material. While monosaccharides are weakly retained on the GCC column, anomeric separation of free reducing end monosaccharides and glycans is readily attained. However, this aspect of the separation is not desired in the quantitative analysis of mixtures of monosaccharides, since each free-reducing end monosaccharide appears as two peaks (α- and β-anomers). Free reducing end monosaccharides and glycans are easily reduced using boran-complex reagent.

Neutral monosaccharide molecules were detected as their alditol acetate anion adducts $[M+CH_3CO_2]^-$ using electrospray ionization in negative ion MRM mode, while sialic acids are detected as deprotonated ions $[M\text{-}H]^-$. Therefore, the MRM transitions for the aldose monosaccharides used in this study correspond to the $[M+CH_3CO_2]^-$ parent ion yielding the $[M\text{-}H]^-$ daughter ion, where M corresponds to the mass of alditol sugar (the molecular mass of the aldose + two hydrogens). The MRM transitions of the alditols and the sialic acids commonly associated with glycoproteins are shown in Fig. 10.7. In the case of sialic acids, the acetate adduct parent ion was not chosen, since they readily deprotonate in the gas-phase yielding $[M\text{-}H]^-$ ions. The new method exhibits very high sensitivity to carbohydrates, and the limits of detection (LOD) of glucose, galactose, mannose, and GlcNAc attained using this method were 1 pg, while that of xylose, fucose, and GalNc was 1.5 pg. The LOD of sialic acids was 5 and 10 pg for NeuAc and NeuGc, respectively. These LODs are significantly lower than what has been previously reported.

Shown in Fig. 10.8 are the overlaid extracted ion chromatograms of 2 ng of glucose, galactose, mannose, *N*-acetylglucosamine, and *N*-acetylgalactosamine following treatment with the ammonia–borane complex. It is clear from the figure that each of these monosaccharides was quantitatively reduced (α- and β-anomeric forms are eliminated) and, more importantly, all diastereomers including the reduced forms of *N*-acetylglucosamine and *N*-acetylgalactosamine are separated from each other with a resolution higher than 1.5, which is adequate for quantification.

The aforementioned approach was validated using model glycoproteins, including fetuin, ribonuclease B, peroxidase, and α1-acid glycoprotein human (AGP).

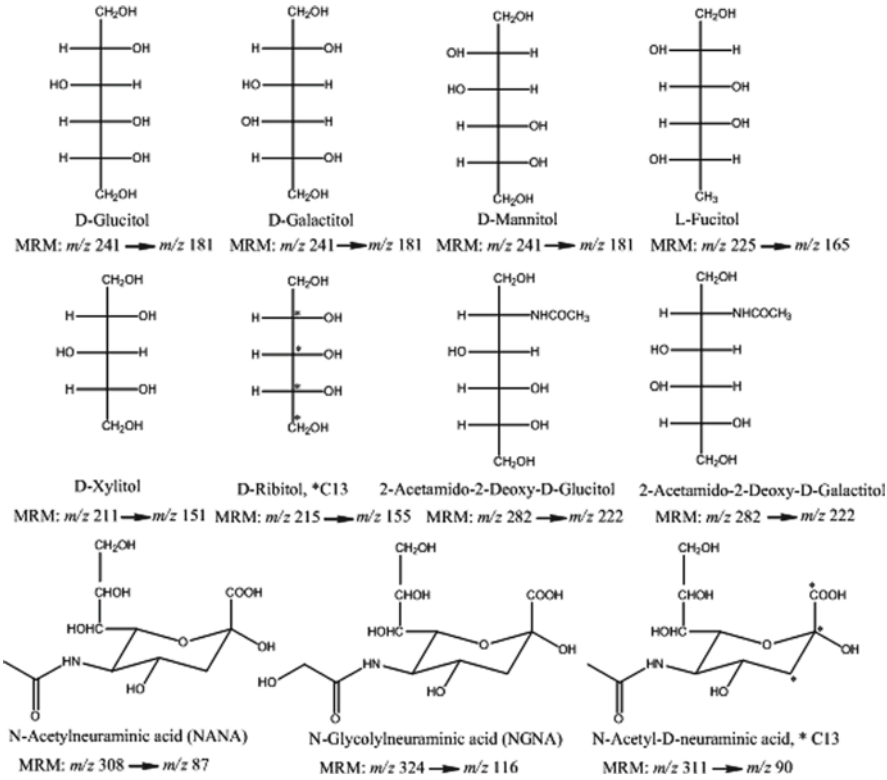


Fig. 10.7 Reduced neutral monosaccharides, sialic acids, and internal standards used in this study and their MRM transitions used for quantification (from Hammad et al. [87], with permission)

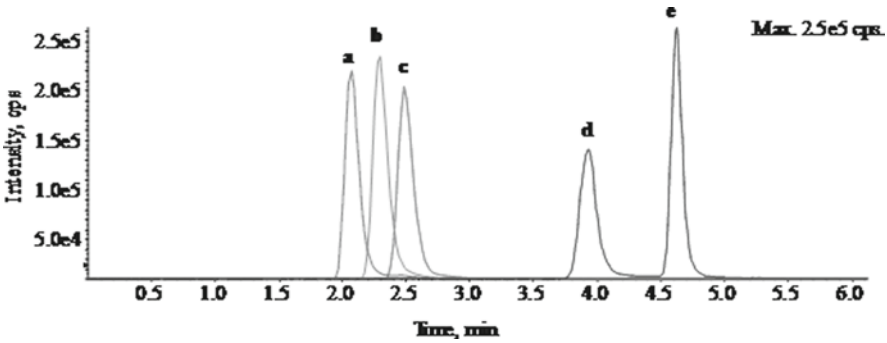


Fig. 10.8 Extracted ion chromatograms (XIC) of the MRM of a 100 pg/μL solutions (2 ng on column) of the reduced alditol forms of galactose (**a**), mannose (**b**), glucose (**c**), *N*-acetylgalactosamine (**d**), and *N*-acetylglucosamine (e), resulting from treating the corresponding aldose with aqueous ammonia-borane complex (from Hammad et al. [87], with permission)

The measured values using the LC-MRM method were consistent with the values cited in the literature. However, the approach described here is ten times more sensitive as suggested by the ability to determine the monosaccharide composition of glycoproteins using only 1-μg aliquots. All other procedures require 5–50 times more glycoproteins [87]. This method is also advantageous because all types of monosaccharides are determined simultaneously under the same chromatographic and mass spectrometric conditions. Since quantification is based on tandem MS data for ions detected at specific retention times and with specific *m*/*z* values, this type of quantification is considered highly accurate and sensitive.

10.3.4 Capillary Electrophoresis with Laser-Induced Fluorescence

Capillary electrophoresis (CE) is a separation technique capable of resolving numerous glycan isomers associated with glycoproteins. It offers inherently high efficiencies and some selectivity in its different formats [88–90]. Various capillary electromigration techniques have been demonstrated to achieve separation of different glycans, including micellar electrokinetic chromatography (MEKC) [91], capillary electrochromatography (CEC) [92–95], capillary gel electrophoresis (CGE) [96, 97], and capillary zone electrophoresis (CZE) [98, 99]. However, the separation efficiency and resolution of glycans achieved through CGE and CZE are superior to those observed in CEC. By introducing a physical gel into a capillary, separation of glycans in CGE results from the sieving of glycans as they migrate through the capillary. This molecular sieving effect is based on the hydrodynamic volume differences of glycans in solution. Due to this mechanism of separation (in addition to the inherent high efficiencies), a distinct resolution of certain structural isomers has been demonstrated using CGE [96, 97]. CZE is the most widely used mode of CE because of its simplicity of operation and versatility. Its simplicity originates from the fact that the separation occurs because solutes migrate in discrete zones at different mobilities in a capillary filled with a buffer [98, 99].

Over the past two decades, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been recognized as a very useful and sensitive method for the analysis of monosaccharides. However, LIF detection necessitates the use of fluorescence tags, whose structures are optimally “tuned” to the output wavelengths of reliable laser technologies; typically, argon-ion lasers are preferred over helium–cadmium (He–Cd) laser. In 1991, the first carbohydrate analysis by CE/LIF combination was demonstrated, using 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) as a derivatizing agent, for amino sugars [100], and *N*-glycans derived from glycoproteins [101–103]. With the detection limits reaching subattomole levels, the oligosaccharide constituents of bovine fetuin (CBQCA-labeled *N*-glycans) were resolved into four major (expected) peaks and some minor components. Since then, additional efforts have been made to exploit the potential of various derivatizing reagents for high-sensitivity glycomic analysis.

The effects of charged groups in several fluorophores, including 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 7-aminonaphthalene-1,3-disulfonic acid (ANDS), and 2-aminonaphthalene-1-sulfonic acid (ANS), on the electrophoretic separation of glycans was investigated [104–107]. A greater charge caused faster analyses and higher resolution, making ANTS one of the most effective derivatizing agents among the aminonaphthalene derivatives for the CE analysis of *N*-glycans derived from glycoproteins. Accordingly, this reagent was successfully employed for characterization of glycans derived from various glycoproteins, including human immunoglobulin G [104], ovalbumin [108-109], fetuin [108-110-111], recombinant HIV gp-120 [111], and α_1-acid glycoprotein [111].

Although ANTS has been found effective in glycomic analysis, the instability and high cost of the required He–Cd laser prompted the need to explore alternative fluorophores, utilizing a more convenient light source such as the argon-ion laser. Today, the most commonly used fluorescence tag for CE-LIF of mono- and oligosaccharides is 1-aminopyrene-3,6,8-trisulfonic acid (APTS, Table 10.1), which was introduced by Evangelista et al. [112] as a novel derivatization agent. APTS offered several distinct advantages over the other fluorescence tags used for derivatizing sugars. The molar absorptivity and quantum efficiency of APTS derivatives are substantially higher than all fluorescence tags. APTS exhibits significant amount of absorption at 488 nm, allowing the use of the stable argon-ion laser. These advantages of this fluorescence tags allows the realization of detection limits as low as 1 pmol for monosaccharide standards.

The initial report by Evangelista et al. [112] demonstrated the ability to separate APTS-labeled monosaccharides using 100 mM borate buffer at pH 12. The separation in such buffer was based on the extent of borate complexation, and allowed baseline separation of several monosaccharide structural isomers such as Man, Gal, and Glc. Later, the same group demonstrated the ability to resolve monosaccharides in other buffers such as acetate (pH 5.0), 3-(*N*-morpholino)-propanesulfonic acid (pH 7.0), and phosphate (pH 7.4) [113]. The separation in such buffers is based on the differences in the hydrodynamic volumes of APTS-labeled monosaccharides. Realizing the low yields of amino sugars labeling with APTS, Evangelista et al. [114] investigated the efficiency of APTS derivatization in seven different organic acids, including the commonly used acetic acid (pKa 4.75). The use of organic acids of higher strength than acetic acid produced substantially higher yields, especially in the case of *N*-acetylamino sugars. This is in accordance with the general acid catalysis of reductive amination reaction. Optimum APTS derivatization yield are recognized using citric acid (pKa 3.13) [114].

APTS is not a suitable fluorescence tag for the CE of sialic acids, since the electrophoretic mobility of APTS-labeled sialic acid is almost equivalent to the electro-osmotic flow (EOF) at pH 10. As a result, the migration time of APTS-labeled sialic acid is a very long migration. Moreover, the yield of sialic acid labeling with APTS is very low. The former caveat is rectified by Guttman [115] through the use of neutral fluorescence tag such as 8-aminoacridone (AMAC, Tables 10.1 and 10.2), which was initially introduced for labeling of oligosaccharide prior to HPLC analysis [115]. Chen et al. [117] eradicated the low yield of sialic acid

derivatization with APTS by employing the treatment of the acid or enzymatic hydrosylate with neuraminic aldolase in a fashion similar to what was initially employed by Yasuno et al. [75]. This enzymatic treatment quantitatively converts *N*-acetylneuraminic acid to *N*-acetylmannosamine, which is more susceptible to APTS labeling, thus prompting higher derivatization yield. This method allows the simultaneous determination of acidic and other type of monosaccharides in a single CE-LIF analysis (Fig. 10.9). Moreover, the detection limits offered by this method are 100 pmol for *N*-acetylneuraminic acid and 50 pmol for all the other monosaccharides.

Currently, Beckman Coulter (Fullerton, CA) offers a carbohydrate labeling and analysis kit based on the above-mentioned methods for monosaccharide composition analysis by CE-LIF. It contains the reagents, buffers, and separation capillaries required to label, separate, and quantify oligosaccharides and monosaccharides derived from glycoproteins. CE with laser-induced fluorescence detection (CE-LIF) appears to be a very useful and sensitive method for the analysis of monosaccharides; however, derivatization of the sugar with a fluorophore is required, and borate is used as a complexing reagent in the separation buffer in order to prompt the separation of closely related monosaccharides [118, 127]. Although attomole sensitivity has been reported for monosaccharides using CE-LIF, the method is limited by the minute amount of sample that can be introduced into the capillary, thus requiring larger sample concentrations [119, 127]. Additionally, as with other methods requiring derivatization, relatively large amounts of the glycoprotein and the derivatization reagent are required in order to efficiently perform the derivatization reactions [120, 127].

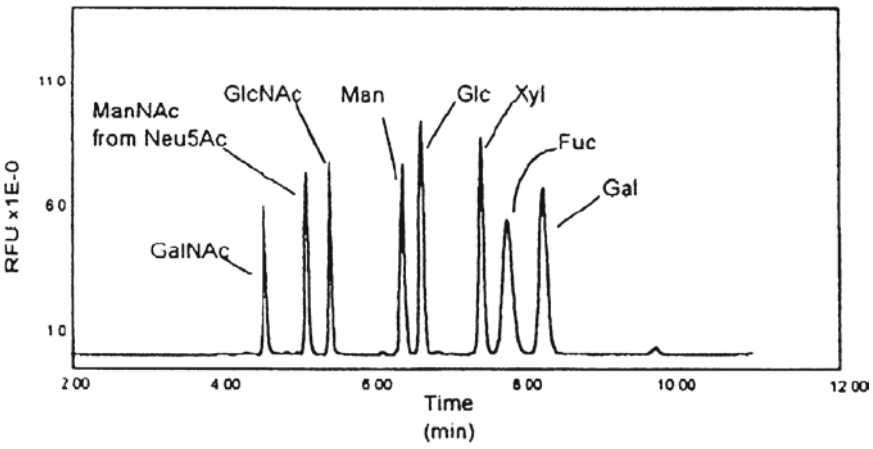


Fig. 10.9 Electropherogram of APTS derivatives of neuraminic acid aldolase-treated mixture of Neu5Ac and seven aldoses separated in an alkaline borate buffer. *N*-acetylneuraminic acid (NeuAc) is converted quantitatively to *N*-acetylmannosamine (ManAc). *GalNAc N*-acetylgalactosamine, GlcNAc *N*-acetylglucosamine, Man mannose, Glc glucose, Xyl xylose, Fuc fucose, Gal galactose (from Chen et al. [117], with permission)

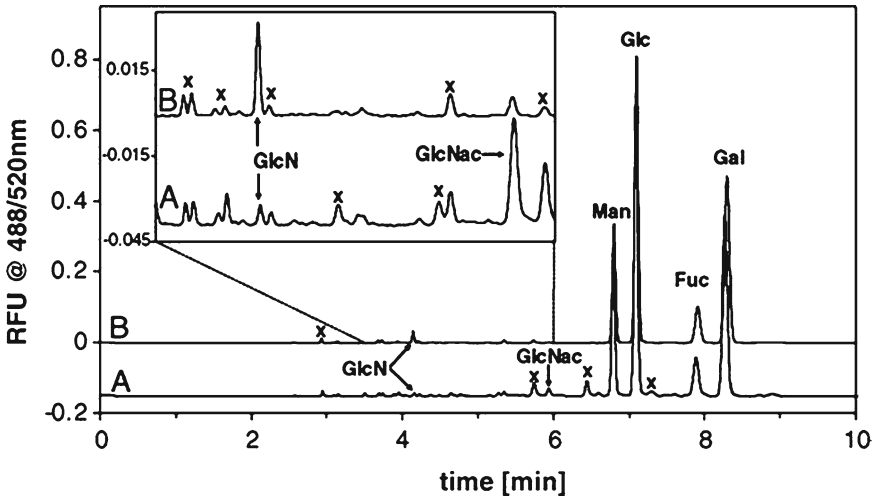


Fig. 10.10 Electropherograms of monosaccharides released from glycoprotein drug substance by acidic hydrolysis, with (A) and without (B) re-*N*-acetylation, followed by labeling with APTS, CE separation buffer: 240 mM borate pH 9.0. The sample was supplemented with 25 nmol Glc for peak area normalization before acidic hydrolysis (from Racaityte et al. [70], with permission)

Recently, Racaityte et al. [70] compared the performance of CE-LIF with APTS labeling and RP-HPLC with AA labeling for the quantitative determination of the neutral and amino monosaccharides derived from a therapeutic glycoprotein. Both methods demonstrated comparable sensitivity, reproducibility, and robustness. The CE-LIF method is fast, lacks any side reaction (Fig. 10.10), and uses inexpensive capillaries, while RP-HPLC method using AA-labeling does not require re-*N*-acetylation derivatization of amino monosaccharides, a step that is required in the case of CE-LIF to enhance the sensitivity of amino monosaccharide detection. A major drawback of the RP-HPLC method using AA-labeling is epimerization of amino monosaccharides, which originates from the use of the methanol–acetate–borate reaction medium as described above. The CE-LIF method can be adopted to analyze both acidic and neutral amino monosaccharides, while this is not conceivable in the case of RP-HPLC method using AA-labeling.

10.4 Monosaccharide Compositional Analysis of Glycoproteins and Glycolipids in the Diagnosis/Prognosis of Diseases

Monosaccharide composition analysis by HPAEC-PAD was also utilized to monitor changes in glycosylation associated with several diseases, including rheumatoid arthritis [121]; alcoholic liver diseases [122] and other liver diseases [123]; renal insufficiency [43]; Crohn's disease [124]; asthma [125]; and stomach, breast, and ovarian cancers [124]. Turner's group [121] determined the monosaccharide

compositions of haptoglobin isolated from serum using an antihaptoglobin antibody by HPAEC-PAD. Results were compared for haptoglobin isolated from seven healthy women, eight women with inactive rheumatoid arthritis, and eight women with active rheumatoid arthritis. Monosaccharide composition analyses revealed a statistically significant increase in the fucosylation (fourfold) of haptoglobin isolated from women with active rheumatoid arthritis. This increase in fucosylation is in agreement with the previously reported increase in binding of haptoglobin isolated from women with active rheumatoid arthritis to lotus lectin [126]; active rheumatoid arthritis was also deduced from HPAEC-PAD monosaccharide compositional analysis [121].

Changes in the monosaccharide composition of haptoglobin were also studied by the same group to monitor alcoholic liver diseases [122]. HPAEC-PAD was utilized to determine the monosaccharide composition of haptoglobin isolated from 48 healthy individuals, 15 alcohol abusers, 25 patients with alcoholic liver disease (including alcoholic cirrhosis), and 17 other patients with either chronic active hepatitis or primary biliary cirrhosis. The results indicated a statistically significant increase in fucosylation among alcohol abusers and patients with different alcoholic liver diseases except chronic active hepatitis. Fucose was most elevated in the case of patients with alcoholic cirrhosis, and significantly different from the patients with other alcoholic liver diseases. Also, this increase in fucosylation was accompanied by an increase in *N*-acetylglucosamine, thus suggesting an increasing in branching. This was true for all patients with alcoholic liver diseases except chronic active hepatitis.

Smith's group [123] employed HPAEC-PAD monosaccharide composition analysis to evaluate the change in the glycosylation of another hepatic glycoprotein (α-1-acid glycoprotein) between individual liver diseases. The study also aimed at investigating whether liver damage could be correlated with changes in α-1-acid glycoprotein glycosylation among groups of patients with various liver diseases similar to the groups studied by Turner's group [122]. Hyperfucosylation of α-1-acid glycoprotein was also observed in the case of all patients with the different liver disease groups. Although the results are quite intriguing, considering the simplicity at which one can attain monosaccharide composition by HPAEC-PAD, the fact that the increase in fucosylation was observed in both rheumatoid arthritis and alcoholic liver diseases limits the clinical potential of using this approach for diagnostic or prognostic purposes. Moreover, it appears that the glycosylation machinery in the liver is effected in a manner prompting an increase in the fucosylation of all hepatic glycoproteins, as can be concluded from the increase in fucosylation observed for both haptoglobin and α-1-acid glycoprotein.

Miyazaki's group [43] also utilized HPAEC-PAD to monitor the change in the glycosylation of α-1-acid glycoprotein isolated from plasma collected from patients with renal insufficiency relative to those collected from healthy individuals. The levels of mannose, galactose and *N*-acetylglucosamine in α-1-acid glycoprotein were significantly higher among individuals with renal insufficiency, while the levels of fucose and *N*-acetylneuraminic acid were comparable among the two cohorts [43].

HPAEC-PAD monosaccharide composition analysis was also utilized to characterize the differences between different glycoforms of respiratory mucins [125]. The tracheobronchial epithelium expresses at least eight mucin apoproteins; however, it is not known which, if any, of these major apoproteins is responsible for the formation of the mucus gel. To address this question, monosaccharide composition analysis was performed on mucins isolated from normal, asthmatic, and chronic bronchitic secretions. Four mucin populations were fractionated, and the monosaccharide composition of each fraction was determined. Two populations were significantly richer in fucose and galactose and relative to *N*-acetylglucosamine have higher monosaccharide content, suggesting they have longer oligosaccharide chains. The other two populations have lower neutral monosaccharide residues, but more sialic acid in agreement with their higher charge density [125]. Although monosaccharide composition analysis was performed for the first time on mucin isolated from respiratory secretion, it does not appear that change in the glycosylation of this mucin is correlated to the state of tracheobronchial epithelium.

The levels of Neu5Ac and Neu5Gc sialic acids were evaluated in serum of women before and after surgical treatment of early endometrial cancer using the HPLC method [128]. Although both Neu5Ac and Neu5Gc were evaluated, the former was identified as the major sialic acid in sera from both cancer patients and healthy individuals as well as in tissue specimens. The total sialic acid level in patients with endometrial cancer before surgical treatment (709.5 ± 306.5 mg/L) was significantly higher ($p < 0.0001$) than that of the control group (213.5 ± 88.7 mg/L). The level of serum Neu5Ac (699.4 ± 305.6 mg/L) statistically decreased (305.9 ± 114.5 mg/L) following surgical treatment [128]. CE was recently employed by Lin's group [129] to determine the levels of sialic acids in the serum of cancer patients. Again, Neu5Ac was the major sialic acid detected in the sera of both cancer patients and control. Neu5Ac concentrations in the sera of patients with various malignant tumors, including breast (1,198 ± 379 mg/L), lung (1,248 ± 302 mg/L), gynecological (1,161 ± 287 mg/L), endometrial (1,038 ± 211 mg/L), ovarian (1,145 ± 324 mg/L), cervical (1,138 ± 269 mg/L), and leukocythemia (1,188 ± 380 mg/L) cancers, were significantly higher ($p < 0.001$) than the control (612 ± 146 mg/L). However, there was no significant difference among the various cancers. Monitoring the levels of sialic acid in sera might offer some clues to the presence of a tumor, but it is not able to determine what type of tumor. This is true since the elevations in sera sialic acid levels were comparable among the different types of cancers evaluated [129].

10.5 Conclusion

Aberrant glycosylation is currently recognized as an attribute of malignancy and cancer progression. Different chromatographic and electrophoretic methods have been developed for monosaccharide composition analysis of glycoproteins and glycolipids, allowing simple, sensitive, and specific determination of monosaccharide composition at high repeatability, reproducibility, and accuracy. Although such methods have

witnessed some success in biological and biomedical application as indicated above, none of the described methods has led to its implementation in clinical setting. This might be partially attributed to the inability of any of the methods to distinguish between diseases. For example, an increase in the levels of sera sialic acids has been determined for all cancer samples irrespective of cancer types. In the case of liver diseases, all different liver ailments exhibited an increase in fucosylation. Thus far, it appears that any of the above-mentioned methods has a clinical potential in prognosis as demonstrated in the case of endometrial cancer before and after surgical treatment. However, extensive research is still needed to conclusively determine the clinical applicability of the different methods described above in the diagnosis or prognosis of diseases.

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