

Biomedical Materials and Diagnostic Devices

Scrivener Publishing
100 Cummings Center, Suite 541J
Beverly, MA 01915-6106

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Biomedical Materials and Diagnostic Devices

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Co-published by John Wiley & Sons, Inc. Hoboken, New Jersey, and Scrivener Publishing LLC, Salem, Massachusetts.

Published simultaneously in Canada.

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Illustration on front cover depicts interaction of stem cells into the nanobiomaterials for tissue engineering.

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Library of Congress Cataloging-in-Publication Data:

Tiwari, Ashutosh, 1945-
Biomedical materials and diagnostic devices / edited by Ashutosh Tiwari ... [et al.]
p. cm.

Includes bibliographical references and index.
ISBN 978-1-118-03014-1 (hardback)

[DNLM: 1. Biocompatible Materials. 2. Drug Delivery Systems. 3. Nanotechnology. 4. Tissue Engineering. QT 37]

610.28'4-dc23

2012025753

ISBN 978-1-118-03014-1

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

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Preface

Engineering of advanced biomaterials has resulted in striking solutions to multifarious biomedical and diagnostic conundrums, including cell separation, stem-cell scaffolds, targeted drug delivery, treatments for hyperthermia, automated DNA extraction, gene targeting, resonance imaging, biosensors, tissue engineering and organ regeneration. The biomedical materials with the most promising potential combine biocompatibility with the ability to precisely adjust biological phenomenon in a controlled manner. The world market for biomedical and diagnostic devices is expanding rapidly and is currently valued over US\$1000 trillion. Likewise, academic research has kept pace with the market demand with over 50,000 papers being published in the field last year. While the field of diagnostic devices has achieved considerable success, commercial returns in this sector are dominated by glucose sensing, despite the myriad of other possibilities for novel and useful analytical devices. Key areas such as drug delivery and regenerative medicine, not only represent huge opportunities to improve longevity and quality of life, but will also benefit from the fusion of ideas occurring within the emerging modern field of biomaterials. Molecular design for one application is finding utility across the field in a synergistic combination of solutions that brings together sensing, imaging, therapy and reconstruction in a plethora of exciting medical applications.

This book aims to provide an up-to-date overview of the fascinating field of biomedical materials and devices. This large volume includes twenty chapters divided into four main areas: biomedical materials, diagnostic devices, drug delivery and therapeutics, and tissue engineering and organ regeneration. It covers the latest research and developments in biomedical materials and medical devices: fabrication, performance and uses.

The chapters seek to address progress in successful design strategies for biomedical materials and devices such as the use of collagen, crystalline calcium orthophosphates, amphiphilic polymers, polycaprolactone, biomimetic assembly, bio-nanocomposite matrices, bio-silica, theranostic nanobiomaterials, intelligent drug delivery systems, elastomeric nanobiomaterials, electropun nano-matrices, metal nanoparticles and a variety of biosensors. This book is intended to be suitable for a wide readership including university students and researchers from diverse backgrounds such as chemistry, materials science, physics, pharmacy, biological science and bio-medical engineering. It can

be used not only as a text book for both undergraduate and graduate students, but also as a review and reference book for researchers in the materials science, bioengineering, pharmacy, biotechnology and nanotechnology.

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PART I

BIOMEDICAL MATERIALS

Application of the Collagen as Biomaterials

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Abstract

Collagen is the protein of connective tissue in mammals. The content of collagen in the total protein is approximately 30% of the mammalian tissues. Due to its good cytocompatibility, researchers use this material for the biomedical research application. However, the control of its physical and biological properties is difficult. There are two obstacles in collagen application: 1) difficulty in regeneration of the collagen properties, and 2) difficulties in controlling the properties of the collagen products. The collagen is easily denatured and affected by the environment, which leads to unexpected results. On the other hand, the crosslinker to suppress the denaturation may cause the stiffness of the collagen product. So the researchers are investigating new ways to prepare a collagen product which can be used as a biomaterial for biomedical research application. An important component of the research is the structure and the function of extracellular matrix (ECM). That is, there is biorelevant structure-function-property relationship, which alters its function as an ECM. Recent studies on decellularized tissue is also based on the fact that the native structure of the ECM can be preserved, and therefore may perform the function of the original tissue. So, by replicating its microstructure and producing a collagen fiber complex, it is expected that the function of ECM can be replicated. In this chapter, we will be introducing recent studies on the preparation of a collagen matrix based on fibrillogenesis, orientation, complex formation and layered structure, and how these structures alter the physical and biological properties.

Keywords: Collagen, decellularization, extracellular matrix, fibrillogenesis, microenvironment, regenerative medicine

1.1 Introduction

Collagen is an extracellular-matrix (ECM) protein that plays an important role in the formation of tissues and organs and is involved in various functional expressions of cells [1]. A native ECM is a complex fiber-composite material in which collagen fibrils are a major component [2]. The function of an ECM is to provide support, tensile strength, and scaffolding for the tissue and cells. In addition, it should serve as a three-dimensional structure for cell adhesion

and movement and as a storage depot for growth factors, chemokines, and cytokines; and it should provide signals for morphogenesis and differentiation [3]. Approximately 30% of all vertebrate body protein is composed of collagen. Among these, the highest collagen composition can be found for the tendon, bone and cornea where 90% of ECM is collagen. Mainly, the collagen can be distinguished into two types; fibrillar and non-fibrillar. There are 28 types of collagen and the collagen types I, II and III are the classical fibril-forming collagens and account for 80–90% of all collagens in the human body. Collagen fibril is very important from the aspect that its properties and the morphology provide the key to the scaffolding structures in the body according to the location.

It has been shown that the collagen possesses non-immunogenicity and good cell compatibility, and can be obtained from various sources. These make collagen popular among biomaterials researchers, and diverse methods have been adopted for its application in the biomedical fields. The collagen is purified after being treated with pH adjustment or pepsin digestion. Either way, the collagen should be water soluble in order to process it for use as a collagen matrix for biomaterial applications. There are several kinds of collagen matrix; gel, film, microparticles, conjugates, minipellets or sponge [1, 4]. However, there are still many problems to overcome. For example, the collagen which is available in the marketplace is hydrophilic, which absorbs water at a high rate. So, the uncross-linked collagen matrix possesses low mechanical strength and fast degradation rate in aqueous solution. The collagen matrix degrades by the collagenase, so this makes the collagen applicable in some biomedical products where the biodegradation in the living body is required. However, control of the biodegradation is not easy. The properties of the collagen matrix can be controlled by cross-linking. The cross-linking is executed chemically or physically. Furthermore, using the same cross-linking process, the collagen matrix can be functionalized by immobilization or, blend of a second component. The collagen is composed of amino acid groups where the chemical reaction can be executed. Mainly, the cross-linking is executed using ϵ -amino groups of lysine or hydrolysis, and aspartic acid or glutamic acid residues. These residues are highly reactive and can be easily functionalized. The cross-linking can change physical and biological properties of the collagen matrix and can be applied for the loading of the drugs. For the chemical cross-link, glutaraldehyde, formaldehyde, hexamethylenediisocyanate, polyepoxy compounds, carbodiimides, and acyl azides are commonly used [1, 4–14]. These show a good result *in vivo*, such as suppressing the inflammatory response and promoting the healing response. However, there are still several problems to be overcome. Although the collagen gels, sponges or films that have been cross-linked show an increase in the mechanical strength, the cross-link which consumes the functional groups are consumed for the cross-linking site, which may affect the biological properties. Moreover, the stiffness of the ECM is also a very important parameter, but it is not easy to control the stiffness of the collagen gel by cross-linking or a change in the collagen solution. That is, a stiff collagen gel can be prepared, but a gel with viscoelasticity cannot be prepared.

This is important because most of the native tissue possesses viscoelasticity which contributes to the toughness [15, 16]. A number of collagen matrices were reported, but the collagen products available in the marketplace is still scarce because of the problems mentioned above, so a new approach was required to move to the next step.

1.2 Structural Aspect of Native Tissue

1.2.1 Microenvironment

Before designing a biomaterial, it is necessary to understand the environment of the living body. When the biomaterial is designed for application in tissue engineering and regenerative medicine, the objective is the repair and remodeling of the damaged ECM and tissue, ultimately regenerating its function. The function of the ECMs is deeply related to the behavior of the cells which is affected by the cell-materials interaction. That is, the control of the cells behavior is very important in the aspect of regenerating the function of the ECMs. The cell is immersed in a dynamic landscape composed of insoluble macromolecules of the ECM, soluble bioactive factors and neighboring cells [17]. The environment which controls the fate of the cell inside the living body is called cellular microenvironment. It is very important in the aspect that the ultimate tissue structure and its function are decided by factors contributing to the cellular microenvironment. For this, there needs to be a fundamental understanding on the cellular microenvironment for the materials design. The cellular microenvironment is the environment in the living body which controls the fate of the cells. The microenvironment is composed of signals from the neighboring cells, physical stimuli, soluble factors such as growth factors, and insoluble factors such as ECM. The ECM has been shown to influence cell mitogenesis and chemotaxis [18, 19], direct cell differentiation [20–23], and to induce constructive host tissue remodeling responses [24–26]. The cells from the ECM sense, integrate and proceed the signals to determine behavior and functions, and the information is passed bidirectionally as the microenvironment is remodeled by the cells.

Development of biomaterials for tissue engineering and regenerative medicine has been approached mainly from the aspect of controlling the soluble and insoluble factors. As for the insoluble factor, diverse materials – natural or synthetic – are being investigated. The main goal of using these materials is to replicate the function of the ECM temporarily or permanently. By loading soluble factors in the materials, researchers tried to control the fate of the cells or stimulate the regeneration of the damaged tissues. On the other hand, manipulation of the morphology, microphase, surface physical properties and chemical properties of the material is a major approach for the control of the insoluble factors. These methods show good results and some of them are actually used for clinical practice.

Since the ECM is mainly composed of collagen, use of collagen to replicate its function is actively executed. It should be noted that the function of the

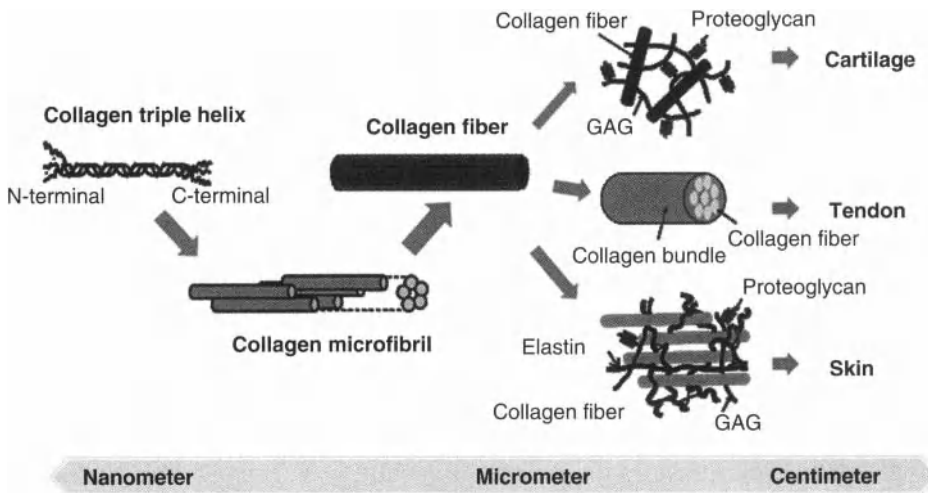


Figure 1.1 The scale from the nano-scale to the macro-scale. The collagen matrices consist of nano-scale no larger than the microfibril, but the actual scale of the ECM or the tissue is much larger.

ECM is different according to the type of tissue such as cornea, brain, skin, tendon, or blood vessel, where they need to perform a certain function. So the design of the ECM using collagen should be different according to the targeted tissue. However, although the tissues perform different functions, the common aspect of the tissue is that all are made up of collagen fibrils. That is, in order to design a material which may replicate the function of ECMs, fibrillized structure should be considered. Furthermore, it should be acknowledged that the design should include a nanometer to centimeter scale. The schematic structural images of respective tissue from the nanometer to centimeter scale are shown in Figure 1.1. Yip discussed the importance of careful consideration of biorelevant structure-function-property relationships in the design of biomaterials [27]. That is, the regeneration of the physical properties of native ECM is important for the regeneration of biological properties. The importance of the structure can be seen in research related to the decellularized tissue which is discussed in the next section.

1.2.2 Decellularization

The decellularized tissue is a native tissue in which the cells are eliminated by certain treatment. Decellularization of tissue is based on the fact that preservation of the native ultrastructure and composition of ECM is possible [26]. The methods for the decellularization include use of chemical agents (ionic detergents, non-ionic detergents, acids and bases, hypotonic and hypertonic solution, and solvents), biological agents (enzymes and chelating agents), and physical treatment (temperature, pressure and electroporation). It should be understood that every cell removal agent and method will alter ECM composition and cause some degree of ultrastructure disruption. For example, the use of some

chemical agents such as sodium dodecyl sulfate (SDS) may cleave the collagen fibrils, but use of physical treatment such as high pressurization would not affect the main structure [26, 28–30]. Furthermore, incomplete rinsing of chemical agents or the cell debris after decellularization process may cause toxicity. However, the minimization of these undesirable effects, rather than complete avoidance by the living body, is the objective of decellularization. So, the focus is set on complete removal of the cells and preservation of the ultrastructure. The methods for the decellularization should be carefully considered according to the density of the fibers, the thickness and the lipid contents. Moreover, the complete washing of the cell debris or the chemical agents after decellularization should be executed because this could cause toxicity.

The mechanical strength after the elimination of the cells is maintained and the regeneration around the implanted decellularized tissue occurs without serious inflammatory response. So, the decellularization can be executed for the partial or full organs. It should be noted that the native tissue possesses complex structure and the whole structure – either macro or micro – is maintained after the appropriate decellularization process. Furthermore, the degradation of the decellularized tissue is slow, and the remodeling of the damaged tissue occurs without any problems. The regeneration within the living body occurs on the implanted decellularized tissue and starts to function as a replacement. Furthermore, the high mechanical strength of the decellularized tissue would endure the physical stress inside the living body [29, 31–32]. So many decellularized tissue products such as dermis, heart valve, blood vessel, bone and so on, have been introduced to the markets and are enjoying success.

1.2.3 Strategy for Designing Collagen-based Biomaterials

The key for the success of the biomaterials for regenerative medicine is control of the cells' fate which depends on the materials characteristics; three-dimensional ultrastructure, surface topology and composition of the ECM [17]. The successful point for decellularized tissue is that the three-dimensional ultrastructure, surface topology and composition of the ECM is maintained after the process. So, in order to reproduce the physical and biological properties of the ECM, we should first mimic its three factors as written above. The key points are the fibril formation, orientation, complex formation with second component such as GAG or elastin, and multiple layers. Since the structure of ECM differs according to the tissue, the mimicking of the structure should also be different according to what kind of tissue the researchers want to make. This is because the key function is different according to the tissue. For example, the tendon should have fibrillar structure with high orientation, the cornea should have fibrillar lattice structure, blood vessels should possess elastin-complex fibril structure with multiple layers and high orientation of collagen fibers, and skin should have elastin-complex fibril structure disregarding the orientation. Such ECM structures allow the various tissues to possess certain physical and biological properties adequate for functional performance. So, the structural consideration for replicating the function of tissue is very important.

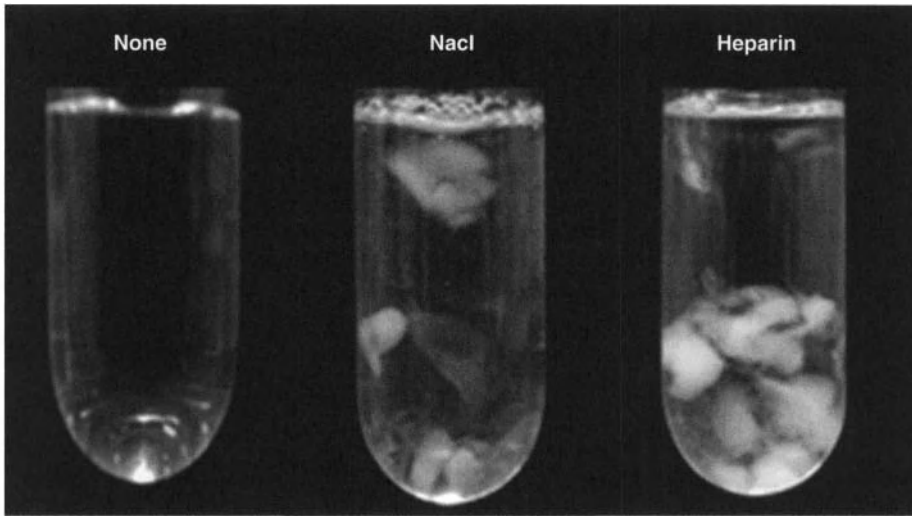
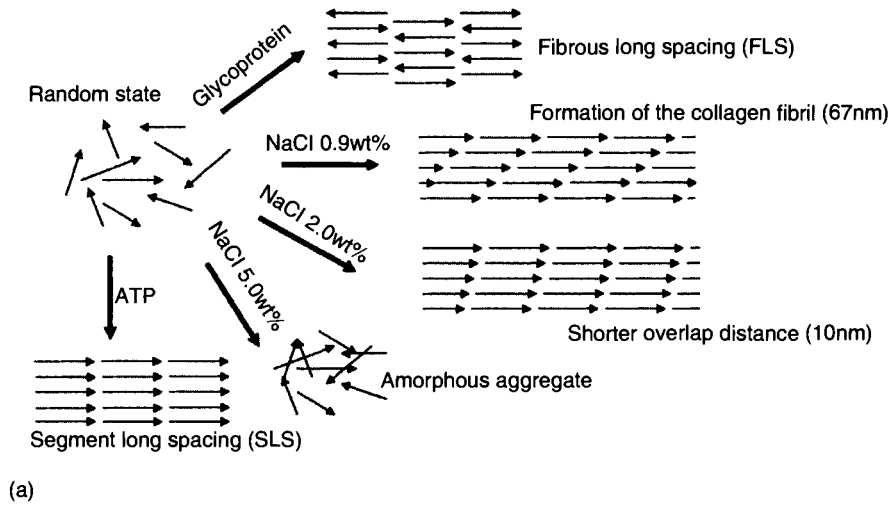
Many articles consider this point and try to create an ECM resembling collagen structure. The ECM structure consists of collagen fibrils with a second component such as GAG or elastin forming collagen fiber complex. The collagen fiber complex is usually aligned and multi-layered. Such structure is very important from the aspect not only of the fate of the cells, but also for mechanical endurance performance against the stress given by the living body. This distinguishes the ECM from the other monolithic structure where single performance is expected. In most cases, a gain of physical properties would result in a loss of desired biological properties. The ECM possesses highly complex structure which is not easy to replicate. However, many trials for the creation of complex structure with controlled physical and biological properties that have been reported on during past decades mainly focused on controlling the ultrastructure, surface topology and composition of the collagen matrix. The next section is divided into four parts: preparation of collagen matrix based on fibrillogenesis, orientation, complex formation and layered structure. We will be discussing the most recent methods for the preparation of collagen matrix focused on these four subjects.

1.3 Processing of Collagen Matrix

1.3.1 Fibrillogenesis

The change of the ionic strength, pH, or hydrophobicity by additives in the aqueous solution may drive the alteration of collagen molecule alignment with certain regularity as shown in Figure 1.2. The alignment of the collagen molecules results in the formation of the fibrils which causes the precipitation (Fig. 1.2b). This is called fibrillogenesis or collagen reconstitution. The Fibrillogenesis is an aggregation of the collagen molecules which is an entropy driven process. The loss of solvent molecules from the surface of protein molecules results in assemblies with a circular cross-section, which minimize the surface area against the volume ratio of final assembly [33]. Hydrophobic residues of collagen (Leu, Ile, Val, Phe and Trp) play the main role in lateral aggregation [34, 35]. The fibrillogenesis occurs in an aqueous condition with a certain amount of salt. It is thought that formation of salt bridges by the salt is also a major driving force for the formation of fibril with certain periodicity [36]. However, it is also argued collagen fibrillogenesis is driven primarily by the formation of hydrogen-bonded water clusters bridging recognition sites on opposing helices, and that and hydrophobic interactions between opposing non-polar amino acid side chains is not a major driving force of collagen self-assembly [37]. However, the fact is that the physiological ionic strength and neutral pH and increasing temperature, induces spontaneous assembly of type I collagen into native-like fibers and hydrophobic interactions, salt bridge and hydrogen-bonded water clusters cannot be ignored.

The preparation of the collagen matrix is based on mixing the collagen aqueous solution with a certain amount of salt to adjust the physiological condition.



(b)

Figure 1.2 The schematic image of fibrillization according to the additives (a) and the photographic images of fibrillogenesis in NaCl aqueous solution and in heparin aqueous solution (b).

The mixture depends on how the collagen matrix is going to be used. For example, when the collagen and cell are mixed together, cell culture medium can be directly put into the container. The resulting material is mainly gel or sponge which has random fibrillized structure. The temperature for the fibrillogenesis is generally 37°C , but diverse temperature can be used if kept under the denaturation temperature. The thickness of collagen fibers can be controlled where the thickness increases in lower temperature [33]. The drawback of producing a gel or sponge with fibrils at 37°C is that the collagen may denature [38, 39]. The cross-linking usually executed for the gel or sponge is mechanically

too weak to support cell growth, proliferation and migration. The most usual phenomenon is the contraction of the matrix caused by the strong cell and matrix surface interaction. However, the cross-linking makes the matrix too brittle [10, 40]. Furthermore, the cross-linked collagen matrix does not show, or at least shows very slow degradation by collagenase [41, 42], which also implies that the possible capsulation *in vivo* might occur. However, a matrix which does not show contraction upon cell culture and slow degradation *in vivo* despite the unuse of cross-linker was successfully created. By trapping the collagen aqueous solution in the dialysis cassette and letting the NaCl diffuse into the collagen dialysis, the collagen aqueous solution turned into fibrillized gel [43]. This gel can be processed into a thin membrane, where the collagen membrane showed a much tougher mechanical strength and slower biodegradation rate *in vivo*. What is interesting is that this matrix showed suppressed inflammatory response *in vivo*. The behavior was almost the same as that of decellularized tissue which makes it possible to claim that the structural aspect is very important.

1.3.2 Orientation

The fibril formation can be obtained by the methods described earlier. But the orientation of the collagen fibrils is another problem. The orientation of the collagen molecules or fibrils is reported to be achieved by applying certain force. That is, if there is a certain driving force which allows the collagen fibrils to align, the collagen matrix with orientation can be obtained. The most commonly used method is flow chamber, which allows the collagen molecules to flow into the chamber and precipitate along the axis of the flow. Lanfer *et al.*, have reported on how shear flow deposition would affect the orientation and density of the fibrils [44]. They have concluded that the degree of collagen fibril orientation increased with increasing flow rates of the solution, while the matrix density increased at higher collagen solution concentrations. The rearrangement of the collagen molecules is also reported to be achieved by controlling the concentration of the collagen solution. This is because the collagen behaves much like a liquid crystal and tends to reorganize in high concentration. The Giraud-Guille research group defined the collagen molecules as spontaneously self-organizing *in vitro* as cholesteric liquid crystal [45, 46]. The fibrillogenesis makes the liquid crystalline phase stable, inducing sol-gel transition. This alignment is especially advantageous for dense collagen, where the direct application for the dermal substitute is expected [47].

An alternative method for collagen alignment is the application of uniaxial elongational strain. This method is advantageous from the aspect that a high orientation percent is obtainable, and direct application on ligaments or tendons is possible. A good example has been reported by Falini *et al.* They reported on applying uniaxial elongational strain and then dehydrating it for 24 hrs [48]. They concluded that the strain of the collagen film or gel would cause the rearrangement of the collagen molecules when higher than 12% of elongational strain is applied and dehydrated. The orientation percent was

approximately 83%, which is very close to the Achilles tendon. However, this method is only applicable to collagen molecules and not to the collagen fibrils. Ross and his group also used an approach for the mechanical strain, but this time they repeated the strain procedure for a longer period of time (2.5% cyclic strain for 2 h per day for 4 days) after seeding the cells [49]. They showed that the alignment of cells along the collagen matrix reflects a response of the cellular environment to the applied strain, concluding that manipulating signal transduction pathways by engineering implantable anterior cruciate ligament grafts or modifying ACL healing response is possible.

Another promising approach is the use of electromagnetic field [50–52]. The collagen molecules tend to align perpendicular to the electromagnetic field upon gelling at above 1T. One great advantage of this method is that a high electromagnetic field can be applied to the collagen aqueous solution containing cells and culture condition. The major target is the regeneration of ECM which requires high tensile strength or the neuron tissue [50, 51]. For these, osteoblast, Schwann cell, glioblastoma cell and erythrocyte were cultured upon the collagen gel in which an electromagnetic field above 8T was applied. Y. Eguchi *et al.* reported that in the mixture of Schwann cells and collagen, Schwann cells oriented in the direction perpendicular to the magnetic field after 2 h of magnetic field exposure. In this case, Schwann cells aligned along the collagen fiber oriented by magnetic fields [52]. This means that the cells and the collagen orientation can be achieved contemporarily. The only problem that remains is how many collagen fibrils would align, because 100% of collagen can not align according to the applied electromagnetic field. Nonetheless, this method remains very promising for tissue engineering.

1.3.3 Complex Formation and Blending

Complex formation of collagen fibrils with a second component is one of the most difficult parts. Complex is mainly executed for the purpose of functionalization. It is generally known that complex formation can be achieved by mixing collagen solution with the second component and cross-link. However, there are some limitations which involve difficulties in collagen molecular control. This is because of the limitation of collagen complex formation with another component. It is known that collagen molecules require hydrogen bond when forming complex with a second component in aqueous solution [53]. Basically, the fibril formation and the complex formation does not occur contemporarily because the second component added to the collagen aqueous solution would function as a defect and prohibit the molecules to aggregate for fibrillogenesis (Figure 1.3). So the complex formation is commonly executed after fibrillogenesis. The most well-known material for collagen complex formation by far is hydroxyapatite, which is designed for bone regeneration. The bone mainly consists of collagen and hydroxyapatite composite where the hydroxyapatite molecules exist between the collagen fibers, providing stiff mechanical strength [54, 55]. The process of collagen-hydroxyapatite composite also targets the creation of such composite structure by diffusing calcium and phosphate ions into

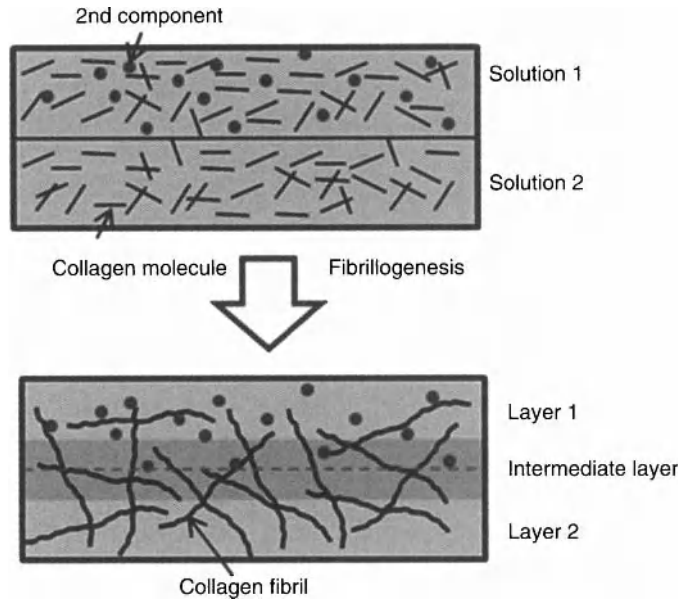


Figure 1.3 The schematic images of layered structure by layer-by-layer deposition.

the collagen fibrils. The process, also called mineralization, is advantageous in the aspect that the collagen orientation may be achieved at the same time. It should be noted that not all collagen-hydroxyapatite composite is composed of collagen fibrils, but it is still one of the most advanced field.

Collagen complex is also executed with synthetic polymers, GAG, proteins or oligo-(poly-) saccharides [9, 14, 56–63]. Fibrillized collagen-hydroxyapatite complex involves the diffusion of calcium and phosphate ions. However, this is not easy when the second component is oligomer or polymer. So, the collagen is not necessarily fibrillized through fibrillogenesis for the complex formation in these cases. Instead, the collagen molecules and the second components are mixed together in certain conditions and chemically cross-linked. This method usually involves a freeze-saw process after mixing two solutions or slurry from a sponge, or absorption of polymer solution into the collagen gel or film. These processes allow the formation of large pores suitable for cell migration and develop into the three-dimensional cell culture. Most often hyaluronic acid is used for the collagen complex, for it is known to enhance the cell migration. Furthermore, the existence of hyaluronic acid may induce the moistening affect, which allows the complex matrix to be applicable for the artificial skin. An alternative method for the collagen complex formation is electrospinning. The electrospinning allows the collagen and second component to form fibril blend [61, 64]. The fibrillized structure provides relatively higher mechanical strength than collagen gel or sponge. Furthermore, various polymers can be applied for the blending and can also be produced in a highly aligned state. However, several researchers argue that although electrospinning is advantageous from the aspect that nanometer scale collagen fibrils can be formed,

the collagen denatures, making it electrospun gelatin fibrils instead [65]. The importance of collagen fibrils possessing regulated *D*-periodicity is also pointed out by some researchers. This is because the lack of *D*-periodicity in the electrospun collagen fibrils may cause diseases such as *osteogenesis imperfecta* and induce cardiovascular disease [2, 66]. The question of using electrospinning for collagen fibrillization is still debated, but diverse blending ability between collagen and the second components, as well as good results of electrospun collagen products *in vivo* cannot be denied, and many products can be found in the market.

One of the most active research areas is collagen-biodegradable polymer complex. Designed to functionalize the collagen matrix, the polymer eventually degrades together with collagen. The favorite polymers are PLLA and PCL which are known for their nontoxicity and good biodegradability [65, 67–70]. The biodegradability is not a mandatory requirement. This sounds like an oxymoron, but the nondegradable polymers of the collagen complex are usually destined for the high mechanical strength. Sionkowska group uses hydrogen bond inducing polymers such as PVA, PVP or PEG to form complex with collagen molecules [53, 71–73]. Biodegradable polymers are usually applied in electrospinning with collagen in organic solvent. Good blending and altering the fibril diameter can be obtained via this method. So far, the electrospinning process is the only confirmed method for producing collagen fibril-polymer complex. The most interesting blend is a collagen-elastin blend designed for blood vessels which showed very good viscoelasticity [74, 75]. However, it should be noted that this is a blend, not a complex, which implies that there is no chemical or physical interaction between the collagen and the elastin. The necessity for the collagen to form complex instead of blend remains a question to be answered in the future.

1.3.4 Layered Structure

One of the most important parts of the native tissue is the layer. The idea of multiple layers was brought up for multi-functionalization. For example, the collagen side possessing different functions on each side can be prepared if such a matrix can be prepared. The best way to prepare a multilayered collagen matrix is to adjoin the collagen matrices using adhesives. However, the adhesive would alter the properties of collagen matrices at the interface of the layers and would consist of pure collagen. So a method for the collagen–collagen integration was investigated. The collagen does not integrate with other collagen once they are in solid form. Furthermore, the collagen matrix with fibrils does not normally absorb the polymer into its matrix. As a result, the collagen–collagen interface with entangled layers with polymer as the intermediate do not form. The immobilization technique is the only method that was actually possible for the collagen layer. The immobilization technique involves cross-linking the collagen.

One simple method is the slow drying process. Nam *et al.* showed that once the collagen matrix with fibrillized structure was formed, the microlayers

formed along an axis perpendicular to the surface by the slow evaporation of water [76]. Unlike the lyophilization or dehydrothermal processes, water is not completely eliminated by dehydration. The fibril rearrangement of collagen fibers by dehydration may induce the stability of the collagen matrix against heat as well as its dimensional stability in water. The dehydration causes the chain rearrangement of the collagen molecules, in which the collagen molecules are brought closer to each other [77, 78]. The stripping of the water bridge, which is connected via hydrogen bonds, occurs contemporarily, but the water bridge itself plays a minor role in the stability of the collagen triple helix, indicating that the air drying process does not cause the denaturation of the collagen matrix [79]. Upon rehydration of water, the water content is approximately 80% and it is no more a matrix with jelly-like property. Similarly, the deprivation of water molecules by compression to form a thick, multilayered structure can be obtained [80]. Both cases result in a collagen matrix with much denser collagen concentration, which possesses viscoelasticity.

The alternative method is the layer-by-layer deposition. This method is advantageous because the collagen aqueous solution is viscose and requires a long time to become a confluent solution when 2 collagen aqueous solutions of different concentrations are deposited on top of one another. The interface between the layers functions as a membrane, allowing water molecules from the sparse collagen layer to move to the dense layer, and the collagen concentration decreases near the interface. Then, the $\text{NaCl}/\text{Na}_2\text{HPO}_4$ salts diffuse into the collagen layers from the bottom, causing fibrillization toward the upper part of the matrix solution [81]. The resulting collagen matrix is one with a multilayered structure without clear boundaries. The cell infiltration differed according to the layers, where the cell infiltration was shown for the less dense side. This method is related to the complex formation as shown in Section 1.3.3 in Figure 1.3.

The integration of collagen matrices with different components was also developed. Tampieri *et al.* have reported that the collagen matrix with different hydroxyapatite concentration at each layer and hyaluronic acid on a specific layer can be prepared [82]. They have developed the layer compatible to cartilage on one side and bone on the other side. This implies that the collagen with different functionality at each side can be prepared. Similarly, Gillette *et al.* have reported the integration of the collagen-based fibril matrix. By increasing the temperature, the collagen solution and the collagen solution with alginate integrate to form a fibrillized collagen matrix with thick interface [83, 84]. This method does not require the layer-by-layer deposition and it shows the importance of collagen–collagen interface, where the actual bonding between the collagen matrices is controlled.

1.4 Conclusions and Future Perspectives

A lot of literature introduces various applications of collagen designed for biomaterials. However, there are not very many successful collagen-based

products that can actually be found in the market. Moreover, scientific information on the physical, chemical and biological aspects of collagen and its behavior upon diverse treatment is still limited. The most difficult problem is lack of control of its physical, chemical and biological behavior. There are many reasons for these problems, but the most critical one is its special structure which is poorly understood. However, collagen still is an important material which challenges researchers. The knowledge about collagen is growing, and there were some very important breakthroughs over the last 10 years. We cannot ignore the good aspects of collagen such as its good biocompatibility and low antigenicity. Also, collagen is soluble in water and possesses functional groups which are relatively easy to chemically or physically modify in an aqueous condition. These benefits will encourage future developments and uses as indicated by the intensification of studies on the utilization of collagen in the growing fields of tissue engineering, regenerative medicine and drug delivery.

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Biological and Medical Significance of Nanodimensional and Nanocrystalline Calcium Orthophosphates

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Abstract

Nano-sized particles and crystals play an important role in the formation of calcified tissues of various animals. For example, nano-sized and nanocrystalline calcium orthophosphates in the form of apatites of biological origin represent the basic inorganic building blocks of bones and teeth of mammals. Namely, according to recent developments in biomineralization, tens to hundreds of nanodimensional crystals of a biological apatite are self-assembled into these complex structures. This process occurs under a strict control by bioorganic matrices. Furthermore, both a greater viability and a better proliferation of various types of cells have been detected on smaller crystals of calcium orthophosphates. Thus, the nano-sized and nanocrystalline forms of calcium orthophosphates have a great potential to revolutionize the hard-tissue engineering field, starting from bone repair and augmentation to controlled drug delivery systems. This chapter reports on current state of the art and recent developments on the subject, starting from synthesis and characterization to biomedical and clinical applications. Furthermore, the chapter also discusses possible directions for future research and development.

Keywords: Calcium orthophosphates, hydroxyapatite, nanodimensional, nano-sized, nanocrystalline, biomedical applications, bone grafts, tissue engineering

2.1 Introduction

Living organisms can create amazing ways to produce various high-performance materials and over 60 different inorganic minerals of biological origin have already been revealed [1]. Among them, calcium orthophosphates are of special importance since they are the most important inorganic constituents of hard tissues in vertebrates [2, 3]. In the form of a poor crystalline, non-stoichiometric, ion-substituted CDHA (commonly referred to as “biological apatite”), calcium orthophosphates are present in bones, teeth, deer antlers

and tendons of mammals to give these organs stability, hardness and function [2, 4, 5]. Though we still do not exactly know why highly intelligent animals use conformable calcium orthophosphates as their crucial biomineral for survival [6], current biomedical questions of persistent pathological and physiological mineralization in the body force people to focus on the processes, including the occurrence, formation and degradation of calcium orthophosphates in living organisms [7, 8, 9].

Biological mineralization (or biomineralization) is a process of *in vivo* formation of inorganic minerals [1, 2]. In the biomineralization processes, organized assemblies of organic macromolecules regulate nucleation, growth, morphology and assembly of inorganic crystals. Biologically formed calcium orthophosphates (biological apatite) are always nanodimensional and nanocrystalline, which have been formed *in vivo* under mild conditions. According to many reports, dimensions of biological apatite in the calcified tissues always possess a range of a few to hundreds of nanometers with the smallest building blocks on the nanometer size scale [2, 4, 5, 10, 11]. For example, tens to hundreds of nanometer-sized apatite crystals in a collagen matrix are combined into self-assembled structures during bone and teeth formation [2, 4, 5]. Recent advances suggest that this is a natural selection, since the nanostructured materials provide a better capability for the specific interactions with proteins [12].

Due to the aforementioned, nanodimensional and nanocrystalline forms of calcium orthophosphates are able to mimic both the composition and dimensions of constituent components of the calcified tissues. Thus, they can be utilized in biomineralization and as biomaterials due to the excellent biocompatibility [13, 14]. Further development of calcium orthophosphate-based biomaterials obviously will stand to benefit mostly from nanotechnology [15], which offers unique approaches to overcome shortcomings of many conventional materials. For example, nano-sized ceramics can exhibit significant ductility before failure contributed by the grain-boundary phase. Namely, already in 1987, Karch *et al.* reported that, with nanodimensional grains, a brittle ceramic could permit a large plastic strain up to 100% [16]. In addition, nanostructured ceramics can be sintered at lower temperatures; thereby major problems associated with a high temperature sintering are also decreased. Thus, nanodimensional and nanocrystalline forms of bioceramics clearly represent a promising class of orthopedic and dental implant formulations with improved biological and biomechanical properties [17].

Many other advances have been made in the biomaterial field due to a rapid growth of nanotechnology [18]. For example, a recent theory of "aggregation-based crystal growth" [19] and a new concept of "mesocrystals" [20, 21] highlighted the roles of nano-sized particles in biological crystal engineering. In this aspect, the study of calcium orthophosphates is a specific area in nanotechnology, because they might be applied readily to repair hard skeletal tissues of mammals [22–24].

Herein, an overview of nanodimensional and nanocrystalline apatites and other calcium orthophosphates in studies on biomineralization and biomaterials is given. The available calcium orthophosphates are listed in Table 2.1. To

narrow the subject of this chapter, with a few important exceptions, undoped and unsubstituted calcium orthophosphates are considered and discussed only. The readers interested in various nanodimensional and nanocrystalline ion-substituted calcium orthophosphates [25–63] are referred to the original publications. Furthermore, details on calcium orthophosphate-based nanodimensional biocomposites [64–85] or nanodimensional calcium orthophosphate-based biocomposites [86–104] are available in references [105, 106].

This chapter is organized into several sections. After a brief introduction (current section), general information on “nano” is provided in Section 2.2. Section 2.3 briefly compares the micron-sized and nanodimensional calcium orthophosphates. Section 2.4 briefly discusses the presence of nano-sized and nanocrystalline calcium orthophosphates in normal calcified tissues of mammals. The structure of nano-sized and nanocrystalline apatites is described in Section 2.5. Synthesis of nanodimensional and nanocrystalline calcium orthophosphates of various dimensions and shapes is reviewed in Section 2.6, while the biomedical applications are examined in Section 2.7. Finally, the summary and reasonable future perspectives in this active research area are given in Section 2.8.

2.2 General Information on “Nano”

The prefix “nano” specifically means a measure of 10^{-9} units. Although it is widely accepted that the prefix nano specifically refers to 10^{-9} units, in the context of nano-sized and nanocrystalline materials, the units should only be those of dimensions, rather than of any other unit of the scientific measurements. Besides, for practical purposes, it appears to be unrealistic to consider the prefix nano to solely and precisely refer to 10^{-9} m, just as it is not considered that “micro” specifically and solely concerns something with a dimension of precisely 10^{-6} m [107]. Currently, there is a general agreement that the subject of nanoscience and nanotechnology started after the famous talk: “There’s plenty of room at the bottom,” given by the Nobel Prize winner in physics Prof. Richard P. Feynman on December 26, 1959 at the annual meeting of the American Physical Society held at California Institute of Technology. This well-known talk has been widely published in various media (e.g., [108]).

In a recent extensive discussion about a framework for definitions presented to the European Commission, the nano-scale has been defined as being of the order of 100 nm or less. Similarly, a nanomaterial has been defined as “any form of a material that is composed of discrete functional parts, many of which have one or more dimensions of the order of 100 nm or less” [109]. Other definitions logically follow this approach such as: a nanocrystalline material is “a material that is comprised of many crystals, the majority of which have one or more dimensions of the order of 100 nm or less” (normally, with presence of neither the micron-sized crystals nor an intergranular amorphous phase), and a nanocomposite is a “multi-phase material in which the majority of the dispersed phase components have one or more dimensions of the order of 100 nm

or less" [107]. Similarly, nanostructured materials are defined as the materials containing structural elements (e.g., clusters, crystallites or molecules) with dimensions in the 1–100 nm range [110], nanocoatings represent individual layers or multilayer surface coatings of 1–100 nm thick, nanopowders are extremely fine powders with an average particle size in the range of 1–100 nm and nanofibers are the fibers with a diameter within 1–100 nm [111, 112]. It also has been proposed to extend the lower size limit to 0.1 nm [113], which would include all existing organic molecules, allowing chemists to rightly claim they have been working on nanotechnology for very many years [114].

Strictly speaking, there are serious doubts that the term "nanomaterial" has a reasonable meaning. For example, let me cite Prof. David F. Williams, the Editor-in-Chief of *Biomaterials*: "... some words which have no rational basis whatsoever become part of everyday language so rapidly, even if so illogically, that it is impossible to reverse the process and their common use has to be accepted, or perhaps, accommodated. Nanomaterial is one such word, where I have argued that it should not exist, but accept that it does through common usage and have to recognise its existence [107]. The discussion about nanomaterial provides a hint of the analysis of a biomaterial that follows, since a prefix, which is an indicator of scale, cannot specify the integer that follows (in this case a material) unless that integer can be qualified by that scale. In other words, it is very clear what a nanometre is because nano means 10^{-9} and a metre is a measure of length. In the case of nanomaterial, what is it about the material that is 10^{-9} . Is it the dimension of a crystal within the material, or of a grain boundary, a domain, or a molecule, or is it a parameter of a surface feature of the sample, or perhaps of the resistivity or thermal conductivity of the material. Clearly this is nonsense, but one has to accept that nanomaterials are here to stay, with even some journal titles containing the word." ([115], p. 5898, left column). Following this logic, such terms as "nanocomposite," "nanocoatings," "nanopowders," "nanofibers" and "nanocrystals" are also senseless and should be replaced, for example, by "composites with nano-sized (or nanodimensional) dispersed phase(s)," "coatings of nano-sized (or nanodimensional) thickness," "nano-sized (or nanodimensional) powders," "fibers of nano-sized (or nanodimensional) thickness" and "nano-sized (or nanodimensional) crystals," respectively. At least this has been done in this chapter.

According to their geometry, all nanodimensional materials can be divided into three major categories: equiaxed, one dimensional (or fibrous) and two dimensional (or lamellar) forms. Selected examples and typical applications of each category of nanodimensional materials and their use in biomedical applications are available in literature [116]. It is important to note, that in literature on calcium orthophosphates there are cases, when the prefix "nano" has been applied for the structures, with the minimum dimensions exceeding 100 nm [44, 83, 117–133].

As a rule, nanodimensional materials can be manufactured from nearly any substance. Of crucial importance, there are two major characteristics conferring the special properties of any nanodimensional material. These are the quantum effects associated with the very small dimensions (currently, this is not applicable

to the biomaterials field) and a large surface-to-volume ratio that is encountered at these dimensions. For instance, specific surface areas for submicron-sized particles are typically 60–80 m²/g, while decreasing particle diameter to tens of nanometers increases the specific surface area up to 5 times more – an amazing amount of surface area per mass! Furthermore, all nanophase materials have unique surface properties, such as an increased number of grain boundaries and defects on the surface, huge surface area and altered electronic structure, if compared to the micron-sized materials [107, 134]. While less than ~ 1% of a micron-sized particle's atoms occupy the surface positions, over a tenth of the atoms in a 10-nm diameter particle reside on its surface and ~ 60% in a 2-nm particle [135]. This very high surface-to-volume ratio of nanodimensional materials provides a tremendous driving force for diffusion, especially at elevated temperatures, as well as causes a self-aggregation into larger particles. Besides, solubility of many substances increases with particle size decreasing [136, 137]. What's more, nanophase materials could have surface features (e.g., a higher amount of nano-scale pores) to influence the type and amount of adsorption of selective proteins that could enhance specific osteoblast adhesion [138]. Finally and yet importantly, the nanodimensional and nanocrystalline materials have different mechanical, electrical, magnetic and optical properties if compared to the larger grained materials of the same chemical composition [139–142].

The nanostructured materials can take the form of powders, dispersions, coatings or bulk materials. In general, nanostructured materials contain a large volume fraction (greater than 50%) of defects such as grain boundaries, inter-phase boundaries and dislocations, which strongly influences their chemical and physical properties. The great advantages of nanostructuring were first understood in the electronic industry with the advent of thin film deposition processes. Other application areas have followed. For example, nanostructured bioceramics were found to improve friction and wear problems associated with joint replacement components because they were tougher and stronger than coarser-grained bioceramics [143]. Furthermore, nanostructuring has allowed chemical homogeneity and structural uniformity to an extent, which was once thought to be impossible to achieve [110]. In calcium orthophosphate bioceramics, the major target of nanostructuring is to mimic the architecture of bones and teeth [144, 145].

2.3 Micron- and Submicron-Sized Calcium Orthophosphates versus the Nanodimensional Ones

The micron-sized calcium orthophosphate-based bioceramic powders suffer from poor sinterability, mainly due to a low surface area (typically 2–5 m²/g), while the specific surface area of nanodimensional calcium orthophosphates exceeds 100 m²/g [146]. In addition, the resorption process of synthetic micron-sized calcium orthophosphates was found to be quite different from that of bone mineral [147].

Although the nanodimensional and nanocrystalline features of natural calcium orthophosphates of bones and teeth had been known earlier [2, 148–153], the history of the systematic investigations of this field was started only in 1994. Namely, a careful search in scientific databases using various combinations of keywords “nano” + “calcium phosphate,” “nano” + “apatite,” “nano” + “hydroxyapatite,” etc., in the article title revealed 5 papers published in 1994 [154–158]. No papers published before 1994 with the aforementioned keywords in the title have been found.

Nanodimensional (size ~ 67 nm) HA was found to have a higher surface roughness of 17 nm if compared to 10 nm for the submicron-sized (~ 180 nm) HA, while the contact angles (a quantitative measure of the wetting of a solid by a liquid) were significantly lower for nano-sized HA (6.1) if compared to the submicron-sized HA (11.51). Additionally, the diameter of individual pores in nanodimensional HA compacts is several times smaller (pore diameter ~ 6.6 Å) than that in the submicron grain-sized HA compacts (pore diameter within 19.8–31.0 Å) [159]. A surface roughness is known to enhance the osteoblast functions while a porous structure improves the osteoinduction compared with smooth surfaces and nonporous structure, respectively [138]. Furthermore, nanophase HA appeared to have $\sim 11\%$ more proteins of fetal bovine serum adsorbed per 1 cm^2 than submicron-sized HA [160]. Interfacial interactions between calcined HA nano-sized crystals and various substrates were studied and the bonding strength appeared to be influenced not only by the nature of functional groups on the substrate but also by matching of surface roughness between the nano-sized crystals and the substrate [161]. More to the point, incorporating nanodimensional particles of HA into polyacrylonitrile fibers was found to result in their crystallinity degree rising by about 5% [162]. In a comparative study on the influence of incorporated micron-sized and nano-sized HA particles into poly-L-lactide matrices, addition of nano-sized HA was found to influence both thermal and dynamic mechanical properties in greater extents [163].

In general, nanostructured biomaterials [164] offer much improved performances over their larger particle-sized counterparts due to their huge surface-to-volume ratio and unusual chemical synergistic effects. Such nanostructured systems constitute a bridge between single molecules and bulk material systems [165]. For instance, powders of nanocrystalline apatites [166–172] and β -TCP [173] were found to exhibit an improved sinterability and enhanced densification due to a greater surface area. This is explained by the fact that the distances of material transport during the sintering becomes shorter for ultra-fine powders with a high specific surface area, resulting in a densification at a low temperature. Therefore, due to low grain growth rates, a low-temperature sintering appears to be effective to produce fine-grained apatite bioceramics [174]. Furthermore, the mechanical properties (namely, hardness and toughness) of HA bioceramics appeared to increase as the grain size decreased from sub-micrometers to nanometers [175].

More to the point, nano-sized HA is also expected to have a better bioactivity than coarser crystals [176–178]. Namely, Kim *et al.* found that osteoblasts

(bone-forming cells) attached to the nano-sized HA/gelatin biocomposites to a significantly higher degree than to micrometer size analog [179]. An increased osteoblast and decreased fibroblast (fibrous tissue-forming cells) adhesion on nanophase ceramics [180–184], as well as on nanocrystalline HA coatings on titanium, if compared to traditionally used plasma-sprayed HA coatings, was also discovered by other researchers [185–187]. Scientists also observed enhanced osteoclast (bone-resorbing cells) functions to show healthy remodeling of bone at the simulated implant surface [177]. Besides, the proliferation and osteogenic differentiation of periodontal ligament cells were found to be promoted when a nanophase HA was used, if compared to dense HA bioceramics [188]. Thus, the underlying material property, responsible for this enhanced osteoblast function, is the surface roughness of the nanostructured surface [18]. Interestingly, an increased osteoblast adhesion was discovered on nano-sized calcium orthophosphate powders with higher Ca/P ratios [189], which points out some advantages of apatites over other calcium orthophosphates. Furthermore, a histological analysis revealed a superior biocompatibility and osteointegration of bone graft substitutes when nano-sized HA was employed in biocomposites [190–192]. However, data are available that nano-sized HA could inhibit growth of osteoblasts in a dose-dependent manner [193]. Furthermore, a cellular activity appeared to be affected by the shape and dimensions of nano-sized HA. Namely, the cellular activity of L929 mouse fibroblasts on nano-sized fibers with a diameter within 50–100 nm was significantly enhanced relative to that on a flat HA surface, while nanodimensional HA needles and sheets with a diameter/thickness of less than 30 nm inhibited cellular adhesion and/or subsequent activity because cells could not form focal adhesions of sufficient size [194].

Obviously, the volume fraction of grain boundaries in nanodimensional calcium orthophosphates is increased significantly leading to improved osteoblast adhesion, proliferation and mineralization. Therefore, a composition of these biomaterials at the nano-scale emulates the bone's hierarchic organization, to initiate the growth of an apatite layer and to allow for the cellular and tissue response of bone remodeling. These examples emphasize that nanophase materials deserve more attention in improving orthopedic implant failure rates. However, to reduce surface energy, all nano-sized materials tend to agglomerate and, to avoid self-aggregation of calcium orthophosphate nano-sized particles [195–198], special precautions might be necessary [54, 60, 120, 199–202].

Finally yet importantly, nano-sized crystals of CDHA obtained by precipitation methods in aqueous solutions were shown to exhibit physico-chemical characteristics rather similar to those of bone apatite [203]. In particular, their chemical composition departs from stoichiometry by calcium and hydroxide ions deficiency, leading to an increased solubility, and in turn bioresorption rate *in vivo* [148, 204–206]. The nano-sized crystals of CDHA also have a property to evolve in solution (maturation) like bone crystals. Namely, freshly precipitated CDHA has been shown to be analogous to embryonic bone mineral crystals whereas aged precipitates resemble bone crystals of old vertebrates [203].

2.4 Nanodimensional and Nanocrystalline Calcium Orthophosphates in Calcified Tissues of Mammals

2.4.1 Bones

Bone is the most typical calcified tissue of mammals and it comes in all sorts of shapes and sizes in order to achieve various functions of protection and mechanical support for the body. The major inorganic component of bone mineral is a biological apatite, which might be defined as a poorly crystalline, non-stoichiometric and ion substituted CDHA [2–5, 204–207]. From the material point of view, bone can be considered as an assembly of distinct levels of seven hierarchical structural units from macro- to micro- and to nano-scale (Figure 2.1) to meet numerous functions [2, 5, 134, 208–210]. Furthermore, all these levels of bones permanently interact with cells and biological macromolecules. At the nanostructural level, tiny plate-like crystals of biological apatite in bone occur within the discrete spaces within the collagen fibrils and grow with specific crystalline orientation along the *c*-axes, which are roughly parallel to the long axes of the collagen fibrils [211]. Type I collagen molecules are self-assembled into fibrils with a periodicity of ~ 67 nm and ~ 40 nm gaps between the ends of their molecules, into which the apatite nano-sized crystals are placed. A biocomposite of these two constituents forms mineralized fibers. The fibers also may be cross-linked, which provides a highly dynamic system capable of modification through the selection of different amino acids to allow for different mechanical properties for different biomaterial applications [212]. This is why bone is usually termed a fiber-reinforced composite of a biological origin, in which nanometer-sized hard inclusions are embedded into a soft protein matrix [213]. Though dimensions of biological apatite crystals reported in literature vary due to different treatment methods and analytical techniques, it is generally around the nanometric level with values in the ranges of 30–50 nm (length), 15–30 nm (width) and 2–10 nm (thickness) [214]. Some details on the stability reasons of nanodimensional apatites in bones are available in literature [215, 216].

Why does the nanometer scale appear to be so important to bones? It was recently demonstrated that natural biocomposites exhibit a generic mechanical structure in which the nanometer sizes of mineral particles are used to ensure the optimum strength and maximum tolerance of flaws [217, 218]. Furthermore, nanodimensional apatite has another crucial function for organisms. It is a huge reservoir of calcium and orthophosphate ions necessary for a wide variety of metabolic functions, which offer or consume calcium and orthophosphate ions through a so-called “remodeling” process because of a continuous resorption and formation of nanodimensional apatite by osteoclasts and osteoblasts, respectively, in a delicate equilibrium [2, 5]. Additional details on the structure, properties and composition of bones might be found in special literature [5, 207, 219].

2.4.2 Teeth

Teeth are another normal calcium orthophosphate-based calcified tissue of vertebrates. Unlike bone, teeth consist of at least two different biominerals: enamel (a crown, the part above the gum line) and dentin (root, the part below the gum line) [220]. Dental enamel contains up to 98% of biological apatite, ~ 1% of bioorganic compounds and up to 2% of water. Typical rods in enamel are composed of rod-like apatite crystals measuring 25–100 nm and an undetermined length of 100 nm to 100 μ m or longer along the *c*-axis [221–223]. However, the apatite crystals in enamel were found to exhibit regular sub-domains or subunits with distinct chemical properties [224]. This subunit structure reflects an assembly mechanism for such biological crystals [225, 226]. Like that for bones (Figure 2.1), seven levels of structural hierarchy have been also discovered in human

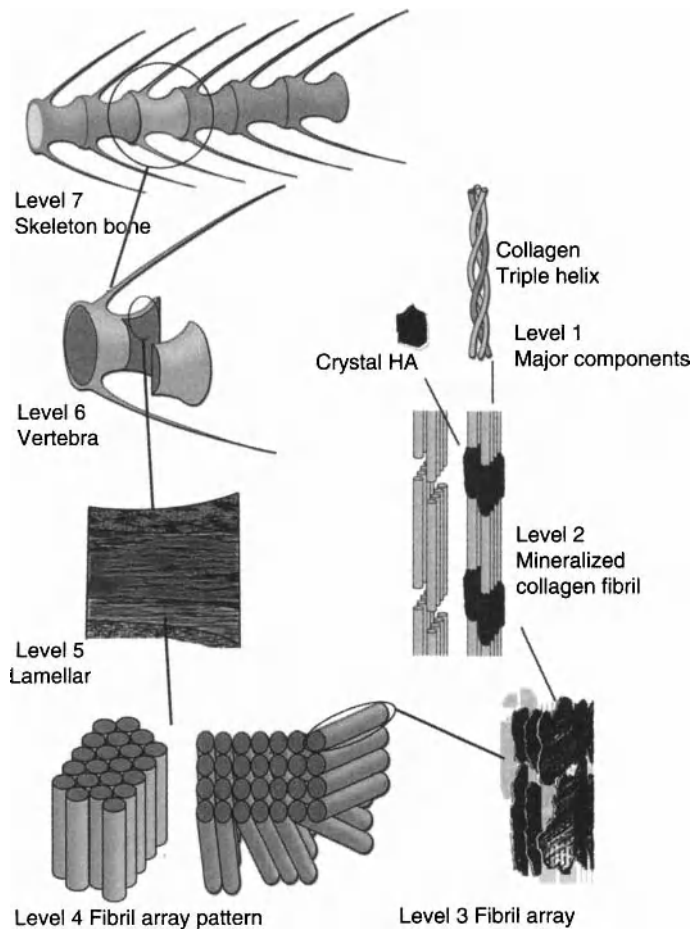


Figure 2.1 The seven hierarchical levels of organization of the zebrafish skeleton bone. Level 1: Isolated crystals and part of a collagen fibril with the triple helix structure. Level 2: Mineralized collagen fibrils. Level 3: The array of mineralized collagen fibrils with a cross-striation periodicity of nearly 60–70 nm. Level 4: Two fibril array patterns of organization as found in the zebrafish skeleton bone. Level 5: The lamellar structure in one vertebra. Level 6: A vertebra. Level 7: Skeleton bone. Reprinted from Ref. [208] with permission.

enamel; moreover, the analysis of the enamel and bone hierarchical structures suggests similarities of the scale distribution at each level [227]. In enamel, nano-sized crystals of biological apatite at first form mineral nanodimensional fibrils; the latter always align lengthways, aggregating into fibrils and afterwards into thicker fibers; further, prism/interprism continua are formed from the fibers. At the micro-scale, prisms are assembled into prism bands, which present different arrangements across the thickness of the enamel layer. These compositional and structural characteristics endow enamel special properties such as anisotropic elastic modulus, effective viscoelastic properties, much higher fracture toughness and stress-strain relationships more similar to metals than ceramics [228].

Dentin contains ~ 50% of biological apatite, ~ 30% of bioorganic compounds and ~ 20% of water. In dentin, the nanodimensional building blocks (~ 25 nm width, ~ 4 nm thickness and ~ 35 nm length) of biological apatite are smaller than those of enamel. Dentin is analogous to bone in many aspects, for example, it has a similar composition and a hierarchical structure up to the level of the bone lamellae [204, 205]. Additional details on the structure, properties and composition of teeth can be found in special literature [229].

2.5 The Structure of the Nanodimensional and Nanocrystalline Apatites

Due to the apatitic structure on natural calcified tissues, apatites appear to be the best investigated compounds among the available calcium orthophosphates (Table 2.1). Thus, nanodimensional and nanocrystalline apatites have been extensively studied by various physico-chemical techniques and chemical analysis methods [197, 230–242] with a special attention to the “nano” effect (i.e., an enhanced contribution of the surface against the volume). Unfortunately, no publications on the structure of other nanodimensional and/or nanocrystalline calcium orthophosphates were found in the available literature.

Due to a nanocrystalline nature, various diffraction techniques have not yet given much information on the fine structural details related to apatite nano-sized crystals (assemblies of nano-sized particles give only broad diffraction patterns, similar to ones from an amorphous material) [230, 231]. Nevertheless, the diffraction studies with electron microprobes of 35 ± 10 nm in diameter clearly indicated a crystalline character of the nano-sized particles in these assemblies. Furthermore, high-resolution transmission electron microscopy results revealed that nano-sized particles of HA behaved like a fine monocrystalline grain structure [197, 230].

Therefore, recent progress on the structure of nanodimensional and nanocrystalline apatites has relied mainly on diverse spectroscopic methods, which are sensitive to disturbances of the closest environments of various ions. Namely, the structure analysis revealed an existence of structural disorder at the particle surface, which was explained by chemical interactions between the orthophosphate groups and either adsorbed water molecules

Table 2.1 Existing calcium orthophosphates and their major properties [204, 205].

Ca/P molar ratio	Compound	Formula	Solubility at 25°C, $-\log(K_s)$	Solubility at 25°C, g/L	pH stability range in aqueous solutions at 25°C
0.5	Monocalcium phosphate monohydrate (MCPM)	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	1.14	~ 18	0.0 – 2.0
0.5	Monocalcium phosphate anhydrous (MCPA or MCP)	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	1.14	~ 17	[c]
1.0	Dicalcium phosphate dihydrate (DCPD), mineral brushite	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	6.59	~ 0.088	2.0 – 6.0
1.0	Dicalcium phosphate anhydrous (DCPA or DCP), mineral monetite	CaHPO_4	6.90	~ 0.048	[c]
1.33	Octacalcium phosphate (OCP)	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	96.6	~ 0.0081	5.5 – 7.0
1.5	α -Tricalcium phosphate (α -TCP)	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	25.5	~ 0.0025	[a]
1.5	β -Tricalcium phosphate (β -TCP)	$\beta\text{-Ca}_3(\text{PO}_4)_2$	28.9	~ 0.0005	[a]
1.2 – 2.2	Amorphous calcium phosphates (ACP)	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$, $n = 3 - 4.5$; $15 - 20\% \text{H}_2\text{O}$	[b]	[b]	~ 5 – 12 [d]
1.5 – 1.67	Calcium-deficient hydroxyapatite (CDHA or Ca-def HA) ^[e]	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ ($0 < x < 1$)	~ 85	~ 0.0094	6.5 – 9.5
1.67	Hydroxyapatite (HA, HAp or OHAp)	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	116.8	~ 0.0003	9.5 – 12
1.67	Fluorapatite (FA or FAp)	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	120.0	~ 0.0002	7 – 12
1.67	Oxyapatite (OA, OAp or OXA) ^[f]	$\text{Ca}_{10}(\text{PO}_4)_6\text{O}$	~ 69	~ 0.087	[a]
2.0	Tetracalcium phosphate (TTCP or TetCP), mineral hilgenstockite	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	38 – 44	~ 0.0007	[a]

^[a] These compounds cannot be precipitated from aqueous solutions.

^[b] Cannot be measured precisely. However, the following values were found: 25.7 ± 0.1 (pH = 7.40), 29.9 ± 0.1 (pH = 6.00), 32.7 ± 0.1 (pH = 5.28) [269]. The comparative extent of dissolution in acidic buffer is: $\text{ACP} \gg \alpha\text{-TCP} > \text{CDHA} \gg \text{HA} > \text{FA}$ [126].

^[c] Stable at temperatures above 100°C.

^[d] Always metastable.

^[e] Occasionally, it is called “precipitated HA (PHA)”.

^[f] Existence of OA remains questionable.

or hydroxyl groups located at the surface of nano-sized apatites [232]. More to the point, infrared (FTIR) spectra of nanocrystalline apatites, in the ν_4 PO_4 domain, revealed the existence of additional bands of orthophosphate ions which could not be assigned to an apatitic environment and which were not present in well-crystallized apatites (Figure 2.2). These bands were assigned to non-apatitic environments of PO_4^{3-} and HPO_4^{2-} ions of the nano-sized crystals. Thus, FTIR spectra can be used to provide a sufficiently accurate evaluation of the amounts of such environments. Furthermore, the non-apatitic environments were found to correspond to hydrated domains of the nano-sized crystals, which were distinct from the apatite domains [234]. Hence, precipitated crystals of nano-sized apatite appeared to have a hydrated surface layer containing labile ionic species, which easily and rapidly could be exchanged by ions and/or macromolecules from the surrounding fluids [233, 232, 241]. For the as-precipitated apatites, such a layer appears to constitute mainly by water molecules coordinated to surface Ca^{2+} ions, approximately in the 1:1 ratio, while the OH groups account only for $\sim 20\%$ of the surface hydration species. The FTIR data indicated that water molecules, located on the surface of nanodimensional apatites, are coordinated to surface cations and experience hydrogen bonding significantly stronger than that in liquid water [240]. The surface hydrated layer is very delicate and becomes progressively transformed into a more stable apatitic lattice upon ageing in aqueous media. Furthermore, it irreversibly altered upon drying [234]. Outgassing at increasing temperatures up to $\sim 300^\circ\text{C}$ resulted in a complete surface dehydration, accompanied by a decrease of the capability to readsorb water. Combination of these data with rehydration tests suggested that a significant part of the surface Ca^{2+} ions, once dehydrated, could undergo a relaxation inward from the surface, more irreversibly as the outgassing temperature increased [239].

In another study, elongated nano-sized crystals of CDHA of ~ 10 nm thick and of ~ 30 – 50 nm length were synthesized followed by investigations with X-ray diffraction and nuclear magnetic resonance techniques. The nano-sized crystals of CDHA were shown to consist of a crystalline core with the composition close to the stoichiometric HA and a disordered (amorphous) surface

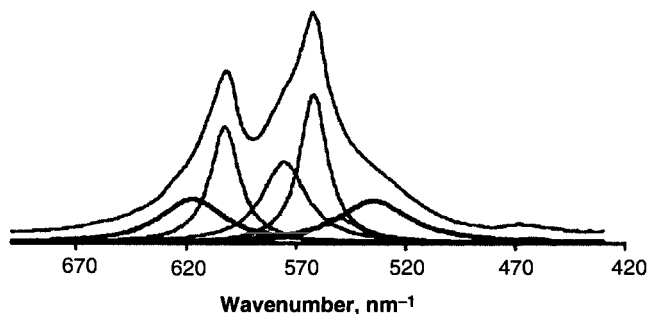


Figure 2.2 FTIR spectra of poorly crystalline apatites showing the non-apatitic environments of the orthophosphate ions (bold lines with peaks at 617 and 534 cm^{-1}) and the apatitic PO_4^{3-} (thin lines with peaks at 600, 575 and 560 cm^{-1}) and HPO_4^{2-} (thin line with peak at 550 cm^{-1}) in the ν_4 PO_4 domain. Reprinted from Ref. [234] with permission.

layer of 1–2 nm thick [238, 239] with the composition close to DCPD [237]. Based on the total Ca/P ratio, on the one hand, and the crystal shape, on the other hand, a thickness of the DCPD surface layer along the main crystal axis was estimated to be ~1 nm [237], which is close to dimensions of the unit-cells (Table 2.2). A similar structure of a crystalline core with the composition

Table 2.2 Crystallographic data of calcium orthophosphates [206].

Compound	Space group	Unit cell parameters	$Z^{[a]}$	Density, g/cm ³
MCPM	triclinic $P\bar{1}$	$a = 5.6261(5)$, $b = 11.889(2)$, $c = 6.4731(8)$ Å, $\alpha = 98.633(6)^\circ$, $\beta = 118.262(6)^\circ$, $\gamma = 83.344(6)^\circ$	2	2.23
MCPA	triclinic $P\bar{1}$	$a = 7.5577(5)$, $b = 8.2531(6)$, $c = 5.5504(3)$ Å, $\alpha = 109.87(1)^\circ$, $\beta = 93.68(1)^\circ$, $\gamma = 109.15(1)^\circ$	2	2.58
DCPD	monoclinic Ia	$a = 5.812(2)$, $b = 15.180(3)$, $c = 6.239(2)$ Å, $\beta = 116.42(3)^\circ$	4	2.32
DCPA	triclinic $P\bar{1}$	$a = 6.910(1)$, $b = 6.627(2)$, $c = 6.998(2)$ Å, $\alpha = 96.34(2)^\circ$, $\beta = 103.82(2)^\circ$, $\gamma = 88.33(2)^\circ$	4	2.89
OCP	triclinic $P\bar{1}$	$a = 19.692(4)$, $b = 9.523(2)$, $c = 6.835(2)$ Å, $\alpha = 90.15(2)^\circ$, $\beta = 92.54(2)^\circ$, $\gamma = 108.65(1)^\circ$	1	2.61
α -TCP	monoclinic $P2_1/a$	$a = 12.887(2)$, $b = 27.280(4)$, $c = 15.219(2)$ Å, $\beta = 126.20(1)^\circ$	24	2.86
β -TCP	rhombohedral $R3cH$	$a = b = 10.4183(5)$, $c = 37.3464(23)$ Å, $\gamma = 120^\circ$	21 ^[b]	3.08
HA	monoclinic $P2_1/b$ or hexagonal $P6_3/m$	$a = 9.84214(8)$, $b = 2a$, $c = 6.8814(7)$ Å, $\gamma = 120^\circ$ (monoclinic)	4	3.16
		$a = b = 9.4302(5)$, $c = 6.8911(2)$ Å, $\gamma = 120^\circ$ (hexagonal)	2	
FA	hexagonal $P6_3/m$	$a = b = 9.367$, $c = 6.884$ Å, $\gamma = 120^\circ$	2	3.20
OA	hexagonal $P\bar{6}$	$a = b = 9.432$, $c = 6.881$ Å, $\alpha = 90.3^\circ$, $\beta = 90.0^\circ$, $\gamma = 119.9^\circ$	1	~ 3.2
TTCP	monoclinic $P2_1$	$a = 7.023(1)$, $b = 11.986(4)$, $c = 9.473(2)$ Å, $\beta = 90.90(1)^\circ$	4	3.05

^[a] Number of formula units per unit cell.

^[b] Per the hexagonal unit cell.

of the stoichiometric HA and a disordered (amorphous) surface layer was found by other researchers [243]; however, in yet another study devoted to nanodimensional carbonateapatites [244], the model of a crystalline core and an outer amorphous layer was not confirmed. Perhaps, this discrepancy could be explained by the presence of carbonates. A lack of hydroxide in nanodimensional apatites was detected; an extreme nanocrystallinity was found to place an upper bound on OH^- possible in apatites [245].

However, it is possible to address the structure of surface terminations of HA nano-sized particles to be amorphous or crystalline by properly selecting the preparation parameters and, in particular, the temperature; thus, nanodimensional HA without the amorphous layer on the surface has been prepared [246]. The two types of surfaces (amorphous or crystalline) of nanodimensional HA appeared to be quite similar in terms of their first hydration layer, as well as Lewis acid strength of exposed Ca^{2+} ions. Both features have a strong dependence on the local structure of surface sites (well probed by small molecules, such as H_2O and CO) that appeared essentially unaffected by the organization at a longer range. But interestingly, once treated at 573 K, the crystalline surfaces of nanodimensional HA was found to adsorb multilayers of water in a larger extent than the amorphous ones [246].

Nevertheless, after summarizing the available data, the following statements on the structure of nano-sized crystals of apatites have been made:

1. They involve non-apatitic anionic and cationic chemical environments (in another study, the researchers mentioned "ordered and disordered HA" [238]);
2. At least part of these environments are located on the surface of the nano-sized crystals and are in strong interaction with hydrated domains;
3. Immature samples show FTIR band fine substructure that is altered upon drying without leading to long-range order (LRO) modifications;
4. This fine substructure shows striking similarities with the FTIR spectrum of OCP [235].

All these elements favor a model in which nano-sized crystals of apatites are covered with a rather fragile but structured surface hydrated layer containing relatively mobile ions (mainly, bivalent anions and cations: Ca^{2+} , HPO_4^{2-} , CO_3^{2-}) in "non-apatitic" sites (Figure 2.3), which is supposed to be of either OCP or DCPD structure. Unfortunately, both the exact structure and the chemical composition of this hydrated layer are still uncertain (regrettably, as the hydrated layer cannot be isolated, it is not possible to standardize the methods for detailed studies) [235, 237–239]. Nevertheless, it is known that the surface layer might adsorb considerable amounts of foreign compounds (molecules and ions) in the percent mass range [247]. Strictly speaking, all the aforementioned apply to both biological apatite of calcified tissues [248] and micron-sized apatites as well [249]; nonetheless, in nano-sized crystals, the

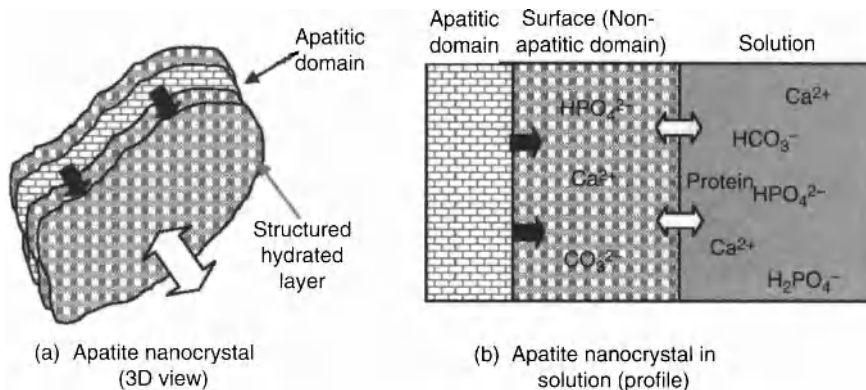


Figure 2.3 A schematic representation of the “surface hydrated layer model” for poorly crystalline apatite nanocrystals. Reprinted from Ref. [235] with permission.

composition of the hydrated surface layer contributes to the global composition for a non-negligible proportion. The results of electron state spectroscopy of nanostructural HA bioceramics are available elsewhere [250, 251].

The hydrated surface layer confers unexpected properties to nano-sized apatite, is responsible for most of the properties of apatites, and, for example, can help to explain the regulation by biological apatites of the concentration in mineral ions in body fluids (homeostasis). These properties are important for living organisms; therefore, they need to be used in both material science and biotechnology [234]. The consideration of this type of surface state can help to understand and explain the behavior of biological apatites in participating in homeostasis due to a very high specific surface area of bone crystals, and in constituting an important ion reservoir with an availability that depends on the maturation state. The important consequences are that the surface of nanodimensional apatites has nothing in common with the bulk composition and that the chemistry of such materials (e.g., binding of protein molecules) must be reconsidered [235, 237]. But interestingly, in response to an electrical potential, the surface of nano-sized HA bioceramics was found to exhibit dynamic changes in interfacial properties, such as wettability. The wettability modification enabled both a sharp switching from hydrophilic to hydrophobic states and a microscopic wettability patterning of the HA surface, which may be used for fabrication of spatially arrayed HA for biological cells immobilization or gene transfer [252].

Furthermore, dry powders of nanodimensional HA were found to contain an X-ray amorphous portion with an unspecified location [253]. After mixing of an initial nano-sized HA powder with a physiological solution (aqueous isotonic 0.9% NaCl solution for injections), this amorphous portion was fully converted into the crystalline phase of HA. The initial crystallite average size (~ 35 nm) was enlarged by a factor of about 4 within the first 100 min after mixing the powder with the physiological solution, and no more structural changes were detected during the following period [253]. In the light of the aforementioned studies, presumably, the discovered X-ray amorphous component of the initial powder was located on the surface of nanodimensional HA.

2.6 Synthesis of the Nanodimensional and Nanocrystalline Calcium Orthophosphates

2.6.1 General Nanotechnological Approaches

The synthesis of nano-scale materials has received considerable attention and their novel properties can find numerous applications, for example, in the biomedical field. This has encouraged the invention of chemical, physical and biomimetic methods by which such nano-sized materials can be obtained [134]. Generally, all approaches for preparation of nanodimensional and nanocrystalline materials can be categorized as “bottom-up” and “top-down” ones [142, 254]. The bottom-up approach refers to the build up of a material from the bottom, i.e., atom by atom, molecule by molecule or cluster by cluster, and then assembles them into the final nanostructured material. An example is production of a nano-sized powder and its compaction into the final product (e.g., hot-pressed or sintered nanostructured ceramics). The top-down approach starts from a bulk material and then, via different dimension decreasing techniques, such as milling, slicing or successive cutting, leads to the formation of nanodimensional materials [134]. Using this approach, a novel 2-dimensional carbon material graphene of just 1 atom thick has been prepared from bulk graphite. Furthermore, environmentally friendly methodologies of nanostructure synthesis have been summarized into a special review [255].

Concerning calcium orthophosphates, presumably, all of them (see Table 2.1) might be manufactured in a nanodimensional and/or a nanocrystalline state; however, not all of them (especially those with low Ca/P ionic ratios) have been prepared yet. The details on the available preparation techniques are given below.

2.6.2 Nanodimensional and Nanocrystalline Apatites

First of all, one should stress that the stoichiometric HA with well resolved X-ray diffraction patterns might be prepared mostly at temperatures exceeding $\sim 700^{\circ}\text{C}$ either by calcining of CDHA with the Ca/P molar ratio very close to 1.67 or by solid-state reactions of other calcium orthophosphates with various chemicals (e.g., DCPA + CaO). Thus, with the exception of a hydrothermal synthesis [256–258], in aqueous solutions only CDHA might be prepared [148, 204–206, 259–263]. As apatites (CDHA, HA and FA) belong to the sparingly soluble compounds (Table 2.1), simple mixing of calcium- and orthophosphate-containing aqueous solutions at $\text{pH} > 9$ results in formation of extremely supersaturated solutions and, therefore, a very fast precipitation of tremendous amounts of very fine crystals [264], initially of ACP, which are afterwards re-crystallized into apatites [204–206, 265–268]. The dimensions of the precipitated nano-sized crystals might be slightly increased by the Ostwald ripening approach (maturation), that is, by boiling and/or ambient aging in the mother liquid (Figure 2.4) [156, 169, 203, 235, 257, 267, 272]. Heat treatment of ACP might be applied as well [273]. Therefore, preparation of nanodimensional



Figure 2.4 Variation of nanocrystalline apatite dimensions with maturation time. Reprinted from Ref. [235] with permission.

and/or nanocrystalline apatites is not a problem at all and has been known for many years [156, 157, 274–276]; however, the prefix “nano” had not been used before 1994. On the contrary, with the exception of a thermally stable FA (thus, big crystals of FA might be produced by a melt-growth process [277, 278]), manufacturing of big crystals of both CDHA and HA still is a challenge.

Many different methodologies have been proposed to prepare nanodimensional and/or nanocrystalline structures [279–286]. Prior to describing them, it is important to stress that in the vast majority of the available literature on apatites, the authors do not tell the difference between CDHA and HA. Therefore, getting through scientific papers, an attentive reader often finds statements, such as: “Because natural bone is composed of both organic components (mainly type I collagen) and inorganic components (HA) ...” [116, p. 357], “The HA nanorods are synthesized via a wet precipitation process ...” [167, p. 2364], “... (TTCP) has been shown previously to be an essential component of self-setting calcium phosphate cements that form hydroxyapatite (HA) as the only end-product. ...” [287, abstract], etc. The matter of distinguishing between CDHA and HA becomes even much more complicated, when researchers deal with nanodimensional and/or nanocrystalline apatites because the assemblies of nano-sized particles give only broad diffraction patterns, similar to ones from an amorphous material [230, 231]. While composing this review, I always tried to specify whether each cited study dealt with CDHA or HA; unfortunately, the necessary data were found in just a few papers. Therefore, in many cases, I was forced to mention just “apatites” without a further clarification. Thus, the readers are requested to be understanding of this uncertainty.

The greater part of the published reports on synthesizing of nanodimensional and/or nanocrystalline apatites is focused on the bottom-up approach. Among the available preparation techniques, a wet chemical precipitation is the most popular one [74, 86, 88, 103, 119, 121, 167–169, 172, 178, 201, 203, 265, 274, 288–332]. Various authors discussed the effects of synthesis parameters, such as temperature [300–303, 322], time [301], calcium ion concentration [303], presents of surfactants [306–308], calcination [301] and the use of different reagents on the morphological properties of nanodimensional apatites. In general, the shape, stoichiometry, dimensions and specific surface area of

nano-sized apatites appeared to be very sensitive to both the reaction temperature (Figure 2.5) and the reactant addition rate [300, 315, 322]. Namely, particle sizes of nanodimensional apatites were observed to increase in a linear correlation with temperature [302, 322], which is a good indication that sizes of nanodimensional apatites can possibly be tailored. Furthermore, the initial pH values and reaction temperatures both play important roles in the morphology of the precipitated apatites, as well as on the phase formation and degree of crystallinity [326]. For example, significant differences in the chemical composition, morphology and amorphous character of nano-sized CDHA produced through the reaction between aqueous solutions of $\text{Ca}(\text{NO}_3)_2$ and $(\text{NH}_4)_2\text{HPO}_4$ can be induced, simply by changing the pH of the reactant hydrogen phosphate solution [327]. Thus, the solvent systems, dispersant species and drying methods appear to have effects on the particle size and dispersibility. However, some conflicting results have been obtained on how certain synthesis parameters can affect the morphological properties of these nano-sized particles. Nevertheless, it was commonly observed that nano-sized crystals of apatites synthesized through the chemical precipitation were often highly agglomerated; however, these agglomerates could be clusters of ultra-fine primary particles [304]. The prepared nanodimensional apatites might be consolidated to transparent bioceramics [330].

A hydrothermal synthesis [69, 72, 156, 157, 257, 258, 288, 322, 323, 333–357] seems to be the second most popular preparation technique of the nanodimensional and/or nanocrystalline apatites. The term “hydrothermal” refers to a chemical reaction of substances in a sealed heated solution above ambient

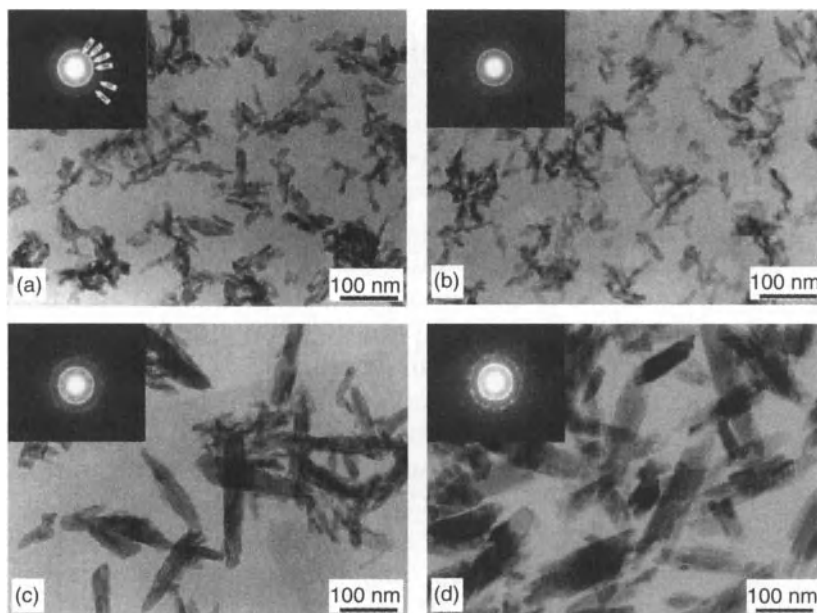


Figure 2.5 The influence of the reaction temperature on the crystal dimensions of precipitated CDHA: (a) 25°C, (b) 37°C, (c) 55°C, (d) 75°C.

temperature and pressure [358]; and this process allows synthesis of highly pure fine-grained single crystals, with controlled morphology and narrow size distribution [333]. Extraneous additives, such as EDTA [351], surfactants [352, 359], anionic starburst dendrimer [353] etc., might be utilized to modify the morphology of nanodimensional and/or nanocrystalline apatites during the synthesis. Most of these techniques produced rod-like crystals or whiskers, while plate-like shapes were obtained in just a few studies [335, 345, 347].

Other preparation methods of nanodimensional and/or nanocrystalline apatites of various states, shapes and sizes include sol-gel [30, 188, 231, 232, 270, 328, 360–376], co-precipitation [271, 333, 334, 377–380], mechanochemical approach [65, 250, 343, 348, 381–387], mechanical alloying [388, 389], ball milling [348, 383, 390–392], radio frequency induction plasma [393, 394], vibromilling of bones [395], flame spray pyrolysis [396], liquid-solid-solution synthesis [397], electro-crystallization [158, 398, 399], electrochemical deposition [400], microwave processing [32, 69, 288, 333, 334, 342, 356, 401–415], hydrolysis of other calcium orthophosphates [416–418], double step stirring [419], emulsion-based [310, 349, 420–433], steam-assistant [434], sonochemical [435] and solvothermal [436] syntheses. However, still other preparation techniques are also known [31, 45, 147, 154, 275, 355, 437–457]. Continuous preparation procedures are also available [200, 458]. Application of both ultrasound [362, 459–461] and viscous systems [462] might be helpful. Furthermore, nanodimensional HA might be manufactured by a laser-induced fragmentation of HA targets in water [463–467] and in solvent-containing aqueous solutions [344, 371, 468], while dense nanocrystalline HA films might be produced by radio frequency magnetron sputtering [469, 470]. An interesting approach using the sitting drop vapor diffusion technique should be mentioned as well [471]. A comparison between the sol-gel synthesis and wet chemical precipitation technique was performed and both methods appeared to be suitable for synthesis of nanodimensional apatite [328]. By means of these methods, a variety of nanodimensional calcium orthophosphate building blocks with various structures and morphologies have been synthesized, including needle-like, spherical, fibrous and mesoporous nano-sized crystals, as well as nano-sized rods, hollow spheres, layered structures and flowers as shown in Figure 2.6 [452].

Table 2.3 presents some data on the chronological development of synthesis of nanodimensional apatites for the period of 1995–2004 [147]. Among the methods described, the thinnest crystals of apatite ($60\text{ nm} \times 15\text{ nm} \times 0.69$ or 0.84 nm) have been prepared by Melikhov *et al.* any have been called “two dimensional crystalline HA” [296]; while the smallest ones (size between 2.1 and 2.3 nm, i.e., around two times the HA unit cell parameters) have been found by Biggemann *et al.* [197]. Liu *et al.* [477, 478] and Han *et al.* [476] synthesized nano-sized HA via a template mediated and a non-template mediated sol-gel techniques, respectively. Both triethylphosphate [477, 478] and other alkylphosphates [122] might be used to produce nanocrystalline apatites. Besides, nanodimensional ion-substituted CDHA might be precipitated from both a synthetic [378] and a simulated [479] body fluids. A relatively simple sol-gel process using ethanol and/or water as a solvent has

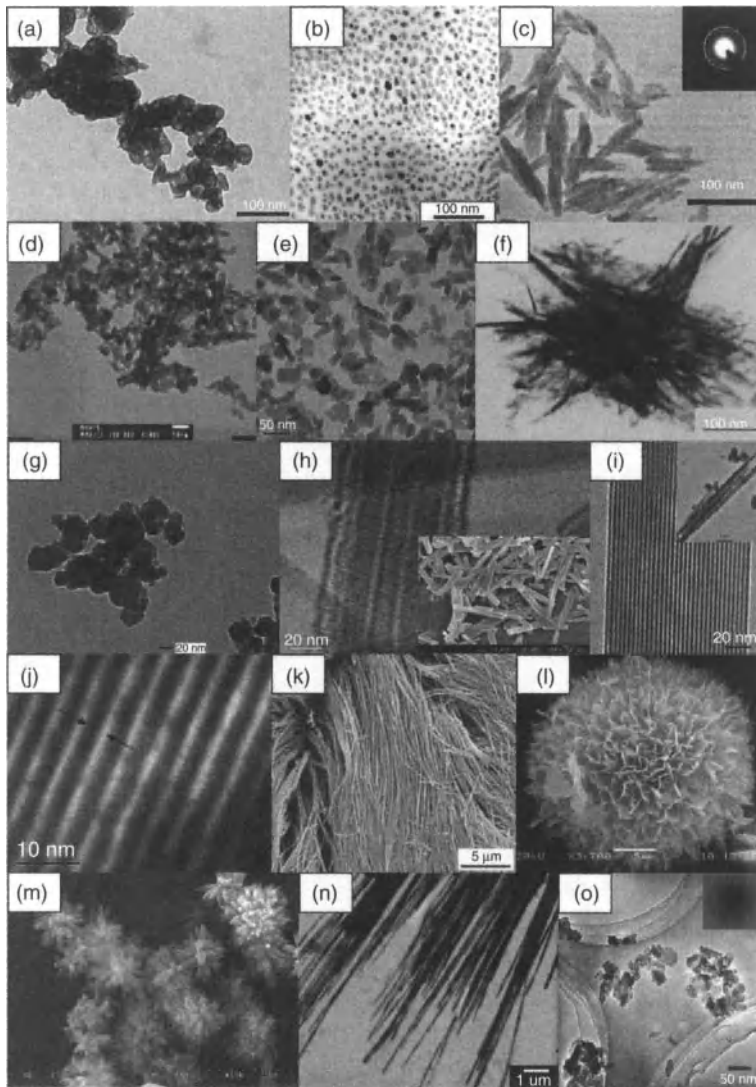


Figure 2.6 A variety of nano-scale calcium orthophosphates with different structures and morphologies synthesized by: (A and B) sol-gel processing, (C) co-precipitation, (D) emulsion technique, (E) hydrothermal process, (F) ultrasonic technique, (G) mechano-chemical method, (H–L) template method, (M) microwave processing, (N) emulsion-hydrothermal combination, (O) microwave-hydrothermal combination. Reprinted from Ref. [452] with permission.

also been reported to obtain the stoichiometric, nanocrystalline single phase HA [371].

Nanocrystalline HA powder was synthesized at a low calcination temperature of 750°C by the citric acid sol-gel combustion method [476]. The attractive features of this method were to synthesize materials with a high purity, a better homogeneity and a high surface area in a single step [476, 480]. An array of highly ordered HA nano-sized tubes of uniform length and diameter was synthesized by sol-gel auto-combustion method with porous anodic

Table 2.3 Synthesis of nanodimensional apatites – a chronological development [147].

Year	Process	Reference
1995	Synthesis of nanocrystalline HA (particle size ~ 20 nm) for the first time using calcium nitrate and diammonium hydrogen orthophosphate as precursors by solution spray dry method.	[472]
2000	Synthesis of biomimetic nanosized CDHA powders (~ 50 nm) at 37 °C and pH of 7.4 from calcium nitrate tetrahydrate and diammonium hydrogen orthophosphate salts in synthetic body fluid using a novel chemical precipitation technique.	[378]
2002	Preparation of nanosized HA particles and HA/chitosan nanocomposite.	[473]
2002	Direct precipitation from dilute calcium chloride and sodium orthophosphate solutions.	[474]
2003	Radio frequency plasma spray process employing fine spray dried HA powders (average size ~ 15 µm) as a feedstock.	[393]
2003	Sol-gel process using equimolar solutions of calcium nitrate and diammonium hydrogen orthophosphate dissolved in ethanol.	[371]
2003	Chemical precipitation through aqueous solutions of calcium chloride and ammonium hydrogen orthophosphate.	[301]
2003	Mechanochemical synthesis of nanosized HA and β -TCP powders using DCPD and CaO as starting materials.	[384]
2003	Synthesis of nano-powders via sucrose-templated sol-gel method using calcium nitrate and diammonium hydrogen orthophosphate as precursor chemicals.	[475]
2004	Hydrolysis method of DCPD and CaCO_3 by 2.5 M NaOH (aq).	[416]
2004	Citric acid sol-gel combustion process using calcium nitrate tetrahydrate, diammonium hydrogen orthophosphate and citric acid.	[476]

aluminum oxide template [370]. Varma *et al.* synthesized nano-sized HA by polymeric combustion method and self-propagating combustion synthesis by using novel body fluid solutions [481]. In another study, nanodimensional HA was synthesized by combustion in the aqueous system containing calcium

nitrate + diammonium hydrogen orthophosphate with urea and glycine as fuels [482]. Furthermore, nano-sized particles of both FA and β -TCP might be synthesized by a simultaneous combustion of calcium carboxylate and tributylphosphate based precursors in a flame spray reactor [483]. Both a flame-based technique [484] and a spray drying approach [125, 485] might be applied as well. Furthermore, crystalline and phase pure nano-sized HA and CDHA were synthesized in a continuous hydrothermal flow system using supercritical water at $t < 400^\circ\text{C}$ and 24 MPa pressure [338].

Nanodimensional powders of the stoichiometric HA of ~ 20 nm particle size were synthesized by hydrolysis of a mixture of DCPD and CaCO_3 performed with 2.5 M aqueous solution of NaOH at 75°C for 1 h. The only product synthesized was nanocrystalline HA and its crystallinity was improved with increasing annealing temperature [416]. Similar results were obtained in other studies [417, 418]. Furthermore, Xu *et al.* used radio frequency plasma spray process to synthesize nanodimensional HA powders with particle size in the range of 10–100 nm [393]. Kuriakose *et al.* synthesized nanocrystalline HA of size ~ 1.3 nm that was thermally stable until 1200°C [371]. Nanocrystalline plate-shaped particles of HA were directly precipitated at ambient temperature and pH ~ 7.4 from dilute aqueous solutions of calcium chloride and sodium orthophosphate. The direct precipitation of nano-sized HA was achieved by submitting the aqueous suspension to microwave irradiation immediately after mixing [474]. A simple and easy approach for synthesizing thermally stable nanostructured stoichiometric HA powder under invariant pH conditions of 7.5, known as the NanoCaP process, was developed. Under these conditions, the synthesized HA not only remained in the nanostructured state but also did not exhibit any compositional fluctuations that were observed in conventional approaches for synthesizing HA [12]. Other preparation techniques of nano-sized apatite might be found elsewhere [275]. Bulk bioceramics made of nanocrystalline HA with a grain size of no more than 50 nm and a near-theoretical density might be prepared by application of a high (~ 3.5 GPa) pressure in uniaxial compaction of nanodimensional powders with subsequent sintering at 640°C [168]. A similar approach was reported by another research group [406].

Mechanochemical processing is another compelling method to produce nanostructured apatites in the solid state [65, 238, 343, 381–386]. For example, Yeong *et al.* used the appropriate amounts of DCPA and calcium oxide. The initial stage of mechanical activation resulted in a significant refinement in crystallite and particle sizes, together with a degree of amorphization in the starting powder mixture. This was followed by steady formation and subsequent growth of HA crystallites with increasing degree of mechanical activation. Finally, a single-phase HA of an average particle size of ~ 25 nm, a specific surface area of ~ 76 m²/g and a high crystallinity was attained after 20 h of mechanical activation [382].

The use of macromolecules as templating agents to manipulate the growth of inorganic crystals has been realized in many biological systems. Namely, in the presence of biological macromolecules (such as collagen), nucleation and growth of nanocrystalline apatite to form highly organized bone minerals is

one of the most fascinating processes in nature. These processes might be simulated. For example, layers of nanocrystalline apatite were formed *in situ* on the surface of various filmt by soaking them in aqueous solutions containing ions of calcium and orthophosphate. The *in situ* synthesized particles were found to be less agglomerated which was believed to be the result of nucleation of apatite crystallites on the regularly arranged side groups located on polymer chains [486, 487]. Another approach comprises precipitation of nanodimensional apatites from aqueous solutions in the presence of dissolved high molecular weight polyacrylic acid [488, 489] that acts as an inhibitor for the crystallization of apatite crystals [490, 491]. A similar inhibiting effect was found for dimethyl acetamide [492], polyvinyl alcohol [291] and several other (bio)polymers [493, 494]. This type of synthesis is expected to lead to the formation of nanodimensional composites, which might be structurally more comparable to bones with closely related mechanical and biological properties. Furthermore, a control of particle size of aqueous colloids of apatite nano-sized particles was described involving a presence of amino acids [495, 496]. The amino acids ensured effective growth inhibition by a predominant adsorption onto the Ca-rich surfaces during the initial stages of crystallization. Thus, the nano-sized particles were formed by an oriented aggregation of primary crystallite domains along the *c*-axis direction. The size of the domains was shown to be governed by the interactions with the amino acid additives, which restricted a growth of the primary crystallites [495, 496]. Furthermore, nanodimensional apatites might be precipitated from aqueous solutions of gelatin [70, 497]. The development of nano-sized apatite in aqueous gelatin solutions was highly influenced by the concentration of gelatin: namely, a higher concentration of gelatin induced formation of tiny (4 nm × 9 nm) nano-sized crystals, while a lower concentration of gelatin contributed to the development of bigger (30 nm × 70 nm) nano-sized crystals. In this experiment, a higher concentration of gelatin supplied abundant reaction sites containing groups such as carboxyl, which could bind with calcium ions. Thitled to the formation of a very large number of nuclei and creation of a large number of tiny nano-sized crystals [70].

Although each of the reported approaches to produce nanodimensional apatites has both a scientific and a practical relevance, a little attention has been dedicated to the physicochemical details involved in the careful control of the particle size distribution and particle shape. Indeed, in the case of particle size distribution, most of the reported ways to synthesize nanodimensional apatites really produced a particle mixture with a wide size distribution from tens to hundreds of nanometers. Moreover, the control of particle shape is another problem for these methods, which commonly result in pin-like or irregular particles. It is well known that bone consists of homogeneous plate-like crystals of biological apatite of 15–30 nm wide and 30–50 nm long, while enamel consists of rod-like crystals of biological apatite of 25–100 nm thick and lengths of 100 nm to microns (Figure 2.7) [2, 5, 207, 208, 210, 219, 227, 229]. The study of higher-level biomineralization and biomimetic assembly involves a search for advanced methods so that the synthesis of nano-sized apatite can be accurately controlled [498]. Namely, the size-controlled synthesis of materials can



Figure 2.7 Scanning electron micrograph of the forming enamel of a continuously growing rat incisor showing ordered rods of calcium orthophosphates. Scale bar: 10 μm . Reprinted from Ref. [2] with permission.

be achieved by using limited reaction spaces. For example, microemulsions have been shown to be one of the few techniques, which is able to produce particle sizes in the range of nanometers and with minimum agglomeration [499]. Thus, microemulsions [349, 423–433], micelles [500] and reverse (inverse) micelles [339, 501–504] have been successfully applied to synthesize nanodimensional apatites with minimal agglomeration. It was found that experimental conditions, such as aqueous/organic phase volume ratio, pH, aging time, aging temperature and ion concentration in the aqueous phase can affect the crystalline phase, surface area, particle size and morphology of nanodimensional apatites.

In some cases, special polymers can be used as spatial reaction vessels for fabrication of CDHA. For example, Shchukin *et al.*, employed a poly(allylamine hydrochloride)/ PO_4^{3-} complex as a source of orthophosphate anions to capture calcium cations and make them react in the capsule volume [505]. Bose and Saha synthesized spherical-like nanocrystalline CDHA powder with particle diameters of ~ 30 and ~ 50 nm using the emulsion route [425]. Furthermore, nano-sized crystals of apatite might be aggregated into microspheres [438, 506]. Hexadecyl (cetyl)trimethylammonium bromide (CTAB) was selected as an efficient agent to modulate the formation of CDHA nano-sized particles [501, 502]. The particle size can be regulated feasibly by changing the concentration of CTAB in the supersaturated by calcium orthophosphates solutions. For example, three different types of spherical particles of nano-sized CDHA with average diameters of 20 ± 5 , 40 ± 10 and 80 ± 12 nm were fabricated using a series of CTAB concentrations to control the particle size. The experimental results revealed that the dimensions of the prepared nano-sized CDHA were relatively uniform. In contrast, nano-sized CDHA grown in the absence of organic additives are typical, rod-like particles with lengths of hundreds of nanometers and width of tens of nanometers [507]. Colloidal formulations are known as well [54, 60, 202] But interestingly, nano-sized apatites might perform crystalline to amorphous phase transformation when powders were aged for 5 months in 30% relative humidity [508].

To conclude this part, the nano-sized particles of apatite might be functionalized and/or doped by various compounds (even by quantum dots [509, 510])

to provide new important properties [495, 511–518], e.g., fluorescence [27–29, 515, 516] and luminescence [54, 60, 510, 517, 518]. Both fluorescence and luminescence can be used as a tracking property for the nano-sized particles to give an observable indication of agent delivery, while the particles are served to protect the agent *in vivo* until it has reached the destination.

2.6.3 Nanodimensional and Nanocrystalline TCP

Many researchers have formulated synthesis of nanodimensional β -TCP. For example, Bow *et al.* synthesized β -TCP powders of ~ 50 nm particle diameter at room temperature in anhydrous methanol as a solvent [519]. With increase in aging time, the phase transformation was found to take place from initial DCPA, to intermediate ACP phases, then to final β -TCP. The authors observed that incorporation of carbonates helped in suppressing formation of ACP phases with apatitic structure and its transformation into poorly crystalline (almost amorphous) CDHA, and favored the formation of β -TCP phase [519]. Nano-sized particles of both FA and β -TCP were synthesized by a simultaneous combustion of calcium carboxylate and tributyl-phosphate based precursors in a flame spray reactor [483]. The same technique was used to synthesize nano-sized particles of amorphous TCP of 25–60 nm size [520–523] after calcinations transformed them into α -TCP or β -TCP. Nanodimensional β -TCP powders with an average grain size of ~ 100 nm [173, 524] and less [525] were prepared by wet precipitation methods, followed by calcining at elevated temperatures. Furthermore, a sol-gel technique [526], reverse micelle-mediated synthesis [527] and a polystyrene template method [528] are also applicable. In wet precipitation techniques, dialysis might be applied as a separation method [524]. When wet precipitation methods were used, initially nanodimensional CDHA with Ca/P ratio of ~ 1.50 was precipitated, that was transformed into nano-sized β -TCP at calcination.

To synthesize nano-sized TCP, other techniques, such as milling [529, 530], a high temperature flame spray pyrolysis [531] and pulsed laser ablation [532], might be employed as well. Afterwards, the nanodimensional β -TCP powders can be compacted into 3D specimens, followed by sintering to achieve the appropriate mechanical strength [173]. The maximal values of the bending strength, elastic modulus, Vickers hardness and compressive strength of the samples fabricated from nano-sized β -TCP powders were more than two-times higher as compared to those of bioceramics obtained from micron-sized β -TCP powders. However, the degradability of bioceramics sintered from nanodimensional powders was just about one fourth of that sintered from micron-sized powders. Thus, the degradability of β -TCP bioceramics could be additionally regulated by the particle dimensions [173].

Nano-sized whiskers of several calcium orthophosphates (HA, β -TCP and biphasic calcium phosphate BCP (HA + β -TCP)) were produced by using a novel microwave-assisted “combustion synthesis (auto ignition)/molten salt synthesis” hybrid route. Aqueous solutions containing NaNO_3 , $\text{Ca}(\text{NO}_3)_2$ and

KH_2PO_4 (with or without urea) were irradiated in a household microwave oven for 5 min at 600 watts of power. The as-synthesized precursors were then simply stirred in water at room temperature for 1 h to obtain the nano-sized whiskers of the desired calcium orthophosphate [533]. Furthermore, nano-structured biphasic (HA + β -TCP) bioceramics was successfully prepared by microwave synthesis [534, 535] and a polymer matrix mediated process [536] in other studies. Good cellular activities of the biphasic bioceramics have been reported.

Layrolle and Lebugle developed a synthesis route of nano-sized FA and other calcium orthophosphates, using calcium diethoxide ($\text{Ca}(\text{OEt})_2$) and H_3PO_4 [154] (+ NH_4F to prepare FA [537]) as the initial reagents and anhydrous ethanol as a solvent. By a simple variance of the ratio of reagents, calcium orthophosphates of various chemical compositions were precipitated in ethanol. The precipitates were characterized and the results indicated that those calcium orthophosphates were amorphous and nanodimensional. Furthermore, they had large specific surface areas and possessed a high reactivity [154, 537].

2.6.4 Other Nanodimensional and Nanocrystalline Calcium Orthophosphates

Nano-sized particles of DCPD (with some amount of CDHA and ACP) of a relatively high monodispersity could be synthesized from aqueous solutions of calcium nitrate and H_3PO_4 in the presence of 2-carboxyethylphosphonic acid. They are produced in a discoid shape with a diameter of 30–80 nm and a height of less than ~ 5 nm. They form stable colloidal solutions displaying minimal agglomeration [538]. Nano-sized rods and nanodimensional fibers of DCPD with average diameters of 25 ± 5 nm (aspect ratio ~ 6) and 76 ± 20 nm (aspect ratio ~ 40), respectively, were synthesized by sucrose-ester-based reverse microemulsion technique [539]. A similar approach was used in another study [431]. Nanodimensional crystals of both DCPD and DCPA were prepared by EDTA-assisted hydrothermal method [351]. An interesting approach comprises precipitation of calcium orthophosphates inside nano-sized pores of another material. For example, nanodimensional clusters DCPD were immobilized into pores of an oxide network by immersion of this network into an acidic ($\text{pH} = 2.7$) calcium orthophosphate solution at 50°C [540]. The acid-base reaction between the calcium orthophosphate solution and the hydroxyl groups of the oxide network resulted in formation of nanodimensional clusters of DCPD immobilized inside the oxide pores. But interestingly, the immobilized nanodimensional clusters of DCPD were further converted into those of ACP and CDHA by supplementary treatment of the oxide network in alkaline solutions [540]. Hollow nano-sized shells of undisclosed calcium orthophosphates (presumably, of ACP) with a size distribution of $(120\text{--}185) \pm 50$ nm and predictable mean shell thickness from 10–40 nm were prepared by crystallization onto the surface of nanodimensional liposomes [541, 542]. Both the suspension stability and shell thickness control were achieved through the introduction of carboxyethylphosphoric acid. Variation of shell thickness and stoichiometry may be

a way of manipulating the dissolution kinetics of ACP coating to control the release of encapsulated materials necessary for drug delivery purposes [541, 542]. Other types of calcium orthophosphate shells with Ca/P ratios of 0.97 (DCPD or DCPD-like ACP) and 1.45 (CDHA or ACP) were prepared using liposome templates [543]. Roughly spherical DCPA particles of approx. 50–100 nm in sizes were synthesized via a spray-drying technique [125, 544–546], while ribbon-like fibers of nano-sized DCPA might be prepared upon hydrolysis in urea [417]. Furthermore, nanodimensional calcium orthophosphate powders with DCPD as the major phase have been synthesized by an inverse microemulsion system using kerosene as the oil phase, a cationic surfactant and a non-ionic surfactant [547]. Microskeletal constructions might be synthesized as well [548].

When it comes to ACP, it is nanodimensional in the vast majority of cases. Approximately spherical nano-sized particles of ACP with a diameter of about 50 nm can be prepared by rapid precipitation from water [549] and subsequent colloidal stabilization by coating with polymers [550]. Nano-sized clusters of ACP [551] or those comprising a spherical core of 355 ± 20 DCPD units with density of 2.31 g/cm^3 and radius of $2.30 \pm 0.05 \text{ nm}$ surrounded by 49 ± 4 peptide chains with a partial specific volume of $0.7 \text{ cm}^3/\text{g}$, forming a tightly packed shell with an outer radius of $4.04 \pm 0.15 \text{ nm}$ were prepared by precipitation using 10 mg/ml of the 25-amino-acid N-terminal tryptic phosphopeptide of bovine β -casein as a stabilizing agent [552]. Nano-sized particles of ACP were prepared by mixing solutions of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (450 mmol/L) in acetone and $(\text{NH}_4)_2\text{HPO}_4$ (30 mmol/L) in deionized water at pH within 10.0–11.0 [553]. Furthermore, nanodimensional particles of ACP might be prepared by electrostatic spray pyrolysis [554, 555], pulsed laser ablation [532], spray drying [125], as well as by flame spray synthesis [457]. By means of the latter technique, one can produce nanodimensional ACPs with a broad Ca/P ratio within 0.5–1.5 [457].

Self-assembled shell cross-linked poly(acrylic acid-*b*-isoprene) micelles and/or cross-linked poly(acrylic) acid nano-sized cages in aqueous solutions, might be used as templates for preparation of polymer/calcium orthophosphate nanodimensional capsules of 50–70 nm in diameter, consisting of spherical polymer nano-sized particles enclosed within a continuous 10–20 nm thick surface layer of ACP [556]. Synthesis of hollow spherical calcium orthophosphate nano-sized particles using polymeric templates has also been reported by other researchers [557]. Furthermore, bundles of surfactant-coated ACP nanodimensional filaments of $\sim 2 \text{ nm}$ in width and $> 300 \mu\text{m}$ in length were synthesized in reverse micelles [558]. Bundles of the nanodimensional filaments were stable in the reverse micelle phase up to around 5 days, after which they transformed into 5 nm-wide surfactant-coated CDHA rods. Discrete filaments of $100\text{--}500 \times 10\text{--}15 \text{ nm}$ in size and a linear superstructure based on the side-on stacking of surfactant-coated ACP nano-sized rods were also prepared [490]. A double reverse-micelle strategy was realized to synthesize amine, carboxylate- and polyethylene glycol surface functionalized calcium orthophosphate nano-sized particles of an undisclosed nature [559]. Furthermore, the reverse micelle technique might be applied to prepare nanodimensional DCPA [501, 560].

Concerning OCP, an oriented growth of nanodimensional belts of OCP with a clean surface has been achieved by a wet-chemical approach using cetyltrimethylammonium bromide [561]. Pulsed laser deposition technique was employed to obtain thin films of nanocrystalline OCP on pure Ti substrates [562]. The deposition was performed by a pulsed UV laser source in a flux of hot water vapors. High-resolution electron microscopy and X-ray diffraction at grazing incidence investigations indicated that the coatings were made of nanocrystalline OCP (unfortunately, the dimensions were not indicated). *In vitro* tests proved that both fibroblasts and osteoblasts adhered, reached a normal morphology, proliferated and remained viable when cultured on the nanocrystalline OCP coatings, supporting a good biocompatibility and absence of any toxicity [562].

Nanodimensional powders of BCP (both HA + β -TCP [563–565] and HA + α -TCP [566]) have been fabricated as well. To get the details, the interested readers are referred to the original publications.

Similar to that for apatites (see above), nano-sized particles of TCP, ACP and other calcium orthophosphates might be functionalized and/or doped by various compounds to provide new important properties [200, 559, 566–572], such as fluorescence [569, 570], luminescence [572] or a good disperseability in organic solvents [566]. Furthermore, nano-sized calcium orthophosphates might be used as templates to manufacture nanodimensional capsules [573].

2.6.5 Biomimetic Construction Using Nanodimensional Particles

Morphological control of bioinorganic materials is another interested issue in biomineralization by which inorganic materials with complex morphologies can be produced. Complex forms or patterns with a hierarchical structure over several length scales are important features of biomineralization. Pattern formation in biomineralization is a process in which self-assembled organic templates are transformed by a material's replication into organized inorganic structures. Needless to mention, researchers try to reproduce these processes in laboratories. For example, Chen *et al.* reported a way to create enamel-like structures by modifying synthetic nano-sized rods of apatite with a surfactant, bis(2-ethylhexyl)sulfosuccinate salt, that allowed the nano-sized rods to self-assemble into prism-like structures at the water/air interface [225]. A nanometer-scale rod array of apatite having preferred orientation to the *c*-axis was successfully prepared simply by soaking calcium-containing silicate glass substrates in Na₂HPO₄ aqueous solution at 80°C for various periods [574]. A biomimetic bottom-up route to obtain the first hierarchical level of bone was reported [212]. A pH-induced self-assembly of peptide-amphiphile to make a nanostructured fibrous scaffold reminiscent of extracellular bone matrix was obtained. After the cross-linking of the scaffold, the fibers were able to direct mineralization of CDHA to form a biocomposite, in which the crystallographic *c*-axes of the nano-sized crystals of CDHA were aligned with the long axes of the fibers. This alignment was similar to that observed between collagen fibrils and crystals of biological apatite in bones [212]. Other attempts to fabricate artificial

materials having both bone-like nanostructure and chemical composition were performed and several significant achievements were obtained [575, 576].

The classical model of biomineralization considers mineral formation as an amplification process in which individual atoms or molecules are added to existing nuclei or templates [1, 2, 577]. This process occurs in the presence of various bioorganic molecules, which deterministically modify nucleation, growth and facet stability. A model involving aggregation-based growth [578] recently challenged this conventional concept for the crystal growth. Inorganic nano-sized crystals were found to aggregate into ordered solid phases via oriented attachment to control the reactivity of nanophase materials in nature [19, 579]. A model of "bricks and mortar" was suggested to explain the biological aggregation of nano-sized apatite [580]. In this model, ACP acts as "mortar" to cement the crystallized "bricks" of nano-sized HA. Meanwhile, biological molecules control the construction process. By using nanodimensional spheres of HA as the building blocks, highly ordered enamel-like and bone-like apatites were hierarchically constructed in the presence of glycine and glutamate, respectively. It is interesting that, during the evolution of biological apatite, the amorphous mortar can be eventually turned into the brick by phase-to-phase transformation to ensure the integrity of biominerals [580].

2.7 Biomedical Applications of the Nanodimensional and Nanocrystalline Calcium Orthophosphates

2.7.1 Bone Repair

Due to advances in surgical practice and a fast aging of the population, there is a permanently increasing demand for bone grafts [581]. Modern grafts should not only replace the missing bones, but also should be intrinsically osteoinductive by acting as scaffolds for guided bone growth. Furthermore, an ability to form a biologically active apatite layer to bond to living bone is an essential requirement to modern biomaterials [582]. In addition, a good graft should provide a framework to support new blood vessels and soft tissues in forming a bridge to existing bones [581].

Calcium orthophosphate bioceramics of micron dimensions have been used in dentistry, orthopedics and surgery for over 30 years because of their chemical similarity to calcified tissues of mammals and, therefore, excellent biocompatibility [148, 204–206, 259, 260]. Due to a rapid development of nanotechnology, the potential of nanodimensional and nanocrystalline forms of calcium orthophosphates has received considerable attention [18] because they produce favorable results in repair of bone defects [583, 584]. For example, due to an improved sinterability, an enhanced densification and a better bioactivity than coarser crystals, they might be chosen as the major components of self-setting bone cements [14, 25, 520, 521, 585–589]. However, there is a study in which an increase of particle and crystallite sizes of TCP did not prolong but shortened the induction time until the cement setting reaction started [523], which was against the common physical rules (generally, smaller particles or crystallites

should enhance reactivity). Nevertheless, two general directions of the biomedical application of nanodimensional and nanocrystalline calcium orthophosphates can be outlined: (i) using them in powder form as filling materials to impart bioactivity to various biocomposites and hybrid biomaterials [64–104, 179, 590]; (ii) manufacturing of either dense compacts or porous scaffolds, possessing the sufficient mechanical properties [74, 96, 294, 295, 575, 576, 591, 592]. As the nanodimensional and nanocrystalline calcium orthophosphates tend to agglomerate at heating (Figure 2.8) [313, 593–595], normally a low-temperature [169, 371] and/or a rapid consolidation [169, 258, 323, 596–602] technique must be employed. The low-temperature approach is comprised of gel hardening (at 4°C) [371] and uni-axial pressing at 150–200°C [169]. The rapid consolidation techniques are comprised of spark plasma sintering [169, 258, 323, 596–599], pressure sintering [597] and microwave sintering over the temperature range 1000–1300°C, using a rapid sintering schedule [600–602]. Furthermore, nanodimensional crystals of calcined HA might be fabricated by calcination at 800°C for 1 h with an anti-sintering agent surrounding the original nano-sized CDHA particles. The agent is subsequently removed by washing after the calcination [603–605]. These consolidation approaches provided a limited alteration of the initial nano-sized crystals, while the final bioceramics possessed the mechanical properties similar to those reached with sintered stoichiometric HA.

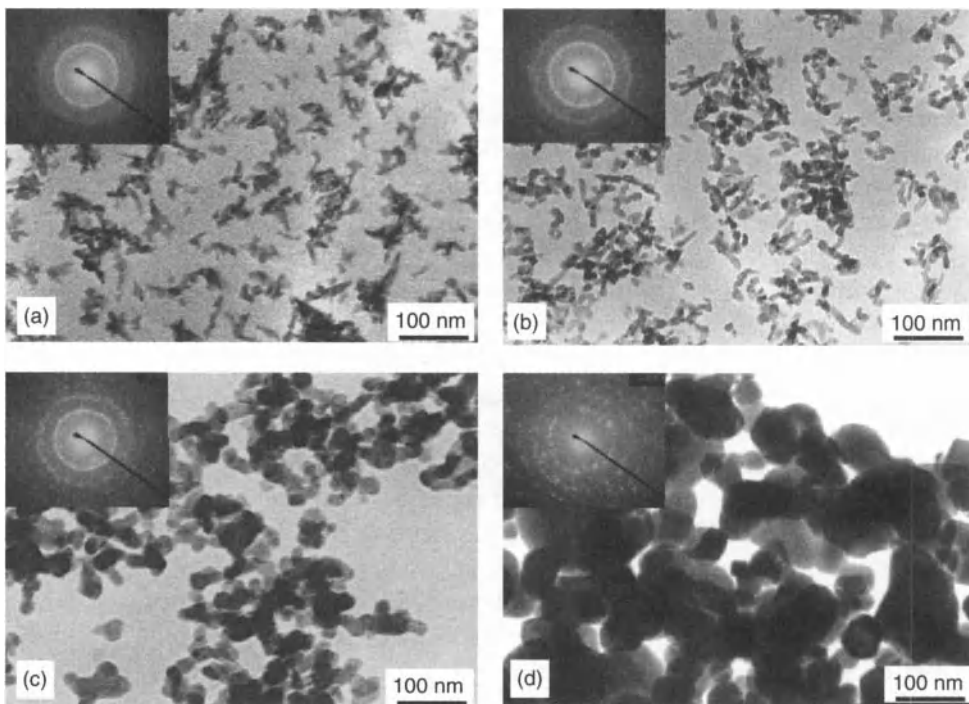


Figure 2.8 Particle sizes and crystallinity of HA powders after a heat treatment at various temperatures: (a) 300°C, (b) 500°C, (c) 700°C, (d) 900°C.

Already in the 1990s, implants prepared from nanodimensional apatites, as well as biocomposites of nanodimensional apatite with organic compounds were tested *in vivo* [606–608]. Cylinders made of both pure nanodimensional apatite and organoapatite containing a synthetic peptide were analyzed 28 days after implantation into spongy bones of Chinchilla rabbits. Both implant types were well incorporated and interface events were found to be similar to those observed on human bone surfaces with regard to resorption by osteoclast-like cells and bone formation by osteoblasts. That study revealed a suitability of such materials for both bone replacement and drug release purposes [606]. Similar results were obtained in other studies [607, 608].

Among the available commercial formulations, NanOss™ bone void filler from Angstrom Medica, Inc. [609] is considered the first nanotechnological medical device to receive clearance by the US Food and Drug Administration (FDA) in 2005. It is prepared by precipitation of nano-sized calcium orthophosphates from aqueous solutions, and the resulting white powder is then compressed and heated to form a dense, transparent and nanocrystalline material. NanOss mimics the microstructure, composition and performance of human bone, as well as being mechanically strong and osteoconductive. It is remodeled over time into human bone with applications in the sports medicine, trauma, spine and general orthopedics markets [609].

Ostim® (Osartis GmbH & Co. KG, Obernburg, Germany) is another popular commercial formulation. This ready-to-use injectable paste received CE (Conformité Européenne) approval in 2002. Ostim is a suspension of synthetic nanocrystalline HA (average crystal dimensions: $100 \times 20 \times 3 \text{ nm}^3$ [a needle-like appearance]; specific surface area $\sim 100 \text{ m}^2/\text{g}$) in water, prepared by a wet chemical reaction [610]. After completion, the HA content in the paste is $\sim 35\%$. Ostim does not harden when mixed with blood or spongiosa, so it is highly suitable for increasing the volume of autologous or homologous material. Simultaneously, its viscosity enables its applications to form-fit in close contact with the bone. Ostim® can be used in metaphyseal fractures and cysts, alveolar ridge augmentation, acetabulum reconstruction and periprosthetic fractures during hip prosthesis exchange operations, osteotomies, filling cages in spinal column surgery, etc. [609–622]. It might be incorporated into bones and a new bone formation is visible after only three months [623]. For a number of clinical applications, Ostim® might be combined with other types of calcium orthophosphate bioceramics, e.g., with a HA bioceramic core (Cerabone®) [610, 624] or with biphasic (β -TCP + HA) granules (BoneSaves®) [625]. Application of such combinations of a nanocrystalline Ostim® with the microcrystalline calcium orthophosphate bioceramics appeared to be an effective method for treatment of both tibia head compression fractures [610] and metaphyseal osseous volume defects in the metaphyseal spongiosa [624]. Besides, such combinations might be used for acetabular bone impaction grafting procedures [625].

Cui *et al.* developed nano-sized HA/collagen biocomposites which mimicked the nanostructure of bones [208, 626]. After implantation, such biocomposites can be incorporated into bone metabolism. Due to processing difficulties and poor mechanical properties of bulk calcium orthophosphates,

their applications are currently confined to non-load-bearing implants and porous bodies/scaffolds. Porous 3D biocomposites of nanodimensional HA and collagen/polymer mimic bones in composition and microstructure and can be employed as a matrix for the tissue engineering of bone [89].

Owing to their low mechanical properties, the use of calcium orthophosphates in load-bearing applications is rather limited: calcium orthophosphates are too stiff and brittle for such use. Today's solutions for weight-bearing applications rely mostly on biologically friendly metals, like cobalt-chromium alloys, titanium and its alloys, as well as stainless steel 31L, but problems with stress-shielding and long-term service can cause failures. All these metals, although nontoxic, are always bioinert and cannot bond to bone directly. In order to improve the biological properties of the metallic implants, nanostructured calcium orthophosphates (mainly, apatites) are generally used as a coating material to accelerate bone growth and enhance bone fixation [186, 187, 316, 439, 562, 627–648]. The coating techniques include thermal spraying, sputter coating, pulsed laser deposition, dynamic mixing method, dip coating, sol-gel method, electrophoretic deposition, biomimetic process, hot isostatic pressing and some other methods [649]. In the majority of cases, the coatings

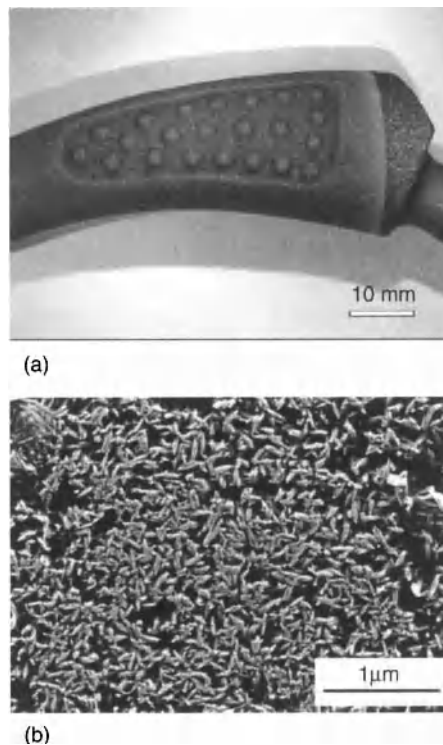


Figure 2.9 (a) A photo of a titanium implant coated with electrochemically deposited HA at 37°C (Cenos® BoneMaster); (b) A micrograph of a titanium implant surface coated with electrochemically deposited HA at 37°C. Reprinted from Ref. [650] with permission. Other micrographs of nano-CDHA coatings biomimetically deposited on NaOH-treated Ti6Al4V surfaces might be found in Ref. [651].

are composed of uniform nanocrystalline apatites (Figure 2.9). They are capable of performing bone formation and promoting direct osseointegration with juxtaposed bone [652–655]. For example, an enhanced new bone formation can be clearly seen on nanophase HA-coated tantalum compared to micro-scale HA-coated tantalum and non-coated tantalum (see figure 2 in Ref. [116]). Furthermore, nanostructured calcium orthophosphates might be used as a coating material to impart surface bioactivity to other materials, e.g., glasses [656] and polymers [657, 658]. Finally but yet importantly, such coatings might be patterned, e.g., by laser direct writing [574] or electrohydrodynamic atomization spraying technique [659].

2.7.2 Nanodimensional and Nanocrystalline Calcium Orthophosphates and Bone-related Cells

It is well accepted that bone-related cells (especially osteoblasts and osteoclasts) play the key roles in the physiological formation of calcified tissues. Bone-related cells not only are speculated to take part in the formation of biominerals and macrostructure constructions of bones, but they also continuously modulate the density, regeneration and degradation of bones. Therefore, understanding the relationship between the bone-related cells and nano-sized calcium orthophosphates has been paid much attention in order to elucidate the formation mechanism of bones, to prevent and cure bone-related diseases and to design novel biomaterials. Better structural biomimicry and osteoconductivity can be achieved using nanodimensional and nanocrystalline calcium orthophosphates [178, 179, 185, 186, 660–663]. Biocompatibility of such biomaterials is the key question for their application possibility for clinical use. For example, adhesion, proliferation and differentiation of mesenchymal stem cells were studied on nano-sized HA/polyamide biocomposite scaffolds. The results indicated that such biocomposites exhibited a good biocompatibility and an extensive osteoconductivity with host bone *in vitro* and *in vivo*, and proved that nano-sized HA/polyamide scaffolds had a potential to be used in orthopedic, reconstructive and maxillofacial surgery [664–666].

Most results demonstrate that nanostructured HA can improve cell attachment and mineralization *in vivo*, which suggests that nano-sized HA may be a better candidate for clinical use in terms of bioactivity [185, 186, 190, 667–669]. The size effects of nanodimensional HA on bone-related cells, as well as the influence of crystallinity of nano-sized HA were studied [507, 670]. Different nano-sized particles of HA, typically of 20 ± 5 , 40 ± 10 and 80 ± 12 nm in diameter, were prepared and their effects on the proliferation of two types of bone-related cells, bone marrow mesenchymal stem cells (MSCs) and osteosarcoma cells (U2OS and MG63) were studied. The cell culture experiments showed an improved cytophilicity of the nanophase HA if compared to the submicron-sized HA. A greater cell viability and proliferation of MSCs were measured for nano-sized HA, remarkably for 20 nm-sized particles. However, the opposite phenomenon occurred for bone tumour cells when nano-sized

HA were co-cultured with cells. Nano-sized HA can inhibit proliferation of U2OS and MG63 cells and the inhibited strengths were inversely proportion to the particle size, i.e., smaller particles possessed a greater ability to prevent cell proliferation. This suggests that nano-sized HA can exhibit favorable cell proliferation to optimize biological functionality in which the particle dimensions are believed to play a key role. These *in vitro* findings are of a great significance for the understanding of cytophilicity and biological activity of nano-sized particles during biomineralization [507]. Furthermore, an early osteogenic signal expression of rat bone marrow stromal cells appeared to be influenced by nanodimensional HA content [671]. On the other hand, there is a study on early bone healing, in which an importance of nanometer thick coatings of nanodimensional HA on titanium implants appeared to be insignificant if compared to the control [672].

Studies confirmed that nano-sized ACP had an improved bioactivity if compared to nano-sized HA since a better adhesion and proliferation of osteogenic cells had been observed on the ACP substrates [673]. However, in order to understand the influence of crystallinity of the nano-sized calcium orthophosphates on the osteogenic cells correctly, it was critical to use nano-sized ACP and HA of the same size distribution [670]. Thus, ACP and HA particles of ~ 20 nm size were synthesized and the effects of crystallinity were studied. The adhesion, proliferation and differentiation of MSC cells were measured on both ACP and HA films and compared at the same size scale. Surprisingly, more cells were adsorbed and proliferated on the films of the well crystallized nano-sized HA than those on the films of nano-sized ACP. Alkaline phosphatase activity assay and RT-PCR assay were also used to evaluate the differentiation of MSC cells. The results showed that the differentiation of MSC cells from osteoblasts was promoted significantly by nano-sized HA. These experimental phenomena clearly demonstrate that the crystallized phase of HA provides a better substrate for MSC cells than ACP when the factor of size effect is removed. This new view on the relationship between the crystallinity of calcium orthophosphates and the responses of cells emphasized the importance of both size and phase control in the application of biomedical materials [670–673].

On the other hand, the chemical composition of the samples appears to be important. Interestingly, in spite of the fact that the biological apatite of bones contains the substantial amount of carbonates, among investigated samples of nanocrystalline apatites, osteoclastic differentiation was found to be constrained on carbonate-rich samples, leading to smaller numbers of osteoclast-like cells and fewer resorption pits. Furthermore, the highest resorption rate was found for nanodimensional HA with a low carbonate content, which strongly stimulated the differentiation of osteoclast-like cells on its surface [674].

Cells are sufficiently sensitive and nano-scale alterations in topography might elicit diverse cell behavior [675–677]. How cells can recognize the particle size and other very small differences in the properties of nano-sized HA in these experiments remains unclear. Actually, determining the mechanisms

whereby nano-sized particles of calcium orthophosphates and their sizes exert effects on bone-related cells will require further systematic studies.

2.7.3 Dental Applications

Dental caries is a ubiquitous and worldwide oral disease. At the initial stage of caries lesions, bacteria cause damage of dental enamel, which is the exterior coating of teeth and possesses remarkable hardness and resistance. As the most highly mineralized structure in vertebrate bodies, enamel is composed of numerous needle-like apatite crystals of nanodimensional sizes, which are bundled in parallel ordered prisms to ensure unique mechanical strength and biological protection. As a non-living tissue, the main constituent (~ 97 wt. %) of mature enamel is inorganic nanodimensional apatite. Therefore enamel is scarcely self-repaired by living organisms after substantial mineral loss. Filling with artificial materials is a conventional treatment to repair damaged enamel. However, secondary caries frequently arise at the interfaces between the tooth and foreign materials [678].

Nanodimensional HA and CDHA are often considered as model compounds of dental enamel due to the chemical and phase similarities [148, 204, 205]. Therefore, enamel remineralization by using nanodimensional apatite or other calcium orthophosphates is suggested in dental research [679]. For example, toothpastes containing nanodimensional apatite could promote a partial remineralization of demineralized enamel [680–684], as well as possess some whitening effect [685]. Furthermore, nano-sized HA might be added to methacrylate-based root canal sealers [686], as well as to a fluoride-containing mouth rinse [687]. A remineralization potential of sports drink, containing nano-sized HA, was also investigated [688, 689]. A positive influence of the addition of nanodimensional β -TCP against acid demineralization and the promotion of remineralization of the enamel surface was detected as well [690]. Unfortunately, these chemically analogous compounds of enamel are not widely applied in clinical practices. The native structure of dental enamel is too complex to be remodeled and the synthesized apatite crystallites often have different dimensions, morphologies and orientations from the natural ones, which result in a poor adhesion and mechanical strength during dental restoration. Recent advances in biomineralization also indicate that features of smaller particles of nano-sized HA might approximate features of biological apatite more closely than features of the larger HA particles that are conventionally used [13]. For example, it has been demonstrated that nano-sized HA can be self-assembled to form enamel-like structures in the laboratory [225]. Therefore, a biomimetic technique is suggested as follows: the localized repair of the enamel surface can be improved by nano-sized HA (dimension of ~ 20 nm) analogues to the basic building blocks of enamel rods. Furthermore, it is found that nano-sized HA can adsorb onto the enamel surface strongly and can even be integrated into the natural enamel structure [691].

It is surprising, but nano-sized HA of ~ 20 nm can inhibit significantly a mineral loss from the enamel surface [228]. Without any treatment, the

demineralization of the natural enamel surface was remarkable in acidic solution ($\text{pH} \sim 4.5 \pm 0.1$, experimental period of 2 days) and damaged sites were observed. The mass loss rate was about $0.12 \pm 0.04 \text{ mg/mm}^2$ per day. In contrast, a layer of nano-sized HA on the treated enamel surface was almost unchanged in acidic solution. The rate of mass loss of enamel coated by nano-sized HA approached zero ($< 0.02 \text{ mg/mm}^2$ per day), which was beyond the sensitivity of the detection methods. Since the coating by nano-sized HA appeared to be insensitive to dissolution, the underlying enamel surface was well protected under slightly acidic conditions. Furthermore, the enamel surface coated by $\sim 20 \text{ nm}$ -sized HA had a hardness of $4.6 \pm 0.4 \text{ GPa}$ and an elastic modulus of $95.6 \pm 8.4 \text{ GPa}$. These data appeared to be very similar to those of natural enamel samples, which are 4.2 ± 0.2 and $94.1 \pm 5.4 \text{ GPa}$, respectively [228].

The similarity between $\sim 20 \text{ nm}$ -sized HA and building blocks of dental enamel results in a good fixation of artificial biomaterials to natural tissues. Moreover, the enamel structure appears to be reinforced by nano-sized HA since secondary caries formation is suppressed and hardness is retained [678, 692, 693]. This strategy may have prospective applications in dentistry as it offers an easy but effective method to reconstruct tooth enamel that is suffering from mineral losses. Generally, these studies also suggest that analogues of nanodimensional building blocks of biominerals should be highlighted in the entire subject of biomineralization.

In the case of nanodimensional DCPA, decreasing of DCPA particle dimensions were found to increase the Ca- and PO_4 -ions releases from DCPA-based biocomposites. Therefore, biocomposites based on nano-sized DCPA, possessing both a high strength and good release of Ca- and PO_4 -ions, may provide the needed and unique combination of stress-bearing and caries-inhibiting capabilities suitable for dental applications [546].

2.7.4 Other Applications

Several other applications of nanodimensional and nanostructured calcium orthophosphates are in progress, some of which are described here. For example, there is a report on a successful preparation of a multi-modal contrast agent based on nano-sized crystals of HA, which was engineered to show simultaneous contrast enhancement for three major molecular imaging techniques such as magnetic resonance imaging, X-ray imaging and near-infrared fluorescence imaging [694]. Furthermore, various compositions based on nanodimensional calcium orthophosphates have already been tested for cancer treatment [48, 60, 199, 354, 570, 695–697]. Besides, nanodimensional HA was found to be effective for proliferation inhibition of highly malignant melanoma cells [698] and human chronic myeloid leukemia K562 cells [699].

Surface modification of nanodimensional calcium orthophosphates was performed in order to modulate their colloid stability, prevent dissolution in the case of low pH, avoid inflammation, serve as an intermediate layer to allow strong bond formation between HA/polymer matrices, and potentially enhance its bioactivity or improve its conjugation ability with special

functional groups [12, 700–706]. Such surface modified nano-sized particles might be applied for oral insulin delivery [707].

In another aspect, many strategies have been employed to load various agents, i.e., therapeutic, bio imaging, etc., to nanodimensional calcium orthophosphates (mainly, apatites) [708]. In summary, these strategies can be broadly categorized into two main approaches. One approach is to load these agents during the synthesis – so called *in situ* loading. This is done by adding the desired agent(s) to the reaction mixture before the formation of a nanodimensional calcium orthophosphate is completed. The second approach is to load the agent(s) only after a nanodimensional calcium orthophosphate has been fully synthesized or, in other words, after the synthesis process – so called *ex situ* loading. This is mainly done through surface adsorption where the agents are adsorbed onto the surfaces of pre-synthesized nanodimensional particles [709]. Therefore, due to established biocompatibility, ease of handling and notorious adsorption affinity, nano-sized calcium orthophosphates have been applied as non-viral carriers for drug delivery and gene therapy [133, 203, 289, 318, 325, 511, 542, 559, 600d, 695, 710–721]. After loading with genes or drugs, nanodimensional calcium orthophosphates provide a protective environment that shields them from degradation while providing a convenient pathway for cell membrane penetration and controlled release of the genes or drugs [512]. The experimental results proved that nanodimensional calcium orthophosphates possessed a higher penetration rate into cell membranes and their transfection efficiency could be 25-fold higher than that of the micron-sized particles. Furthermore, due to the larger specific surface areas, nanodimensional calcium orthophosphates can hold larger load amounts of drugs than coarser particles. These results indicate the potential of nano-sized calcium orthophosphates in gene delivery and as drug carriers [512, 722–725]. Since a charge of the particles influences their ability to pass through the cellular membrane and a positive charge is beneficial, positively charged nano-sized particles of calcium orthophosphate/polymer biocomposites were successfully applied for photodynamic therapy [726]. Furthermore, nanodimensional calcium orthophosphates can be stably loaded with radioisotopes [318, 727].

A transfer of functional foreign nucleic acids (DNA or RNA) into nuclei of living cells (transfection) with the aim of repairing missing cell function and providing a means to enhance or silence gene expression is currently used extensively in the laboratory and is fast becoming a therapeutic reality. As nucleic acids alone are unable to penetrate the cell wall, efficient carriers are required [728, 729]. Nanodimensional calcium orthophosphates can be represented as a unique class of the non-viral vectors, which can serve as efficient and alternative DNA carriers for targeted delivery of genes [289, 696, 697, 708, 714, 730–742] and cells [567, 743–749]. The standard transfection method using calcium orthophosphates, first introduced by Graham and van der Eb in 1973 [748], is still used in biochemistry. It involves a straightforward *in situ* precipitation of calcium orthophosphate/DNA aggregates. A similar experimental approach is used to load calcium orthophosphates by drugs [719]. But interestingly, the

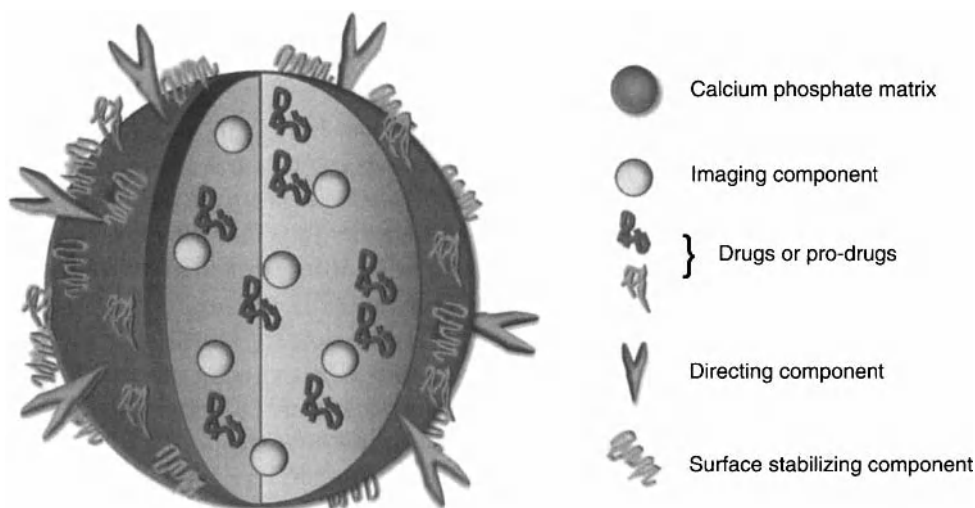


Figure 2.10 A generalized schematic setup of a nanodimensional particle of a calcium orthophosphate suitable for both imaging and drug delivery purposes. Reprinted from Ref. [756] with permission.

transfection efficiency of nanodimensional calcium orthophosphates were found to depend on Ca/P ionic ratio: namely, calcium orthophosphates with Ca/P = 1.30 ratio exhibited a fourfold increase in the transfection efficiency over the ones with Ca/P = 1.65 ratio composition [289]. This data emphasizes the importance of understanding the interaction between calcium orthophosphates and DNA to optimize the DNA uptake and its channeling to the nucleus of the cell. Besides, it has been demonstrated that surface modified particles of nano-sized calcium orthophosphates can be used *in vivo* to target genes specifically to a liver [750]. Attachment of galactose moiety onto the particle surface has increased the targetability of the nano-sized particles. Furthermore, this surface modification makes it possible for site-specific gene delivery [750, 751]. Assemblies of block-copolymer/nano-sized calcium orthophosphate were prepared and used for cell transfection; a high biocompatibility of this system was emphasized [752]. Structures that are even more complex are known as well [753–755]. A schematic drawing of a functionalized nano-sized particle suitable for both imaging and drug delivery purposes is shown in Figure 2.10 [756]. Furthermore, vaccination to protect against human infectious diseases may be enhanced by using adjuvants that can selectively stimulate immunoregulatory responses, and nano-sized particles of calcium orthophosphates were found to be suitable for such purposes [757, 758].

In all these new applications of nano-sized calcium orthophosphates, knowledge of the exact internalization pathway into the cells represents the first necessary step towards the detailed investigation and optimization of the functional mechanism. The main groups of pathways into the cell are diffusion, passive and active transport, as well as a number of endocytic mechanisms [695]. Bigger particles of far above 10 nm are internalized by eukaryotic cells

through the endocytic pathways including phagocytosis, macropinocytosis, clathrin-mediated endocytosis and non-clathrin-mediated endocytosis such as internalization via caveolae. To date, the exact internalization pathway of nano-sized calcium orthophosphates into cells has not been determined and there are many questions that remain to be answered, particularly concerning possible interactions of calcium orthophosphates with nucleic acids. Furthermore, the mechanisms of cellular uptake and transport to the cell nucleus of calcium orthophosphate/DNA nanodimensional complexes remain unclear. Therefore, there is a need to conduct a focused study on the synthesis of various forms of nano-sized calcium orthophosphates that could elucidate the mechanisms of binding, transport and release of attached plasmid DNA for understanding the gene delivery method. Research is also warranted to understand the tracking of DNA intracellularly [745] to understand the release and transport of DNA into cellular nuclei.

Concerning the healing abilities of nano-sized calcium orthophosphates, an *in vitro* inhibiting effect and even apoptotic action of unfunctionalized nano-sized HA of about 50 nm diameter on a hepatoma cell line in the concentration range of 50–200 mg/1 was reported [759]. A similar inhibiting effect was discovered for discrete nano-sized particles of HA, which appeared to cause apoptosis of leukemia P388 cells [119] and rat macrophages [760]. This effect might be due to a harmful increase in the intracellular calcium concentration. However, the correlation between the particle dimensions and the apoptotic action of nano-sized calcium orthophosphates appears not to be straightforward. Namely, the apoptosis efficacy of nanodimensional particles of HA of various sizes was found to decrease in the order of 45 nm > 26 nm > 78 nm > 175 nm [761].

Hollow nano-sized spheres are extremely attractive constructions because they can greatly enhance the load quantity. Though these novel biomaterials can improve the total intake of drugs, they also bring new problems, e.g., uncontrolled release kinetics and unreasonable metabolism pathway of the carriers [762]. In order to solve these problems, calcium orthophosphates were selected as suitable biomaterials to construct nanodimensional spheres that were hollow inside [198, 541, 542, 717, 763, 764]. Such spheres with dimensions ranged from 110–180 nm were synthesized by an ultrasonic-assisted wet chemical reaction in the presence of a modifier [765]. In addition, they might be prepared through emulsions [766]. Transmission electron microscopy investigations revealed that the uniform nanodimensional spheres were formed and they were well dispersed in the solutions. Thickness of the shells was about 45 nm; thus, they always had ~ 60 nm-sized internal cavities, which could be used to load drugs. The hollow spheres appeared to be stable in both air and aqueous solutions without ultrasonic application. However, when an ultrasonic treatment (40 kHz, 150 W) was applied, the hollow structures deconstructed to form pin-like nano-sized crystals of calcium orthophosphates [765]. During this transformation, the encapsulated drugs and chemicals are released [559, 765]. Different from a free and slow diffusion of encapsulated drugs from the cavity through the shells [199], the release kinetics in this system was triggered

and controlled by ultrasound. Furthermore, the power density of ultrasound can conveniently regulate the release dynamics. Besides, the formed pin-like nano-sized crystals of calcium orthophosphates had similar behavior to the biological apatite of bones. Thus, a combination of the hollow calcium orthophosphate nanospheres and ultrasonic treatment might provide a good system for drug delivery and release [765].

2.8 Other Applications of the Nanodimensional and Nanocrystalline Calcium Orthophosphates

Just a few publications are available on non-biomedical applications of the nanodimensional and nanocrystalline calcium orthophosphates. For example, nano-sized particles of calcium orthophosphates with a mean size of 150 ± 20 nm filled with a solution containing luminol, haematin and fluorescein were found to improve the ease and accuracy of H_2O_2 sensing [767]. Besides, nanodimensional HA particles were tested as a component of a green slow-release fertilizer composition [768]. Also, addition of nanodimensional HA remarkably inhibits desorption of heavy metals from soils, which increases their geochemical stability in metal contaminated soils [769]. Furthermore, nanodimensional HA was found to hold a great potential to remove cationic heavy metal species from industrial wastewater [770–774]. Finally yet importantly, nanodimensional and nanocrystalline calcium orthophosphates occasionally might possess a catalytic activity [775, 776].

2.9 Summary and Perspectives

As the basic building blocks of calcified tissues of mammals, nano-sized calcium orthophosphates of the apatitic structure play an important role in the construction of these biominerals. Therefore, they appear to be almost the ideal biomaterials due to their good biocompatibility and bioresorbability. Even more enhanced applications are expected in drug delivery systems [777]. However, there is still an unanswered question concerning their structure: do the majority of nanodimensional calcium orthophosphates appear to be almost amorphous (according to numerous results of X-ray diffraction studies) due to their nanoscopic dimensions of well-crystallized structures or due to a really amorphous (i.e., retaining only a short-range order at the scale of few atomic neighbors) matter? A good attempt to discuss this topic is available in literature [778], to which interested readers are referred.

In the future, an ability to functionalize surfaces with different molecules of varying nature and dimensions by means of their attachment to cells will enable them to act selectively on biological species such as proteins and peptides. The capability of synthesizing and processing of nanodimensional and nanocrystalline calcium orthophosphates with the controlled structures and

topographies, in attempts to simulate the basic units of bones and teeth, will provide a possibility of designing novel proactive bioceramics necessary for enhanced repair efficacy. The various primary positive results on the biocompatibility and biomimicry of novel nanostructured bioceramics merit further confirmations. Namely, much work remains to be undertaken to address the following key challenges and critical issues of nanodimensional and nanocrystalline calcium orthophosphates [779]:

- Consistency of the processing technologies;
- Optimization of the structure and properties mimicking bones;
- Matching the strength of nanodimensional and nanocrystalline constructs with those of bones in order to provide a uniform distribution of stresses (load sharing);
- Optimizing bioresorption without comprising the mechanical properties;
- Assessing the inflammatory response to validate their biosafety.

Furthermore, substantial research efforts are required in the analysis of cells and their different behaviors with regard to their interactions with nanodimensional and nanocrystalline calcium orthophosphates [779]. An important but still unsolved question is how the cells can recognize the particle dimensions and crystallinity of nano-sized calcium orthophosphates. What is the signal for nanodimensional biomaterials to promote cell proliferation and differentiation and how can the pathways be found out? According to the results on experiments on transfection, nano-sized particles can enter into cells readily but many details of this process remain unclear. Namely, the pathways for the nano-sized particles to enter the cells through the membranes should be revealed [780]. A greater influence of the hydrated surface layer with labile ionic species of smaller particles and crystals (see Section 2.5, The Structure of the Nanodimensional and Nanocrystalline Apatites, for the details) might be another possible option to be confirmed experimentally. Then it is important to examine the metabolism process of nano-sized calcium orthophosphates inside cells, so the existing forms of these particles during the biological processes can be understood. Further, a critical step will be the investigation of possible changes of gene or protein expression in the absence and presence of various nano-sized calcium orthophosphates, which may directly be related to cell proliferation and differentiation [13].

Understanding of the interactions between nano-sized particles and living cells is still a great challenge [779]. Namely, elucidating mechanisms, by which cells internalize and process nanodimensional particles, is of great importance for understanding their potential toxicity and for improving the targeted delivery of nanodimensional particles for biomedical applications. Already, some data are available that clathrin-mediated endocytosis might be responsible for the uptake of nano-sized HA [695]. In another study, nanodimensional particles of HA were sequestered within a specialized membrane-bound surface-connected compartment, directly connected to the extracellular space [781].

Future studies will focus on 1) the detailed interfacial structure of nanodimensional calcium orthophosphates and the specific adsorption of proteins or other matrices; 2) an uptake processes of the nano-sized particles by cells; 3) metabolism of nano-sized calcium orthophosphates inside the cells and its possible interference with physiological reactions. Another important topic is a biological security of nano-sized particles in general [144, 145, 782, 783] and those of calcium orthophosphates particularly [178, 376, 784]. For example, toxicity of nano-sized HA was found to vary considerably, which was related to their physico-chemical properties. Furthermore, cell death correlates strongly with the load of nano-sized particles. Namely, the biological effects of rod-shaped apatite, 50–80 nm in length, were investigated on human monocyte-derived macrophages [178]. High concentrations of apatite (200 nano-sized particles per cell) were incubated for 24 hours with the macrophages in both serum and serum-free conditions. This induced high levels of lactate dehydrogenase release, which is an indicator of cellular damage. However, lower concentrations (20 and 2 nano-sized particles per cell) of the rod-shaped apatite did not affect the cell viability similarly to the control group that did not contain nano-sized apatite [178]. Similarly, intracellular dissolution of nano-sized HA as a function of time suggests that increased cytoplasmic calcium load is likely to be the cause of cell death [784]. Furthermore, nano-sized calcium orthophosphates were found to interfere with the cell cycle of cultured human ovarian granulosa cells thus increasing cell apoptosis [785]. That pilot study suggested that effects of nano-sized particles on ovarian function should be extensively investigated. Additional examples of cytotoxicity experiments of nanodimensional calcium orthophosphates are well described in a recent review [709].

To finalize this topic, one should stress that *in vivo* evaluation of nano-sized particles includes the particle's activity, biodistribution and pharmacokinetic properties [786]. Ultimately, all these properties are determined by dimensions, surface charge, morphology and surface chemistry. Furthermore, nano-sized particles penetrate and leave biological organisms more readily using a number of pathways. Namely, very small (< 10 nm) particles are generally eliminated from the body via renal clearance, i.e., being filtered through the kidneys and eliminated through urine; while nano-sized particles of larger dimensions are phagocytized by tissue macrophages of the reticuloendothelial system in the liver and spleen [713]. For example, intravenously administered nanodimensional (~ 40 nm and ~ 200 nm) rod-shaped crystals of apatite showed clearance from the bloodstream within two hours, with ~ 90% of them being cleared in the first 10 minutes post injection; those nanodimensional crystals of apatite were observed primarily in the liver with a minority seen in the spleen [318]. These results indicate that bloodstream clearance occurs rapidly for a wide range of nanodimensional sizes. The accumulation of nanodimensional (50–100 nm in size) apatite in the liver was also noted in another study [510].

Thus, understanding the biological influence of nano-sized and nanocrystalline calcium orthophosphates is essential for a future development of bionanotechnology [787]. This interdisciplinary approach is very complicated

and the effective collaboration of scientists from different disciplines is the key [13].

2.10 Conclusions

With a high surface area, un-agglomerated nanodimensional and nanocrystalline bioceramic particles are of interest for many applications including injectable or controlled setting bone cements, high strength porous or non-porous synthetic bone grafts, and the reinforcing phase in biocomposites that attempt to mimic both the complex structure and superior mechanical properties of bone. Therefore, nano-sized and nanocrystalline calcium orthophosphates have already gained much regard in the biomedical field due to their superior biocompatibility and biomechanical properties. This is easily seen from the permanent increase in the amount of publications. At present, apatites (HA and CDHA) and β -TCP are the major calcium orthophosphates used in clinics. Currently, nanodimensional apatites are used primarily as bioactive coatings on bioinert materials like titanium and its alloys, in bone tissue repairs and implants, as well as for drug delivery purposes. The nano-sized β -TCP exhibits a significant biological affinity and activity and responds very well to the physiological environment. A lot of research is expected for much enhanced applications of the nanodimensional and nanocrystalline calcium orthophosphates for both drug delivery systems and as resorbable scaffolds that can be replaced by the endogenous hard tissues with the passage of time [147, 787].

Although the nanostructured biomaterials may have many potential advantages in the context of promoting bone cell responses [534–536, 677], it is important to remember that studies on nanophase materials have only just begun; there are still many other issues regarding human health that must be answered. Since particles of very low size have higher reactivity and effectiveness, a rapid technical development of nanometer-scaled particles in the biomedical field leads to concerns regarding the unknown risks of such materials [782, 783]. These nano-sized particles might induce inflammatory reactions, cytotoxicity, oxidative stresses or thrombogenesis when injected for drug delivery purposes. Namely, nano-sized particles may enter the human body through pores and may accumulate in the cells of the respiratory or other organ systems (when becoming dislodged through wear debris) and the health effects are yet to be largely known. This could happen during commercial-scale processing of the nano-sized particles as well as using these materials as implants [790]. Besides, nano-sized particles might be the objects whose existence has not been assumed by living body defense system [18, 144, 145]. Up to now, only a small number of short-term and small-scale health effects of single nanodimensional materials have been examined in toxicological studies, usually of the lungs [783]. Therefore, prior to clinical applications, any toxicity concerns of the nanophase materials [791–796] need to be overcome.

In summary, despite the challenges that lie ahead, significant evidence now exists elucidating that nanophase biomaterials represent an important growing

area of research that may improve bonding between the implants and the surrounding tissues. It has proven to be a versatile approach that can increase bone cell functions on a wide range of orthopedic implant chemistries. Even if the nanodimensional and nanocrystalline calcium orthophosphates do not provide the ultimate answer for increasing bone cell responses (due to some potential problems as mentioned above), researchers have learned a tremendous amount of information concerning bone cell recognition with nanostructured surfaces that will most certainly aid in improving orthopedic implant efficacy [144, 145].

Closing Remarks

According to Prof. D. F. Williams [115], the term "nanomaterial" should not exist because it is senseless (see Section 2.2, General Information on "Nano"). Following this logic, the term "nanoapatite" is senseless as well. However, it is presented in the titles of several publications, namely Refs. [606, 629, 653, 667]. In a slightly modified form, the term "nano-apatite" is presented in the titles of several other publications, namely Refs. [89, 100, 226, 511, 631, 735, 789]. Furthermore, similar terms "nano-HA" [103, 575, 608, 659, 681, 692], "nanohydroxyapatite" [42, 87, 91, 92, 96, 97, 102, 119, 190, 191, 294, 295, 314, 338, 451, 459, 486, 492, 578, 583, 606, 627, 628, 641, 664, 666, 677, 682, 685, 687, 693, 698, 701, 703, 705, 770, 773], "nano-fluorapatite" [104, 684] and "nanohydroxyapatite" [86, 93, 99, 162, 201, 239, 240, 242, 246, 253, 274, 320, 329, 362, 462, 576, 656, 664, 764] are presented in the titles of still other publications. Presumably, it is wiser not to use these terms anymore.

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Layer-by-Layer (LbL) Thin Film: From Conventional To Advanced Biomedical and Bioanalytical Applications

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Abstract

Development of nanoscale structured materials is of importance in the fields of micro-electronics, microdevices, biomedical, biosensors and pharmaceutical applications. The development of thin film technologies for modifying the surface properties and construction of multilayer structures is the key step in creating novel materials for emerging applications. Among the existing thin film technology, LbL thin film coating provides a general method for fabricating multicomponent films on solid supports. Moreover, the LbL technology allows thin films designed with nanometer precision to meet different requirements such as controlled permeability, integrated multiple functionalities and enhanced biocompatibility. This chapter will review the conventional and emerging applications of LbL thin film for biomedical implantation materials, biosensors, drug delivery and micro-encapsulated reactor.

Keywords: Layer-by-layer, encapsulation, microcapsule, biomedical implant, drug delivery, biosensor, microreactor

3.1 State-of-the-art LbL Technology

The alternate LbL self-assembly for the creation of multilayer thin film was developed on charged flat surfaces in 1991 by Decher *et al.* [1, 2]. The LbL self-assembly process is basically conducted through electrostatic interaction between oppositely charged species. The fabrication of multilayered structured thin films by LbL coating only involves a number of simple adsorption steps by incubating a template without limitation on the morphology such as flat surface, irregular surface or colloid particles in a polyelectrolyte solution followed by several washing steps under mild physiological condition. The thin films thickness, or even a single layer thickness, can be adjusted by

altering the adsorption conditions (e.g., ionic strength, temperature and pH condition). The permeability of LbL films can be adjusted by simply controlling the layer numbers. The thin film fabrication process for a single layer polyelectrolyte deposition usually required only several minutes [3, 4]. The deposition kinetic also depends on the polyelectrolyte concentration and molecular weight. Moreover, other properties such as magnetic and optical properties can be introduced to LbL film by simply incorporating magnetic nanoparticles or dye-labelled polyelectrolyte into the multilayer [5, 6, 7]. Furthermore, the use of natural polymers or synthetic biocompatible polymers for the film thin deposition can modify the surface attribute of the template materials with improving biocompatibility and low cytotoxicity for biomedical applications [8].

LbL thin film coating in principle has no restrictions with respect to the substrate size and topology. Moreover, various charged materials (building blocks) such as polyions [9,10], inorganic nanoparticles [11, 12], proteins [13, 14], aggregated small molecules [15, 16], deoxyribonucleic acid (DNA) and charged polysaccharides [17, 18, 19] can be assembled. Moreover, recent studies showed that LbL adsorption can be performed under a water-solvent mixture or pure organic solvent. This can further extend the use of weakly charged polymer which is poorly soluble in water as the building block of multilayer film. Besides, performing LbL deposition under pure organic solvent allows direct encapsulation of water soluble materials such as proteins, organic salts, and inorganic salts in their solid state [20, 21]. This opens a new direction for applications in drug formulation and delivery; stabilization of powders and suspensions.

3.2 Principle of Biomaterials Based LbL Architecture

Among the above mentioned adsorbents, LbL adsorption of biomaterials such as proteins, poly-nucleic acids and polysaccharides for construction of biofunctional multilayer film gives the most prominent advantages for the development of a new type of biomaterials for bioengineering, biomedical and bioanalytical applications. The LbL technology has the remarkable advantage of providing a simple procedure for the construction of biomaterials based multilayer thin films with integrated biological functions and activities. One major concern for the biomaterials based LbL architecture is regarding the preservation of their biological functions after the LbL deposition process. The LbL film construction is a simple adsorption procedure which does not require chemical reactions and severe physical conditions; therefore protein denaturation during the multilayer film formation process is minimized. The biological functions of the resultant protein film can be tailored by controlling the amount of biomolecules being deposited per single layer. Moreover, layer structure and the biomolecules layering sequence can be easily controlled. The combination of various substrates and wide range of adsorbents made the LbL technique a versatile method for biological thin film design.

Polysaccharides and poly-nucleic acids are polyelectrolytes which consist of repeating units of monomers bearing ionisable groups, which can dissociate into either positively charged or negatively charged polymer chains, while proteins are polyampholytes which carry both positively and negatively charged amino acid monomers. The overall charges on the protein arise from association or dissociation of acidic and basic residue groups along the polypeptide chain depending on the pH environment. The isoelectric point of protein was defined as the pH value where the overall net charge of the protein is zero.

The deposition of polyelectrolyte onto a surface leads to the reduction of the conformational entropy (i.e., thermodynamically unfavorable). Thus, the deposition process takes place only if the loss in conformational entropy is compensated by a sufficient amount of energy and gain in entropy elsewhere [22]. The adsorption of polyelectrolytes onto an oppositely charged template is mainly driven by the longer-range electrostatic attractive coulomb forces between oppositely charged species [5, 6]. Depending on the nature of the polyelectrolyte and surface of the template, other short range forces such as Van der Waals interaction [23], charge transfer [24], hydrophobic interaction [25] and stereo-complex assembly [26] contribute to the adsorption process.

Proteins are polyampholytes made up of polypeptide chain folded into a three-dimensional structure. This structure is stabilized by hydrogen bonds, hydrophobic interaction, coulomb interaction and Van der Waals interaction. Due to the compact and ordered structure, the rotational freedom along the polypeptide chain is restricted. As a consequence, proteins experience low conformational entropy [27]. In contrast to polyelectrolytes, deposition of protein onto a sorbent surface leads to slightly structural rearrangements in the protein structures [28, 29]. This causes an increase in conformational entropy of the protein molecule. The gain of entropy causes protein thermodynamically favorable for the deposition process [30]. Besides conformational entropy, the adsorption of proteins is pH-dependent. The amount of proteins adsorbed to a surface is maximized around the isoelectric point of the protein/sorbent surface complex. In other words, the deposition process is maximized at a condition where the charge on the protein and the sorbent surface just compensate each other [31].

3.3 LbL Thin Film for Biomaterials and Biomedical Implantations

The major criterion for biomedical implantations is the biocompatibility between the implanted materials and the surrounding tissue. Construction of biointerfaces as thin films would provide a tool to achieve enhanced biocompatibility and lower cytotoxicity. LbL deposition enables modifying the surface attribute of the implanted materials. It retains the physical properties such as mechanical strength or elastic property of the biomedical implant while modifying only the outermost surface of the implanted materials to influence the biointeraction. Natural polymers have advantages for thin film construction

because they are nontoxic, naturally available and biocompatible. Natural polymers such as proteins (e.g., albumin, collagen and gelatin) [32, 33, 34]; polypeptides (e.g., poly(L-lysine), poly(aspartic acid) and poly(L-glutamic acid) [35, 36, 37]; polysaccharides (e.g., hyaluronic acid, dextran and chitosan) [38, 39, 40] and heparin [41] have been widely applied in biomaterial-based LbL film thin construction.

The development of nonfouling materials which can reduce the affinity of proteins adsorption and cells adhesion is of interest for biointerface construction. Non-specific fouling can promote inflammation and infection that influence the lifetime of a biomedical implant. The use of polyethylene glycol (PEG)-coated surface has shown a reduced adsorption for serum and cell-borne proteins [42]. The mechanism of the nonfouling property of PEG can be explained by steric repulsion arising from the unfavourable entropy when protein molecules or cells attempted to fill the space between adjacent PEG molecules [43, 44]. The use of poly(L-lysine) and poly(L-glutamic) multilayer followed by a single outermost poly(L-glutamic) grafted PEG coating showed a reduction of 72% adhesion for *Escherichia coli* bacteria, while coating of three layers of poly(L-glutamic)-grafted PEG showed a reduction of 92% adhesion [35]. The antifouling property of the PEG modified LbL films can reduce the bacteria adhesion and thus reduce the chance of microbial infection. Moreover, other antifouling LbL films by assembly of poly(L-lysine) and alginate on a gelatin substrate showed a 200-fold decrease in the adsorption of human fibroblast cells when compared with the untreated substrate [36].

When biomedical implants come into contact with blood, it induces a sequence of processes that result in thrombus formation or blood coagulation. When the implant materials contact with the blood, plasma protein adsorbed onto the surface of the implanted materials. It induces the adsorption of fibrinogen and platelet adhesion stimulating proteins to trigger irreversible platelet adhesion, thus leading to the formation of thrombus. The thrombus formation protects the organisms against bleeding and infection. However, thrombus formation will influence the performance and lifetime of implanted biomedical devices. Surfaces coated with hyaluronic acid, heparin, albumin and dextran showed an improvement in thrombo resistance and lower platelet adhesion [38, 39]. These surface coatings can be easily achieved by LbL self-assembly technique. Brynda *et al.* demonstrated the construction of LbL films by alternating deposition of human serum albumin (HSA) and heparin multilayers onto polystyrene substrates. Results showed an 80% drop in fibrinogen adsorption onto the LbL modified surfaces when compared with the blank surfaces. Moreover, LbL films with blood anticoagulation property have been reported by Serizawa *et al.* [40]. Chitosan/dextran and chitosan/heparin multilayer was constructed onto a sterilized cell disk by LbL self-assembly technique. For chitosan/dextran films, anticoagulation property resulted when dextran coating was the outermost layer, while for chitosan/heparin films, anticoagulation property was found with both chitosan and heparin as the outermost layer.

Besides biomedical devices, silicone rubber is widely used as biomedical implant material for vascular grafts, catheters, heart valves, artificial joints

and breast implants. However, the hydrophobic properties of silicone rubber has the disadvantages of nonspecific protein and lipid adsorption on contact lenses, lipid infiltration in breast implants and lipid adsorption on silicone heart valves. Introduction of hydrophilic property to silicone rubber by LbL assembly was demonstrated by Hi *et al.* [45, 46]. Surface modification was performed by alternating deposition of polyethyleneimine (PEI) and sodium polystyrene sulfonate (PSS) layers followed by alternating gelatin and poly-D-lysine (PDL) layers. Surface modified silicone rubber provides a good interface for the adhesion and growth of bovine coronary artery endothelial cells, and biocompatibility for cerebellar neurons adhesion with active viability indicated by lactate dehydrogenase (LDH) assay and fluorescence cellular metabolism observations; while no cell growth was observed on unmodified silicone rubber.

3.4 LbL Thin Film for Biosensors and Bioassays

The construction of biosensors involves the immobilization of enzymes or antibodies. The electrostatic self-assembly LbL technique provides a possible way to immobilize enzymes and build up ordered protein multilayer structures. For construction of amperometric or potentiometric biosensors, protein immobilization is performed on platinum, gold or glassy carbon electrode surfaces which are usually inert, thus LbL assembly of multilayer is prohibited. Various surface modified electrodes have been introduced to achieve multilayer construction. Modified electrodes with positively charged surfaces, such as gold/3-mercaptopropyl sulfonic acid (MPS) electrodes [47] and indium tin oxide (ITO) electrodes [48, 49]; and modified electrodes with negatively charged surfaces, such as pyrolytic graphite [50, 51] and gold/cystamine dihydrochloride (Cys) [52], have been applied for protein multilayer immobilization. Besides surface charge modification, avidin functionalized platinum electrodes followed by deposition of biotin-labelled enzymes have been reported for construction of biospecific LbL assembly for biosensor applications [53]. Furthermore, LbL assembly can be applied for immunobiosensors based on the surface plasmon resonance technique. Gold substrates are first modified with avidin followed by alternate deposition of biotin-labelled antibodies and avidin for the construction of a multilayer of antibodies for the detection of human immunoglobulin [14]. Various LbL constructed biosensors are summarized in Table 3.1.

LbL assembly for the construction of biosensors has the advantage of increasing the sensitivities of the biosensors due to the ultrathin property of the protein layers. The sensitivities and linear ranges of biosensors can be tailored by controlling the layer numbers and the amounts of protein adsorbed. Moreover, multilayer incorporation of polyelectrolytes with redox property allows the construction of reagentless biosensors. Beside the applications of protein immobilization, LbL acting as a peripheral coating of implanted biosensors has been demonstrated. Implanted glucose biosensors coated

Table 3.1 Various biosensors constructed by LbL technique.

Modified electrodes / substrates	Surface charge	Type of LbL assemble	Proteins assembled	Detected analytes
Gold / MPS	Negative	Electrostatic interaction	Horseradish peroxidase, alcohol oxidase, fluctose dehydroganse	Hydrogen peroxide, fluctose, methanol
Gold / Cys	Positive	Electrostatic interaction	Horseradish peroxidase, alcohol oxidase, fluctose dehydroganse	Hydrogen peroxide, fluctose, methanol
ITO	Positive	Electrostatic interaction	Glucose oxidase, cholesterol oxidase, cholesterol esterase	Glucose, cholesterol
Pyrolytic graphite	Negative	Electrostatic interaction	Glucose oxidase, horseradish peroxidase, myoglobin	Glucose, hydrogen peroxide
Platinum / avidin	Nil	Biospecific assembly	Glucose oxidase, lactate oxidase, ascorbate oxidase	Glucose, lactate
Gold / avidin	Nil	Biospecific assembly	Anti-human immunoglobulin antibodies	Human immunoglobulin

with humic acid by LbL assembly showed an increased *in vivo* biocompatibility [54]. Moreover, silicon surfaces coated with Naflon/Fe³⁺ multilayers showed a calcification resistance property which may prolong the lifetime of implanted biosensors [55].

A new concept utilizing LbL thin film encapsulated organic micro/nanocrystals conjugated with antibodies as a probe for amplified affinity bioassay has been described by Trau *et al.* [56]. Organic nanocrystal is a hyper-dense nanometer-sized crystalline organic compound that can be triggered by chemical or physical induction to give a thousandfold increase in signal detection. One of the typical applications is using organic nanocrystals as biolabel for signal amplified affinity bioassays as shown in Figure 3.1.

Organic nanocrystals conjugated with specific antibodies, biotin-binding protein (avidin) or complementary oligonucleotides by LbL deposition technique are served as the probes for affinity bioassays. Following the affinity

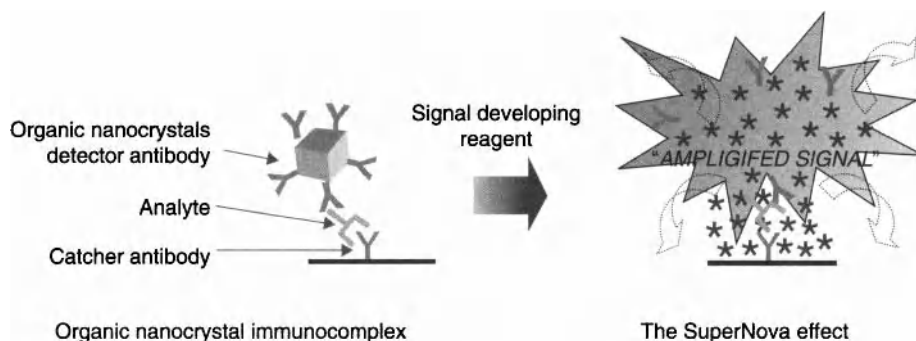


Figure 3.1 Working principle of organic nanocrystals for signal amplified affinity bioassays. Following the affinity reaction, the organic nanocrystals were dissolved by exposure to a releasing reagent leading to the instant release of a large number of signal generating molecules in the surrounding medium to create a so-called "SuperNova Effect".

reaction, the organic nanocrystals were dissolved by exposure to a releasing reagent leading to the instant release of a large number of signal generating molecules ($\sim 10^7$ to 10^9 molecules per microcrystal with a size of 100 nm) in the surrounding medium to create a so-called "SuperNova Effect". Moreover, a high molar ratio of signal-generating molecules to biomolecules can be achieved. Organic nanocrystals with different chemical and physical properties have been applied for various bioassays such as electrochemical bioassays utilizing electrochemical active organic crystals [57] or encapsulated enzyme crystals [58, 59], fluorescence immunoassays/nucleic acid assays utilizing fluorogenic nanocrystals [60, 61, 62] and aggregation-induced emission based bioassays utilizing silole nanocrystals [63]. More recently, we have extended the application of organic nanocrystals biolabel to enhance sensitivity for point-of-care lateral flow assay [64]. In this study, an LbL surface modified indigo derivative organic compound was employed as biolabel, which can be triggered to released an intense visible blue color that can be recognized by the naked eye without the need of instrumentation. In addition, the indigo biolabel shows a higher signal-to-noise ratio (sensitivity) compared with the conventional colloidal gold label. This novel class of organic nanoparticle may offer an alternative biolabel system for the development of point-of-care immunodipsticks. The encapsulated organic nano/microcrystal biolabel technology provides a new strategy for signal amplification over the traditional enzymatic amplification system.

3.5 LbL Thin Film Architecture on Colloidal Materials

The concept of the alternate LbL deposition of polyelectrolyte multilayers onto colloidal particles was first proposed by Keller *et al.* in 1995 [65]. Various colloidal templating particles including silica [65], polystyrene (PS) microspheres [66, 67], melamine formaldehyde microparticles [68],

and organic microcrystals [69, 70] were introduced as a template. Besides inorganic and synthetic organic colloidal templates, LbL assembly on bio-colloids such as catalase microcrystals, glucose oxidase microcrystals, red blood cells, white blood cells, *Escherichia coli* bacteria and yeast cells were described [71, 72, 73]. Moreover, varying assembly species such as polyelectrolytes [67], inorganic nanoparticles [74, 75], protein [76, 77, 78] or DNA [79] can be assembled onto the colloidal surface to fabricate various functional core shell materials.

The creation of hollow polymer microcapsule via LbL thin film architecture on colloidal materials is the most significant discovery with promising biomedical applications especially in the field of drug delivery. The fabrication of polymer capsules is simply done by sequential deposition of multiple layers of polyelectrolytes onto sacrificial template particles, followed by removal of the template [80]. Homogeneous microcapsule can be fabricated with colloidal gold, polystyrene or melamine formaldehyde sacrificial template with well-characterized diameter [81, 82, 83]. The wall thickness and permeability of the resulting microcapsules can be precise through varying the number of polymer layers deposited, and the type of polyelectrolytes used for LbL deposition which provide a promising carrier for controlling drug retention, degradation and drug release rates.

3.6 LbL Thin Film for Drug Encapsulation and Delivery

Encapsulation technologies are utilized in the field of medicine, pharmaceuticals, agriculture, and cosmetics as controlled-release delivery systems for encapsulated drugs, vaccines, hormones, antibodies, pesticides, DNA fragments, enzymes and other substances. Effective loading of the interested drug substances into polymer microcapsules are especially important for drug delivery application. Depending on the properties of the polyelectrolyte, such as the ionic strength of the associating/dissociating group and molecular weight, polymer microcapsules can be designed with reversible controlled permeability in response to changes in the pH, ionic strength or solvent to achieve drug loading and release.

The pH-controlled encapsulation and release of macromolecules (α -chymotrypsin) from polymeric capsules have been introduced by Tiourina *et al.* [84]. Hollow capsules were prepared via alternative adsorption of oppositely charged polyelectrolytes onto decomposable melamine formaldehyde cores. The resultant hollow capsules contain pores that were opened for macromolecule penetration at below pH 6 and closed at above pH 8. The operation principle of opening/closing the capsule walls was further studied by Rubner *et al.* who discovered that varying the pH of the solution induced a charge balance between polycations and polyanions in the multilayer, thus resulting in opening of pores with a diameter of 50 nm [85]. Beside a pH-controlled pore opening/closing system, a pore opening/closing system based on varying the

solution dielectric constant has been studied. The hollow capsules exposed to a water/ethanol mixture leads to pores opening, while there is a closing of the pores in water solution. The encapsulation of urease into hollow polymeric capsules based on solution dielectric controlled pore opening/closing system has been demonstrated by Lvov *et al.* [86].

Apart from controlling drug loading and release, cellular uptake is another major challenge for drug delivery. The typical size of a polymer microcapsule ranges from hundreds of nanometers to several micrometers, which is relatively large for effective cellular uptake. The first demonstration on the interaction of multilayered polymer capsules with cells was carried out by Sukhorukov *et al.* They showed that polymer capsules with a diameter of $\sim 5\mu\text{m}$ can be internalized by mammalian cells [87]. Following this breakthrough, LbL microcapsules fabricated with different polyelectrolytes such as PSS/PAH, PLL/PGA, dextran/poly-Arg and PMA HC were applied in cellular studies to investigate their binding and internalization behavior in various cell types such as white blood cells, mammalian cells and VERO-1 cells [88, 89]. All these studies demonstrated polymer capsules can be internalized by various cell types. A comprehensive study on the surface properties of polymer capsules composed of different outermost polymer layers, such as PAH, PSS, PEI, PEG and albumin, was investigated against their efficiency towards cellular uptake [90]. Results showed that all the polymer capsules with different surface properties can be internalized by the cells.

Polymeric capsules composed of synthetic polymer are stable, however their cytotoxicity is a major concern for *in vivo* application. Among different polyelectrolyte combinations for making polymer capsules, the PSS/PAH capsule was well characterized and was shown to exhibit high colloidal stability [91, 92]. Most cytotoxicity studies that focus on PAH/PSS capsules concern the viability of macrophages and dendritic cells [93]. All studies show that the cytotoxicity of PAH/PSS capsules is dependent on the effective dosage, and the PAH/PSS capsules does not significantly affect cell viability at a concentration of several tens of capsules per unit cell. Besides the concentration of the capsule dosage, the size of the capsule is also an important factor on cytotoxicity and influences the cell viability. The study was performed by comparing the cytotoxicity of PSS/PAH capsules with a large diameter of $\sim 8\text{--}10\mu\text{m}$ and capsules with a small diameter of $\sim 1\text{--}2\mu\text{m}$; while keeping the capsule-to-cell ratio constant. Results indicated that the larger-size polymer capsules will significantly decrease the cell viability. It has been proposed that the increase in cytotoxicity of large-size capsules is related to the sedimentation of capsules onto adhered cells rather than the cytotoxicity of the materials that form the capsules [94]. Other studies on cytotoxicity with different polymer capsules, including dextran/poly-Arg capsules, showed over 80% of dendritic cells remained viable with an effective concentration of 30 capsules per unit cell [95]; PGA capsules showed no significant decrease in LIM1899 cell viability with an effective concentration of 24 capsules per unit cell [96].

3.7 LbL Thin Film Based Micro/Nanoreactor

The advantage of LbL self-assembly technique applied to colloidal particles is that different functional species can be incorporated in the multilayer films. This results in a high concentration of functional groups or biomolecules that can be in ultimate contact with the core colloidal templates. The large surface areas to volume ratios of the colloidal particles increase the contact areas between the biomolecules and the outer environment, enhancing the efficiency of the bioreaction. Nanometer-diameter latex particles with surface functionalized glucose oxidase and urease multilayer have been reported for the construction of nanobioreactors [76, 77, 78, 97]. The LbL technology has the advantage of providing a simple procedure for the construction of nanoscaled bioreactors with tailored biological functions and activities. Moreover, Fang *et al.* demonstrated the incorporation of magnetic nanoparticles in the micro-bioreactors to introduce a self-stirring property which can further enhance the effectiveness of the bioreactors [7]. Besides surface functionalized latex particles, the concept of encapsulating enzyme crystals such as catalase and glucose oxidase microcrystals as the core materials for construction of microbioreactors has been demonstrated by Trau *et al.* [58, 59]. The resulting functionalized nano- or microbioreactors have potential applications in catalysis, separation and bioremediation.

Recently, research has been focused on using LbL polymer capsule as an individual compartment to perform complicated chemical and biochemical reaction. Dähne *et al.* demonstrated the concept of using polymer microcapsules as compartments for chemical polymerization reaction in order to synthesize copolymer [98]. Due to the controlled permeability property of the polymer capsule, small molecular weight monomers permeate the capsule wall easily, while the synthesized copolymer (cargo) has a larger molecular weight loaded in the capsule cage. The physicochemical properties of the capsule interior such as ion strength, pH, light absorption, and fluorescence could be controlled independently from the surrounding solvent by means of the chemical nature of the captured polymer.

The cargo-loading concept into microcapsules was further extended to include biochemical reactions. Mak *et al.* developed an evolutionary way to perform millions of individual polymerase chain reactions (PCR) in parallel by using diffusion controlled microcapsules as reaction compartments, termed "Microcapsule-PCR" as shown in Figure 3.2 [99]. Microcapsules were fabricated through matrix assisted entrapment of the PCR reagent mixture followed by formation of polymer capsule via LbL method. The microcapsules were found to be temperature stable when subjected to 35 times PCR thermal cycles up to a temperature of 95 degree. A multiplex Microcapsule-PCR experiment also demonstrated that microcapsules are individual compartments and do not exchange templates or primers between microcapsules during PCR cycling. A similar approach was employed for the synthesized RNA within LbL microcapsules [100]. RNA was successfully transcribed from an entrapped

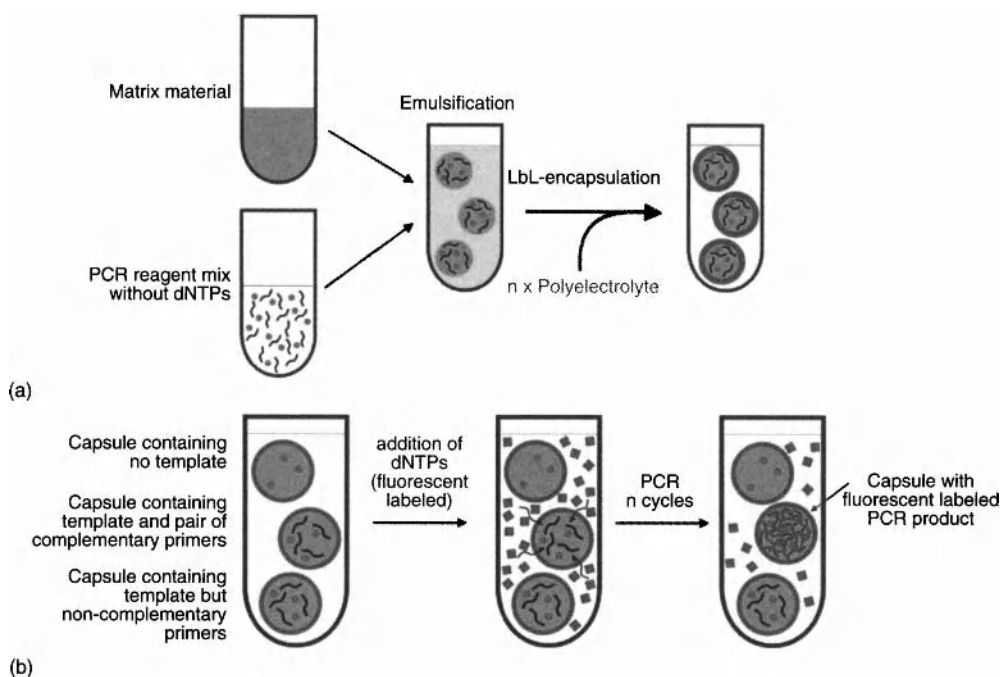


Figure 3.2 (a) Schematic illustration of the "Matrix Assisted LbL-Encapsulation" process. Agarose microbeads entrapping PCR reagents are created by emulsification and are consecutively encapsulated with polyelectrolyte multilayers, creating LbL capsules with controlled permeability. (b) Principle of the Microcapsule-PCR method. A PCR reaction only occurs in capsules containing template and a complementary primer pair, resulting in accumulation of a fluorescent labeled PCR product.

DNA template upon external introduction of RNA polymerase and by feeding nucleosides from bulk solution into the capsule interior. Significant progress on performing biochemical reaction using LbL polymer capsules inspired a new research direction to mimic the biochemical reaction in LbL polymer capsules and to create artificial cells.

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Polycaprolactone based Nanobiomaterials

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Abstract

Polymeric biomaterials have been used extensively in the medical, personal care and pharmaceutical applications including drug delivery, artificial implants, and functional materials for tissue engineering, organ regeneration and food applications across disciplines. This chapter will emphasize polymers used in medicine and specifically those designed as scaffolds for use in tissue engineering and regenerative medicine. Nanocomposites have emerged in the last two decades as an efficient strategy to improve the structural and functional properties of most synthetic polymers. Aliphatic polyesters such as polylactide (PLA), poly(glycolides) (PGA), and poly(ϵ -caprolactone) (PCL) have attracted wide attention for their biodegradability and biocompatibility with the human body. A logic consequence has been the introduction of organic and inorganic nanofillers into biodegradable polymers to produce nanocomposites based on hydroxyapatite, metal, inorganic filler and clay nanoparticles or carbon nanostructures, in order to prepare new biomaterials with desired and more importantly tuned properties. In this chapter, different results on the fabrication of nanocomposites based on polycaprolactone (PCL) biomaterials for the specific fields of tissue engineering and drug delivery systems are presented. Different types of nanocomposites with polycaprolactone (PCL) and its copolymer are reported on, specifically their cytotoxicity or biocompatibility with improved mechanical properties and biodegradation. Several specific advantages of using PCL nanocomposites as biomaterials in selected applications are also highlighted.

Keywords: Polycaprolactone, nanocomposites, nanoparticles, preparation, properties, structure, crystallization, biodegradation, biocompatibility

4.1 Introduction

In the last few decades, wide varieties of biomaterials have been developed with different physico-chemical, mechanical and biochemical properties depending on the biomedical applications. Biocompatibility is directly related to the chemical and biochemical characteristics of the material with the quality of not having toxic or injurious effects on biological systems [1]. Recently,

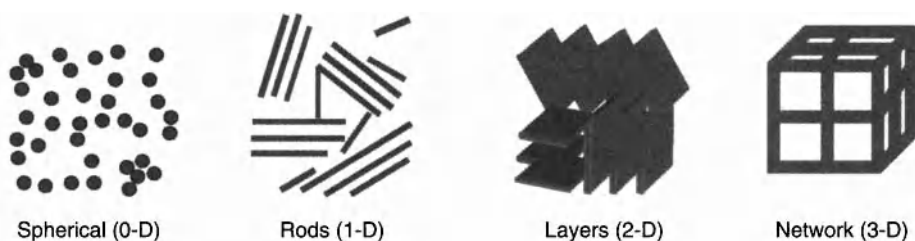
biocompatibility has been considered as the ability of a material to perform within an appropriate host responding to a specific application, taking into account the activity of the biomaterial in the host. Amongst the prominent applications for biomaterials are orthopedics, cardiovasculars, ophthalmics and drug-delivery systems. Depending on the application, biodegradable polymers are classified as the second generation bioimplant materials due to their advantages over the first generation metallic and ceramic implants, as they do not leave any undesired foreign elements in the body. Further, a second operation is not required and applications are made simple where implant removal is not feasible. One of the main applications of the biodegradable polymers is temporary scaffolds in tissue engineering (2–10). Amongst the biodegradable polymers, the most commonly used are poly(ϵ -caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PLG) and their copolymers, because of their excellent bioresorbability and biocompatibility [11–15].

Polycaprolactone is regarded as both soft and hard tissue compatible bioresorbable material [16] and it has been considered as a potential substrate for wide application, such as in drug delivery [17–20], tissue-engineered skin [21], axonal regeneration [22] and scaffolds for supporting fibroblast and osteoblast growth [23]. These advances in biomaterial research have made possible the rapid emergence of tissue engineering. This new interdisciplinary field applies principles of engineering and life sciences toward the development of biological substitutes with many different applications [24]. Among these applications, the development of vascular grafts require the selection of the appropriate biomaterial, and the culture of specific cell types on this material, in order to reproduce the characteristics of the vascular tissue. The fundamental requirements of the biomaterials used in tissue regeneration are biocompatible surfaces and favorable mechanical properties. Conventional single-component polymeric materials sometimes cannot satisfy these requirements. In fact, although various polymeric materials are available and have been investigated for tissue engineering, no single biodegradable polymer can meet all the requirements for biomedical scaffolds. Therefore, the design and preparation of multi-component polymeric systems represent a viable strategy in order to develop innovative multifunctional biomaterials. In particular, this review deals with the introduction of nanostructures in biodegradable polymer matrices to obtain nanocomposites with specific properties able to be used in tissue engineering. Nanotechnology enables the development of new systems that mimic the complex and hierarchical structure of the native tissue. Therefore, a confluence of nanotechnology and biology can address several biomedical problems and can revolutionize the field of health care and medicine [25].

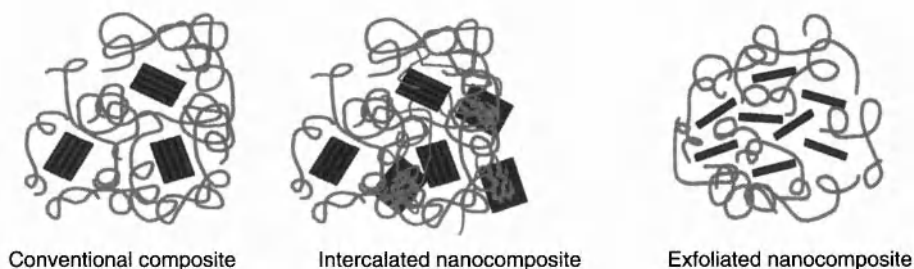
Nanocomposites are the new class of composites consisting of two component systems where one of the components must have at least one dimension in nanometer length scale. Most often the inorganic components act as filler for the polymer matrix. The reinforcing agent/filler can be of any dimension, e.g.: zero dimensional, such as metal oxides and their clusters; one dimensional like nanotube or nanofiber; two dimensional, such as layered materials including layered silicates; so-called nanoclay, and; three dimensional

framework systems such as zeolites. A typical classification of fillers is represented in Scheme 4.1. Depending on the dispersion of nanoparticles, polymer nanocomposites are usually divided into three general categories (Scheme 4.2): *conventional composites* where the clusters of nanoparticle act as conventional macro-filler; *intercalated nanocomposites* consisting of a regular insertion of polymer between the layers, and; *exfoliated nanocomposites* where 1 nm thick layers are dispersed forming a monolithic structure at microscale. Because of their nanometer-size dispersion, the nanocomposites exhibit markedly improved properties as compared to the pristine polymer [26–30]. The improvement in properties includes high modulus, strength and toughness, decreased gas permeability, increased and regulated biodegradability and thermal stability [31–34]. A basic reason for those improvements in properties is the interfacial interaction between polymer matrix and nanoparticles. As a matter of fact, natural bone is an organic/inorganic composite material of collagen and apatite. From this viewpoint, composite materials are excellent choices as bone tissue engineering scaffolds.

In this chapter, different results on the fabrication of nanocomposites based on polycaprolactone (PCL) as biomaterials for focused application of tissue engineering and drug delivery are presented. Different types of nanocomposites of polycaprolactone (PCL) and its copolymers have been reported, especially their cytotoxicity or biocompatibility with tuned mechanical properties and biodegradation. Several specific advantages of using polycaprolactone (PCL) nanocomposite as biomaterials in selected applications are also highlighted. The chapter also examines the critical issues and scientific challenges that require further research and development of PCL nanocomposite materials for their increased acceptance in the biomedical industry.



Scheme 4.1: Different types of nanofillers



Scheme 4.2: Three possible combinations of filler and polymer composites

4.2 Preparation of Polycaprolactone Nanocomposites

Several techniques have been reported to prepare PCL nanocomposites. The preparation techniques are divided into three types based on the starting materials and processing conditions.

4.2.1 Solution Casting Method

In this technique, the polymer is first dissolved in a solvent. Then, the nanoparticles are dispersed in the polymer solution. The nanoparticles can nicely be distributed in the polymer matrix after removing the solvent by fast evaporation. Maiti *et al.* [35] have prepared PCL nanocomposite by solution casting technique. The organically modified nanoclay was sonicated in chloroform to achieve a good dispersion. The nanocomposites of PCL were prepared through solution route by dissolving PCL in the dispersion of nanoclay in chloroform, followed by removing the solvent at a fast rate. The solution was stirred for 30 min to ensure proper mixing. Liu and his coworkers [36] have prepared PCL/LDH nanocomposite by solvent-casting method. Firstly, a desired amount of OCoAl-LDH was sonicated at a frequency of 40 kHz for 0.5 h and refluxed in 50mL cyclohexanone for 12 h under flowing nitrogen. Subsequently, this solution was added to the PCL solution in 50mL cyclohexanone and refluxed for another 12 h. Finally, the solution was poured into 300ml cool methanol. The precipitates, PCL/CoAl-LDH nanocomposites, were filtered and dried under vacuum at 40°C for 48 h. Tasdelen [37] prepared the polycaprolactone/clay nanocomposites by solution casting using "click" chemistry. In this method, ring-opening polymerization of ϵ -caprolactone using propargyl alcohol as the initiator has been performed to produce alkyne-functionalized PCL and the obtained polymers were subsequently attached to azide-modified clay layers by a CuAAC "click" reaction.

4.2.2 Melt Extrusion Technique

In this method, the nanoparticles are mixed with the polymer matrix in the molten state, preferably in a single/twin screw extruder. Solvent is not required in this technique. Powdered polymer and nanoparticles were mixed in a high speed mixer to prepare the premixture prior to extrusion. Chen and Evans [38] prepared the composites using melt processing. A preweighed amount of clay were gradually added to PCL melt in a heated twin roll mill. The mixture was stripped from the rolls and refed at least five times to ensure proper mixing. The average processing temperature was 128°C. Perrin *et al.* [39] have prepared the PCL-30B nanocomposites by melt intercalation using twin screw extruder. The developed screw configuration allowed them to obtain an intercalation/exfoliated nanocomposite. The processing temperature was set at 140°C with the screw speed of 250 rpm and the polymer flow rate at 3kg/h. Dubois *et al.* [40] have prepared the nanocomposite of PCL with natural Na⁺ montmorillonite

(MMT) and MMT modified with hydrogenated tallow alkyl (HTA) based quaternary ammonium cations, such as dimethyl 2-ethyl hexyl HTA ammonium and methyl bis(2-hydroxyethyl) HTA ammonium by mechanical kneading with an agila two-roll mill at 130°C for 10 min. The collected molten materials were compression molded within thick plates by hot pressing at 100°C. The composites of PCL and organically modified MMT (25A, 30B) were also prepared in the same fashion through mechanical kneading [41, 42].

4.2.3 In Situ Polymerization

This technique involves the dispersion of nanometer-size inorganic particles in monomer followed by polymerization using simple bulk or solution method in the presence of a small amount of catalyst. Ultimate dispersion of nanoparticle in resulting nanocomposites is better in this process as the nanoparticles can be distributed homogeneously in low viscous monomer. There are lots of examples of this category for PCL and other polymers. In 1993 Messersmith and Giannelis [43] reported the first preparation of PCL based nanocomposites by *in situ* intercalative polymerization method. They used Cr³⁺ exchanged fluorohectorite (FH) for the synthesis of nanocomposites. In a typical synthesis process, a mixture of 0.1g of Cr³⁺ FH and 1g of CL was stirred at 25°C for 12 h, followed by heating at 100°C for additional 48 h. The reaction mixture was solidified upon cooling to room temperature. Unintercalated PCL fractions of the composites were recovered by dissolving a portion of the product in acetone followed by centrifugation at 3000 rpm for 2 min. Similarly, Dubois and his coworkers [44] have prepared poly(ϵ -caprolactone)/clay nanocomposites using *in situ* ring-opening polymerization of ϵ -caprolactone by using dibutyltin dimethoxide as an initiator/catalyst. An unmodified Na⁺-montmorillonite, dimethyl 2-ethylhexyl (hydrogenated tallow alkyl), and methyl bis(2-hydroxyethyl) (hydrogenated tallow alkyl) ammonium cations surface-modified MMT were used. Several other reports have been published on the preparation of PCL nanocomposites using *in situ* intercalative polymerization techniques [45–48].

4.3 Characterization of Poly(caprolactone) Nanocomposites

After the formation of nanocomposites, both the nature of nanoparticles and polymer may change relating to structure and morphology. In general, the structure of nanocomposites has typically been established by using wide angle X-ray diffraction (WAXD) analysis and transmission electron microscopic (TEM) observation to probe the nanostructure of the nanocomposites. The structure of nanocomposites based solely on WAXD patterns is only tentative. On the other hand, TEM allows a qualitative understanding of the internal structure, spatial distribution of various phases and views of the defect structure through direct visualization.

4.3.1 Nanostructure

Several articles have reported the nanostructure of PCL layered silicates nanocomposites [36, 38, 40]. Messersmith and Giannelis characterized PCL layered silicate nanohybride by using XRD [43]. Intercalation of the CL monomer was revealed by powder XRD, which shows an increase in the silicate d -spacing from 1.28 to 1.46 nm. The d_{001} spacing observed prior to polymerization was found to be consistent with the orientation of the CL ring perpendicular to the silicate layers. XRD analysis of the nanocomposite after polymerization indicates a reduction in the silicate d -spacing from 1.46 to 1.37 nm as presented in Figure 4.1. The decrease in the d -spacing is consistent with the dimensional change accompanying polymerization of CL monomer. Opening of the lactone ring in the monomer to produce a monolayer of fully collapsed PCL chains is accompanied by a decrease in layer spacing as observed in the XRD measurement. The observed layer spacing of 1.37 nm correlates as well with the sum of the thickness of the silicate layer (0.96 nm) and the known interchain distance (0.4 nm) in the crystal structure of PCL. Repeated washing with a solvent for PCL did not alter the silicate layer spacing, indicating that the interaction between the intercalated polymer and the silicate surface is strong and that intercalation of the PCL is irreversible. Dubois *et al.* (44) investigated the nanostructure of PCL nanocomposites prepared using pure MMT and organically modified nanoclay (MMT- C_8H_{17} , 30B). Figure 4.2a,b show the PCL-MMT and PCL/ MMT- C_8H_{17} nanocomposites with intercalated nanostructure as evidenced from the shifting of the (001) peak of the nanoclay. Further, the intercalation is higher in PCL/MMT- C_8H_{17} as compared to PCL/ MMT with both having 3 wt% of clay content in the respective composites. Maiti *et al.* studied the nanostructure of both oligo-poly(caprolactone) [41] and PCL with different layered silicates. The nature and extent of intercalation of the oligomer strongly depends on the type of interactions between the oligomer and the

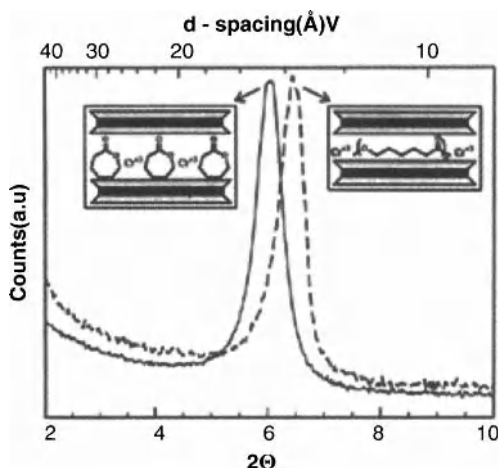


Figure 4.1 Powder XRD of the composite before (solid line) and after (dashed line) polymerization. Insets are schematic illustrations (not drawn to scale) corresponding to the intercalated monomer (left) and intercalated polymer (right) (43).

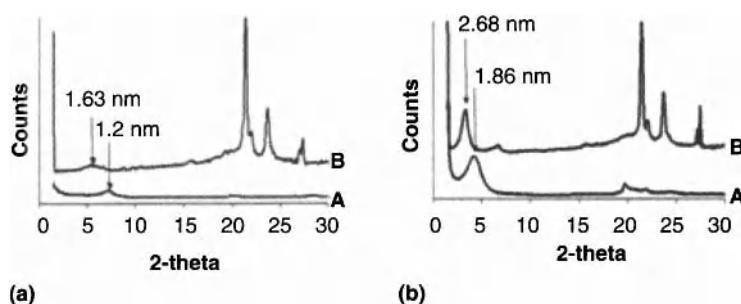


Figure 4.2 a) XRD profiles for (A) native montmorillonite (MMT-Na) and for (B) a PCL nanocomposite containing 3 wt % MMT. b) XRD profiles for (A) the montmorillonite modified by dimethyl 2-ethylhexyl (hydrogenated tallow alkyl) ammonium (MMT-C₈H₁₇) and for (B) a PCL nanocomposite containing 3 wt % of this modified clay (44).

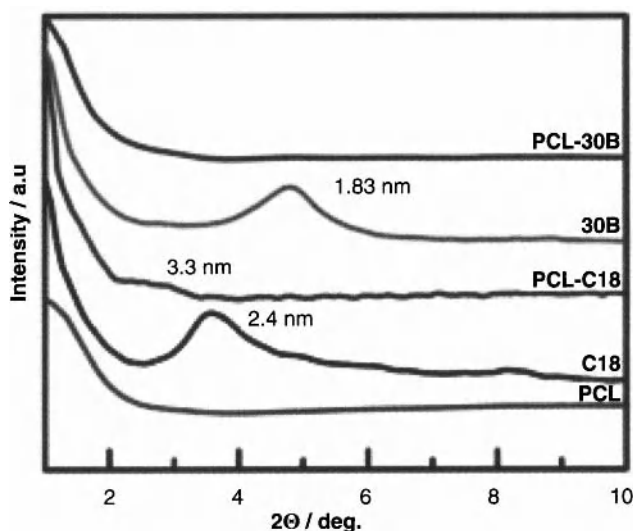


Figure 4.3 Wide-angle X-ray diffraction patterns of indicated organically modified nanoclays and PCL nanocomposites. The numbers in nanometers represent the gallery spacing calculated using Bragg's equation (35).

organically modified silicate. WAXD patterns of the poly(caprolactone) nanocomposites of 30B and C18 are presented in Figure 4.3 [35]. The peaks in the X-ray diffraction patterns are indicating intercalated pattern for PCL-C18 in which the gallery spacing increases as a result of polymer intercalation while the PCL-30B exhibits exfoliated nanostructure.

4.3.2 Microstructure

The qualitative idea of nanoparticles dispersion in different polycaprolactone matrices has been shown in this section. Dubois *et al.* have investigated the nanoclay dispersion by using transmission electron microscopy [44, 40] of methyl bis(2-hydroxyethyl) (hydrogenated tallow alkyl) ammonium (30B) modified

MMT in PCL matrix (36) and dimethyl 2-ethylhexyl (HTA) ammonium modified MMT-alk [40]. The exfoliated structure has been confirmed using TEM as illustrated in Figure 4.4a showing a typical micrograph for the nanocomposite containing 3 wt% of nanoclay. Individual clay platelets are randomly distributed in PCL matrix. In addition, the dispersion of modified MMT-alk presented in Fig. 4.4b shows small stacks of intercalated nanoclay. The dispersion depends on clay concentration and the distribution has been presented in Figure 4.5a while the unmodified clay exhibit clusters of clay shown in Fig. 4.5b indicate lesser interactions [50]. These clay aggregates are larger, less diffuse and darker compared to the intercalated and partially exfoliated stacks seen in Fig. 4.5a.

Several other reports on microstructure of PCL nanocomposites with MMT and layered double hydroxide have been investigated [36, 51, 52].

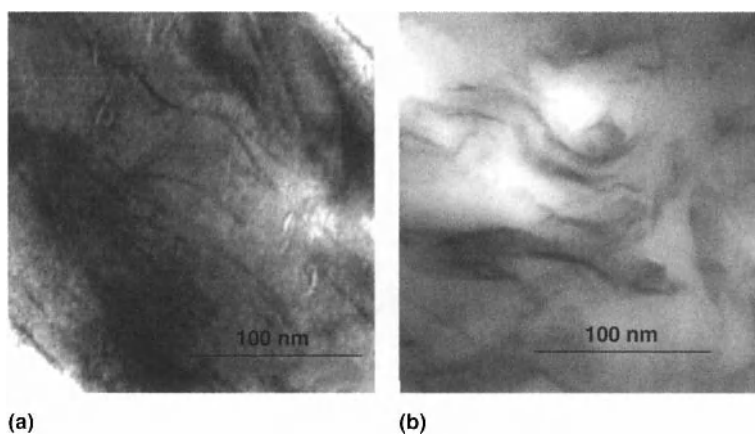


Figure 4.4 TEM images for PCL nanocomposites containing 3 wt% of organomodified montmorillonite (44) and (b) MMT-alk (40).

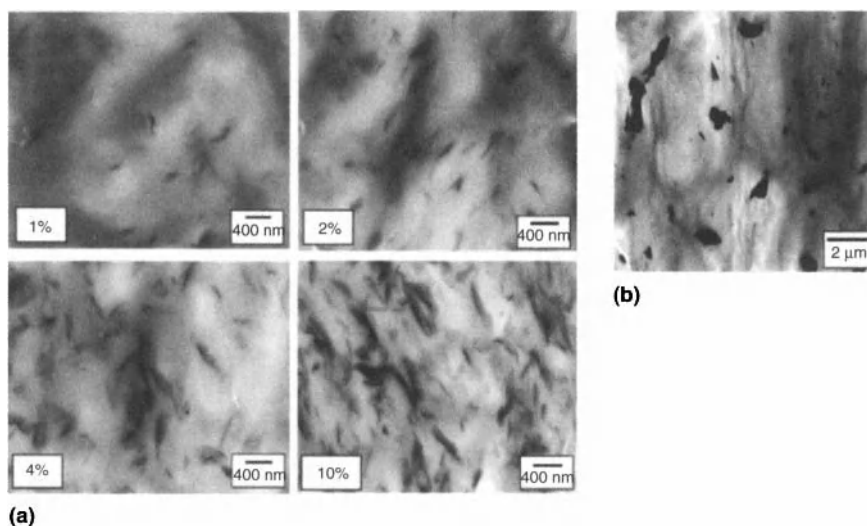


Figure 4.5 (a) Transmission electron micrographs of PCL-MMT composites at the indicated nanoparticle concentrations. (b) Transmission electron micrographs of PCL composite (8% unmodified MMT) (50).

4.4 Properties

4.4.1 Mechanical Properties

4.4.1.1 Dynamic Mechanical Analysis

Dynamic mechanical analyzer (DMA) measures the response of a given material to an oscillatory deformation (usually in tension or torsion mode) as a function of temperature. DMA measurements are of use mainly for three parameters: (i) the storage modulus (E'), related to the elastic response of the material under deformation, (ii) the loss modulus (E''), corresponding to the plastic response to the deformation, and (iii) $\tan \delta$ or loss tangent useful for determining the occurrence of molecular mobility such as glass transition temperature or other transitions. DMA analysis has been used to study the temperature dependence of the storage modulus of pure PCL and its nanocomposites under different experimental conditions. Figure 4.6 shows the storage modulus as a function of temperature for modified sisal whiskers (panel A) and microfibrillated Cellulose (MFC) (panel B) reinforced PCL composites [53]. The decrease of modulus around -60°C is associated with the glass transition and the irreversible polymeric chain flow around 50°C is due to the melting of the PCL matrix. However, the storage modulus has increased upon increasing filler concentration. DMTA for PCL/fumed silica nanocomposites have been performed to see the effect of silica on the thermomechanical properties in the linear viscoelastic region [54]. The addition of HDK D05 (fumed silica nanoparticle) to PCL decreases the modulus below glass transition (Figure 4.7), whereas, slight increases have been found by the addition of other variety of fumed silica particles (Table 4.1). The decrease observed for HDK D05 is attributed to a hindered stress transfer from the polymer to the inorganic silica particle. On the other hand, above T_g (at 25°C) a reinforcement effect of the silica

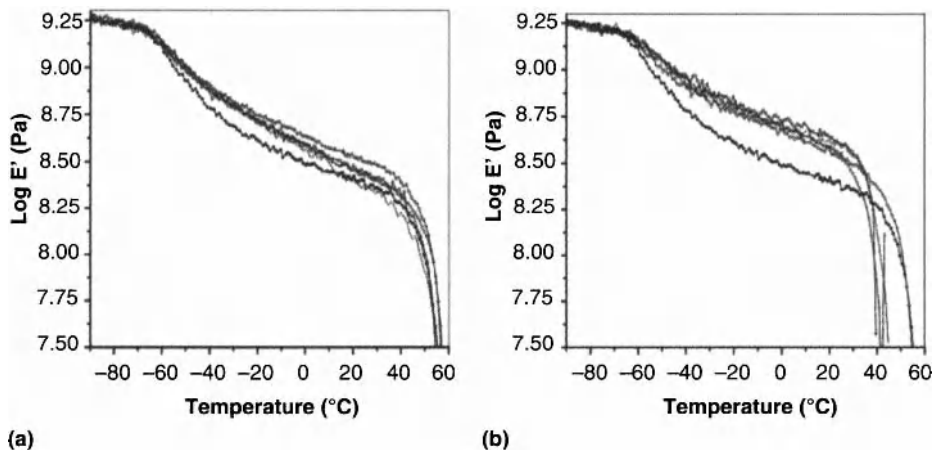


Figure 4.6 Evolution of the storage modulus ($\log E'$) vs. temperature at 1 Hz for PCL based nanocomposites reinforced with modified sisal whiskers (A) or modified MFC (B) 0 wt % (■), 3 wt % (○), 6 wt % (+), 9 wt % (x), and 12 wt % (Δ) [53].

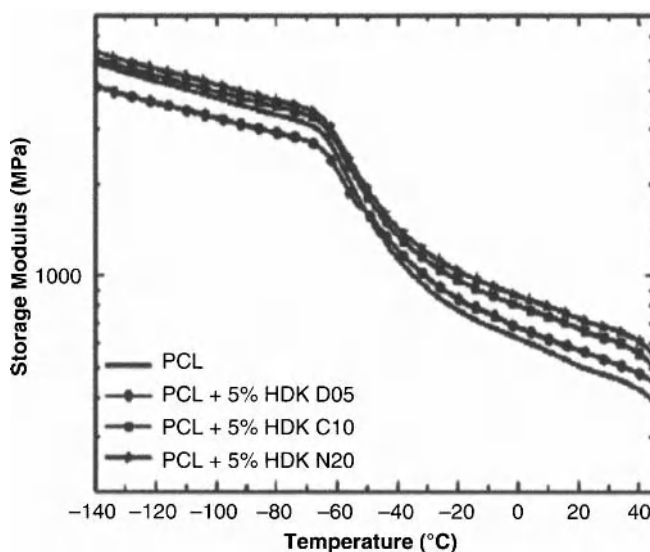


Figure 4.7 Temperature dependence storage modulus (E') for *PCL* and its fumed silica composites with varying concentration (54).

Table 4.1 Storage modulus values of *PCL* based composites at different temperatures (54).

Sample	E' at -125°C (MPa)	E' at -25°C (MPa)	E' at 25°C (MPa)	$\tan \delta$ (°C)
<i>PCL</i>	4427	836	488	-49
<i>PCL</i> +5% <i>HDK</i> D05	3750	903	552	-46
<i>PCL</i> +5% <i>HDK</i> D10	4594	1038	642	-48
<i>PCL</i> +5% <i>HDK</i> D20	4839	1104	698	-49

particles is observed, leading to increases in modulus. The addition of silica in *PCL* matrix was found to have a slight effect on $\tan \delta$. The increase in storage modulus indicates the reinforcement effects of fumed silica in *PCL* matrix. This reinforcement effect is more evident with increasing the temperature and concentration of the fumed silica with higher surface area and state of dispersion.

4.4.1.2 Tensile Properties

The tensile modulus of polymeric materials has been shown to be remarkably improved in nanocomposites as compared to pure *PCL*. The influence of clay content on the tensile properties of the nanocomposites has been shown in Table 4.2 with different clay and its content [55]. For the sake of comparison, Young's modulus, ultimate tensile strength and elongation at break have been measured for the microcomposites prepared by direct melt blending of the unmodified montmorillonite (MMT-Na) with *PCL* under the same conditions.

Table 4.2 Tensile properties of neat *PCL* and *PCL*-based nanocomposites containing native montmorillonite (*Na*-*MMT*) prepared by direct melt blending and by the masterbatch (M1) process (55).

Sample	Clay content (wt%)	Ultimate tensile strength (MPa)	Elongation at break (%)	Young modulus (MPa)
Neat <i>PCL</i>	0	37	746	217
Melt blending	1	35	715	201
	3	35	714	197
	5	32.5	666	207
	10	28	569	238
Masterbatch process	1	27	454	252
	3	26	448	253
	5	22	363	270
	10	22	397	365

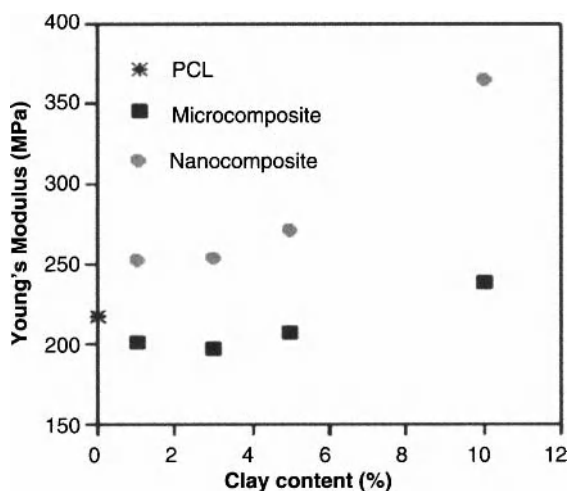


Figure 4.8 Variation of the Young's modulus on the clay content for composites containing *Na*-*MMT* Comparison of microcomposites (direct melt blending of *PCL* with *Na*-*MMT* and nanocontposites (melt blending of *PCL* with the masterbatch, M1) (55).

The difference in properties observed in two sets of composites is in terms of the difference in their morphology. *PCL* is a ductile polymer, able to sustain large deformations consistent with a rather low elastic modulus. This characteristic feature is improved from 217 MPa for the unfilled *PCL* to 365 MPa for the *PCL* nanocomposite that contains 10 wt% of clay prepared by melt blending. In contrast, microcomposites prepared from the native montmorillonite show only a small increase in stiffness (Figure 4.8). *PCL* nanocomposites

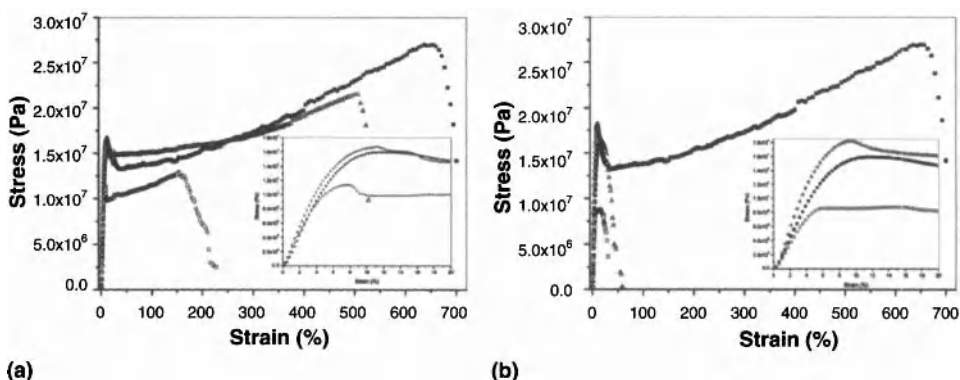


Figure 4.9 Typical stress-strain curves obtained from tensile tests for PCL-based nanocomposites reinforced with sisal whiskers with 3 WI % (A) and 12 WI % (B) unmodified (O) and chemically modified (Δ). The behavior of the neat PCL matrix is added as reference. The inset is an expanded view of the low strain region (53).

remain rather ductile, with an elongation at break higher than 300%. The stress at break decreases when the clay content is increased but remains at an acceptable level. Similar observations have been reported for two other organo-modified clays where stiffness has increased while retaining the ultimate tensile properties. Similarly, Evans and his coworkers [38] also prepared the PCL nanohybrid with pure and modified MMT and reported the increased tensile modulus by 50% at 8 wt% clay loading. Further, dramatic improvement was observed for elongation at break which increased from 165% to 550% by the addition of 10 wt% clay. Dufresne *et al.* reported the effect of cellulose nanowhisker content on the tensile properties [53]. Typical stress-strain curves of PCL-based nanocomposites are shown in Figure 4.9. Fig. 4.9a shows that the film reinforced with 3 wt% of modified sisal whiskers display higher tensile modulus, strength, and strain at break as compared to its unmodified filler counterpart. Moreover, the addition of 3 wt% of modified nanoparticles enhances the tensile modulus without detrimental effect on the ultimate mechanical properties (strength and strain at break) contrary to unmodified nanoparticles. At this loading level, the nanocomposite films display a ductile and rubber-like behavior similar to the PCL matrix. On the other hand, highly filled composites (12 wt%, Fig. 4.9b) show a completely different behavior. No plastic deformation was observed and their extreme brittleness is clearly noticed. Recently, Gorrasi and his colleague prepared ZnAl-layered double hydroxide (LDH) and incorporated it into PCL matrix using high-energy ball milling and studied the tensile testing of PCL/LDH nanocomposites; improved mechanical properties were shown [56].

4.4.2 Thermal Properties

The response of polymeric materials under heat treatment is usually characterized by using several techniques like TGA, DTA, DSC, and TMA.

4.4.2.1 Thermal Stability

The thermal stability of polymeric materials is usually studied by thermogravimetric analysis (TGA). Both intercalated and exfoliated nanocomposites are degraded at higher temperatures compared to pure PCL and PCL/clay microcomposites [44]. Thermograms for nanocomposites containing 5 wt% MMT- C_8H_{17} are compared in Figure 4.10a with pure PCL. The temperature at which 50 wt% loss noted is considered as degradation temperature which is higher by 25°C for the nanocomposites as compared to pristine PCL. This shift to higher temperature might be explained by a lower permeability/diffusivity of oxygen and volatile degradation products through the sample as a result of barrier properties imparted by highly anisotropic two dimensional nanoparticles and to char formation. Resistance to thermal degradation has improved up to 5 wt% of nanoclay in the composites. Fig. 4.10b shows the thermograms for nanocomposites containing 1–5 wt% of MMT- C_8H_{17} . Similarly, other literature reports are also available for the thermal stability of PCL nanocomposites showing improved thermal stability of PCL nanocomposites in comparison to pure PCL [36, 37, 40, 53, 56]. Wu *et al.* [57] studied the effect of CNTs on the thermal stability of PCL showing bit improvement in stability. Recently, Liu *et al.* investigated the thermal stability in exfoliated CoAl-layered double hydroxide (CoAl-LDH)/PCL nanocomposites (Figure 4.11) [36]. It is obvious to note that the degradation of nanocomposites occurs at lower temperature as compared to neat PCL and the thermal degradation temperature decreases gradually with the increase of OCoAl-LDH loading level. The thermal decomposition temperatures for neat PCL and its nanocomposites containing 1, 2 and 4 wt% LDH were determined to be 402, 384, 374, 370°C, respectively. Decrease in thermal stability is due to the presence of OCoAl-LDH.

4.4.2.2 Crystallization Behavior

Crystallization of PCL nanocomposites is an excellent technique for controlling the structure and various properties of the nanocomposites. Oligopoly(caprolactone) (o-PCL) nanocomposites with layered silicates having a

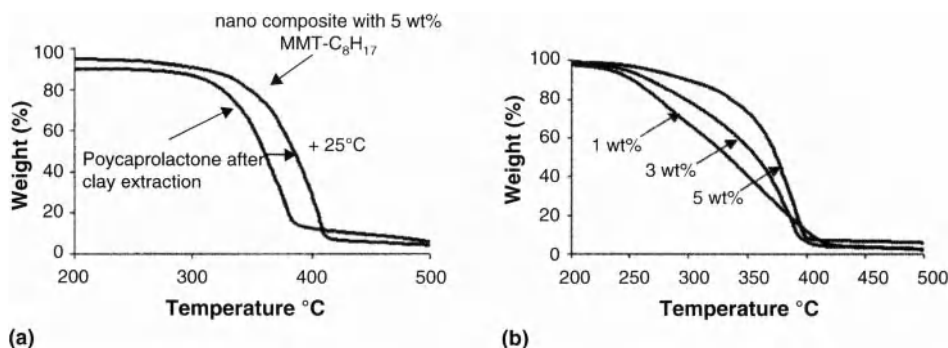


Figure 4.10 (a) Temperature dependence of weight loss for PCL nanocomposite containing 5 wt% (relative to inorganics) of MMT- C_8H_{17} and for pure PCL matrix. (b) Temperature dependence of weight loss for nanocomposites containing 1, 3, and 5 wt% (relative to inorganics) of MMT- C_8H_{17} (44).

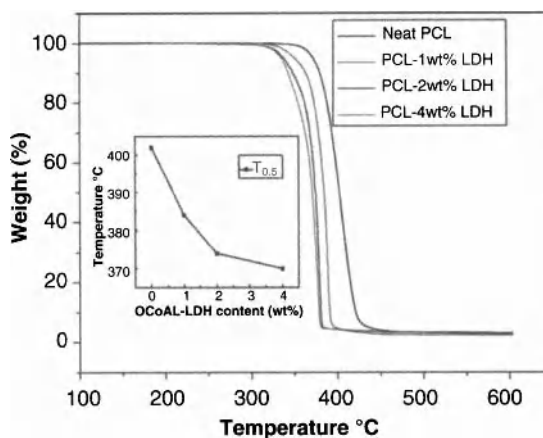


Figure 4.11 TGA profiles for neat PCL and PCL/CoAl-LDH nanocomposites with different loadings of OCoAl-LDH in nitrogen atmosphere with a heating rate of 10°C/min. The inset shows the thermal decomposition temperature at 50% weight loss ($T_{0.5}$) of neat PCL and its nanocomposites (36).

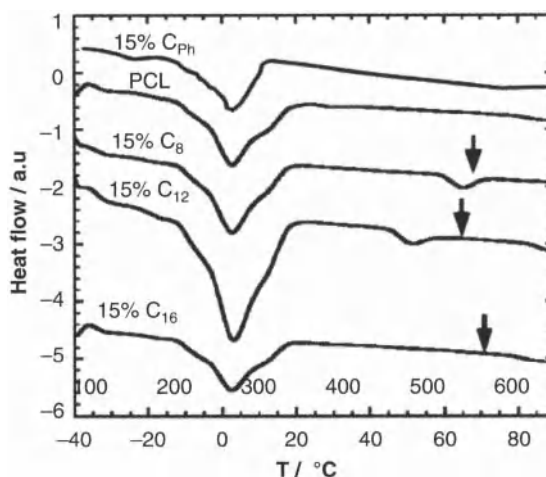


Figure 4.12 DSC thermograms of pure *o*-PCL and its nanocomposites with synthetic smectites with different phosphonium modifiers as indicated in the figure. The arrows indicate the melting temperatures of the corresponding organic modifiers (49).

variety of organic modifiers, by simple mechanical mixing have been studied to observe the effect of various modifier chain length on miscibility [49]. Figure 4.12 shows the DSC thermograms of *o*-PCL and 15 wt% of the various modified silicates revealing the chain length dependence of miscibility indicating greater compatibility of silicate with higher alkyl chain lengths with *o*-PCL. Hence, the C_{16} modified silicate is completely miscible with *o*-PCL, while the C_8 modified silicate is slightly miscible and the C_{12} modified silicate is only partially miscible with *o*-PCL. The enthalpy of fusion, ΔH gradually decreases with increasing chain length; 22, 21.3, 19.2, 18, and 17.0 J/g for PCL, C_{Ph} , C_8 , C_{12} and C_{16} , respectively. Wu and his coworkers [57] studied the effect of MWCNTs on the non-isothermal crystallization behavior of PCL, indicating higher crystallization temperature and enthalpy of crystallization with the addition of few weight

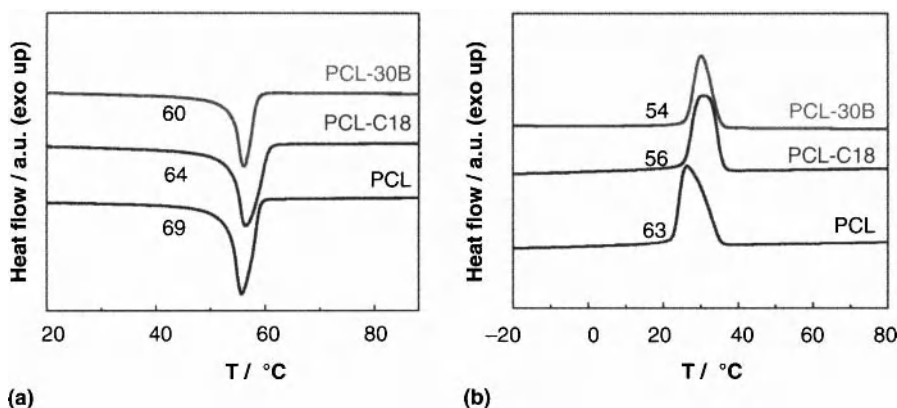


Figure 4.13 DSC thermograms of PCL and its indicated nanocomposites **a)** heating curves, **b)** cooling curves. The numbers represent the heat of fusion (a), and heat of crystallization (b) in joules/gm (35).

percentages of MWCNTs (1–3%) in PCL matrix. Recently, PCL nanocomposites with organically modified commercial nanoclays (30B, C18) have been studied to observe the effect of nanoclay on crystallization behavior of PCL in filled systems [35] (Figure 4.13a). Both pristine PCL and nanocomposites exhibit distinct endothermic melting peaks (T_m) at 55.7°, 56.3° and 56.4°C for PCL, PCL-C18 and PCL-30B, respectively. The heat of fusion (ΔH) decreases in nanocomposites in the presence of nanoclays (69, 64 and 60 J/g for pristine PCL, PCL-C18 and PCL-30B, respectively) due to interaction between polymer and nanoclays. The extent of interaction is more in the case of PCL-30B nanocomposite as revealed from the lowest heat of fusion and lowering of melting temperature. The cooling curves exhibit an increase in crystallization temperature (T_c) for nanocomposites as compared to pristine PCL. The crystallization peak is slightly broad for pristine PCL against a rather sharp peak for nanocomposites (Fig. 4.13b). The shifting of T_c to 31°C for nanocomposites against 26°C broader peak for pure PCL suggests that the clay particles act as nucleating agent [58]. The nanocomposites also appear to be less crystalline during cooling as compared to neat polymer ($\Delta H_c = 63, 56$ and 54 J/gm for PCL, PCL-C18 and PCL-30B, respectively).

Quantitative interaction parameter between polymer and nanoclays (χ) has been calculated using the depression of melting point of PCL in presence of nanoclay following the equation of Nishi and Wang [59]:

$$\frac{1}{T_{NC}^o} - \frac{1}{T_{PCL}^o} = -\frac{RV_{PCL}}{\Delta H_u V_{clay}} \phi_{clay}^2 \chi \quad (4.1)$$

where, T_{NC}^o and T_{PCL}^o are the equilibrium melting points of the nanocomposite and that of the pure PCL, respectively. V_{PCL} and V_{clay} are the molar volumes of the repeating unit of PCL and the molar volume organoclay, respectively, f_{clay} is the volume fraction of clay, and ΔH_u is the enthalpy of fusion per mole of repeating unit of PCL. The extrapolation technique of $T_m - T_c$ plot to the $T_m = T_c$ line (Hoffman-Weeks plot [60]) has been used to measure the equilibrium melting point. Figure 4.14a shows the representative plot of melting point against heat of fusion of pure PCL crystallized at different temperatures mentioned for

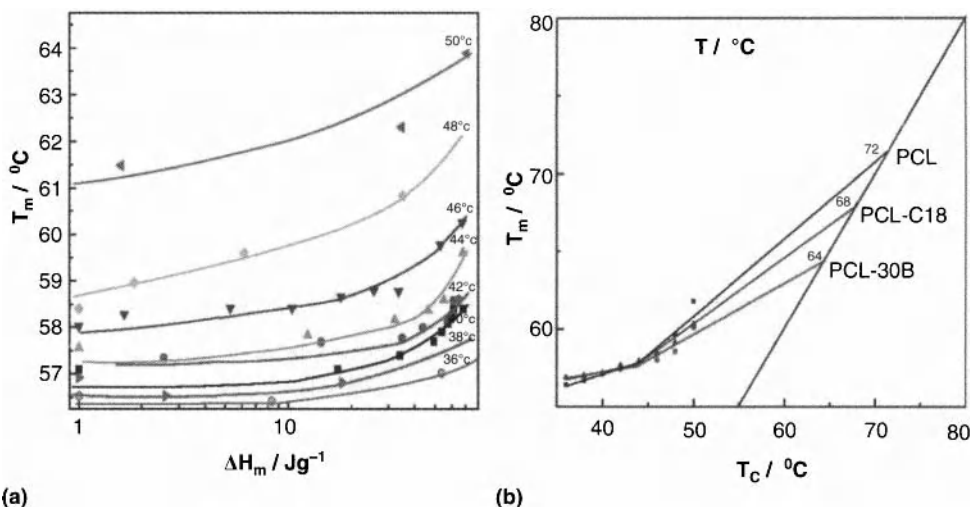


Figure 4.14 Representative plot of melting temperatures (T_m) vs. heat of fusion (ΔH) of pure PCL at the indicated crystallization temperatures (T_c). (b) $T_m - T_c$ plot at 5% ΔH for indicated specimens. The numbers show the corresponding equilibrium melting temperatures T_m° (35).

various time. The melting point increases with increasing crystallization temperature (T_c) and this phenomena is followed for the nanocomposites as well. The $T_m - T_c$ plot has been shown in Fig. 4.14b, where the melting points of the nanocomposites are slightly lower than that of pure PCL for any particular T_c . The equilibrium melting points, T_m° of pure PCL and its nanocomposites have been evaluated after extrapolation to $T_m = T_c$ line and the values are 72°, 68° and 64°C for neat PCL, PCL-C18 and PCL-30B, respectively. The equilibrium melting temperatures of the nanocomposites decreased as a result of interaction between matrix and organoclays. The calculated values of χ 's (using equn. 4.1) were thus obtained -4.91×10^{-5} and -9.95×10^{-5} for PCL-C18 and PCL-30B, respectively. The lower χ value for PCL-30B indicates stronger interaction between its components as compared to PCL-C18 nanocomposite. Similar value of χ (-8.0×10^{-5}) between PCL and other miscible polymer has been reported in the literature (61). Further, Liu and his coworkers also studied the effect of LDH on the crystallization behavior of PCL (Figure 4.15 [36]). The melt crystallization temperature increases gradually with OCoAl-LDH loading and the addition of 4 wt% OCoAl-LDH results in an increase in the crystallization temperature of PCL nanocomposite by 36°C. The increase in crystallization temperature with the incorporation of LDH into PCL matrix is due to the fact that LDH can act as nucleating agent which has a heterogeneous nucleation effect.

4.4.3 Biodegradation

Tuning of biodegradation rate is still a challenge to the scientific community especially for biomedical application of polymers. Tetto *et al.* [62] first reported the biodegradability of nanocomposites based on PCL showing improved biodegradability as compared to pure PCL. The improved biodegradability

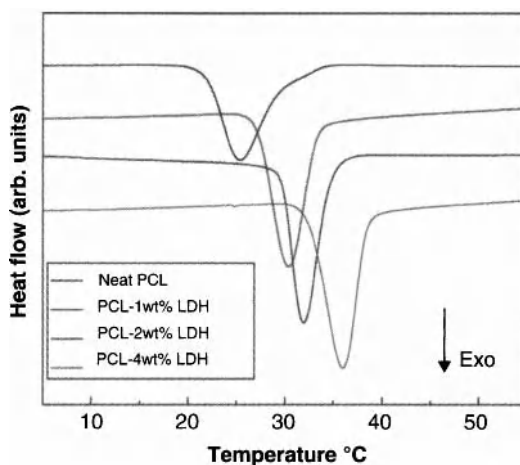


Figure 4.15 DSC cooling curves of neat *PCL* and *PCL/CoAl-LDH* nanocomposites with different filler contents (36).

of PCL in the presence of nanoparticles was explained on the basis of the catalytic activity of *OMLS* but the biodegradation mechanism was still not clear. Xanthos and his coworkers studied the degradation of polycaprolactone composites with bioglass in PBS buffer solution. Degradation rate of composites was slightly enhanced relative to pure PCL [63]. Thereafter, controlled mechanism of biodegradation of PCL layered silicate nanocomposites have been studied in detail in different media-like compost, microorganism, enzyme, etc. [35]. Biodegradation tests have been carried out at 30°C with the samples quenched to room temperature in enzyme media (lipase: type XIII from *Pseudomonas* sp. in buffer solution). The enzymatic degradation rates of nanocomposites are much faster than that of pure PCL (Figure 4.16a) and amongst the nanocomposites, PCL-30B exhibits higher degradation rate than that of PCL-C18. The matrix PCL degrades at a regulated higher rate in presence of organically modified nanoclay raising a clay dependency phenomenon. The morphology as observed in scanning confocal images (Fig. 4.16b) also exhibits highest enzymatic degradation in PCL-30B and lowest in pure PCL. The morphology at various Z-sections of the specimen also supports the similar nature of degradation as seen in confocal images. Perforated images are evident for PCL-30B nanocomposite while small holes are observed in pure PCL after biodegradation. The phase contrast images of pristine PCL and its nanocomposites (before and after enzymatic degradation) also support the relative degradation rate (Fig. 4.16c) of pure polymer and different nanocomposites.

Studies have been carried out to observe the effect of nanoclay on biodegradation rate of PCL in a microorganism (fungi: *Aspergillus fumigatus*-NCIM 902) medium. A marked increase in biodegradation rate has been observed for PCL-30B nanocomposite (more than 90% weight loss) in pure fungi medium as compared to pristine PCL (~40 wt%) (Figure 4.17a). Moreover, the rate of biodegradation of PCL-30B is higher as compared to PCL-C18, the same trend observed in case of enzymatic degradation. Biodegradation up to 95% weight

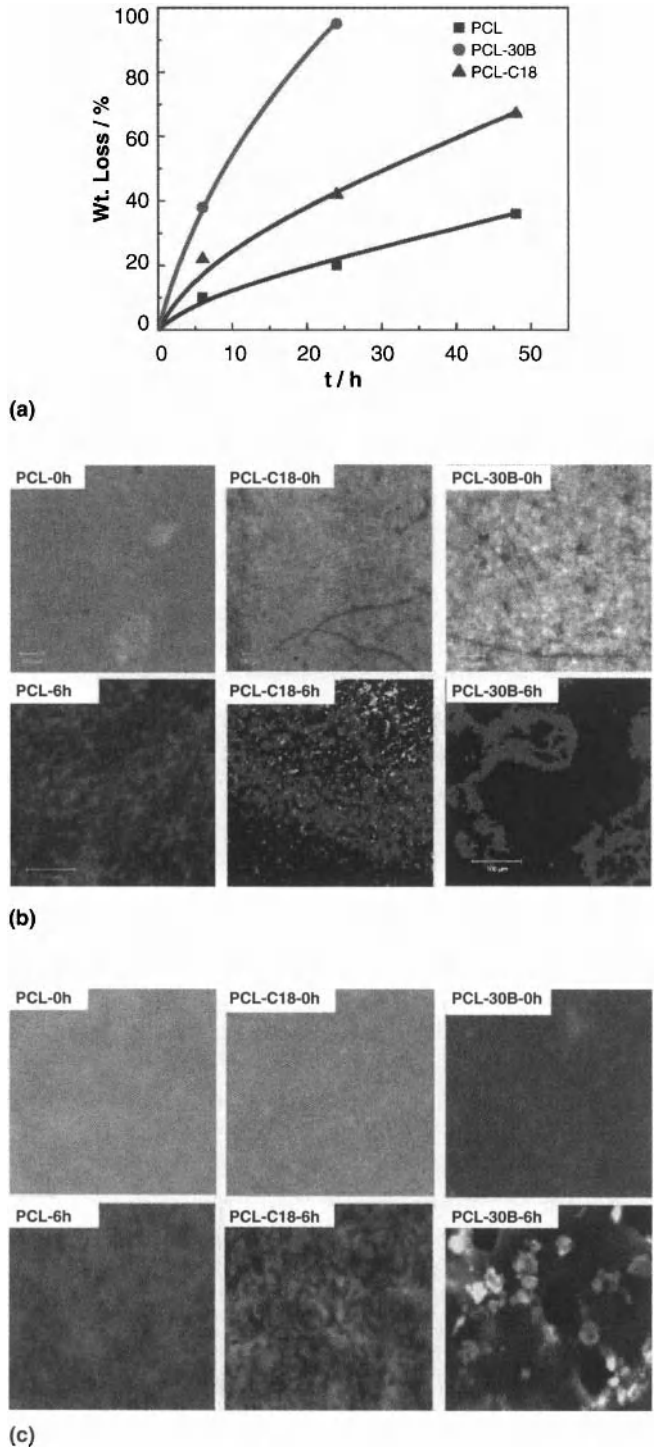
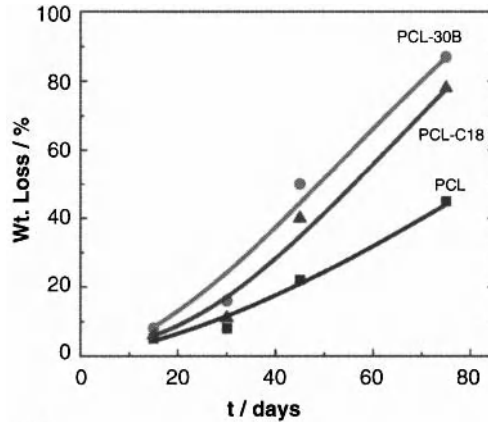
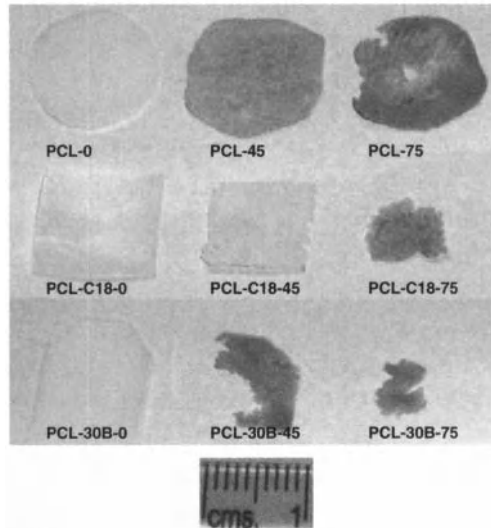


Figure 4.16 (a) Percentage weight loss of *PCL* and its indicated nanocomposites during enzymatic (Lipase from *Pseudomonas* sp., Type XIII) degradation at 37°C, (b) LCM images of *PCL* and its indicated nanocomposites before and after enzymatic degradation, and, (c) Phase contrast images of *PCL* and its indicated nanocomposites. The numbers after the specimens name represent the biodegradation time in hours. The number "0" indicates the sample before starting enzymatic degradation (35).



(a)



(b)

Figure 4.17 (a) Percentage weight loss of *PCL* and its indicated nanocomposites during biodegradation at room temperature in pure *fungi* medium (b) the optical micrographs of *PCL* and its indicated nanocomposites before and after biodegradation in *fungi* medium. The last numbers (0, 45 and 75) represent the days of exposure in biodegradation (*fungi*) medium. The number "0" indicates the sample before starting biodegradation. The shape of the samples were so chosen that after exposure we could locate the individual sample as they were put together in single media in order to keep the environment same. The scale has been given to show the dimension of the film used for biodegradation and their shape after biodegradation for various time (35).

loss has been achieved as the sample recovery was relatively easier in pure *fungi* medium and the integrity of the sample was quite fair as compared to compost degraded specimens discussed below. The optical images of the specimens before and after biodegradation in *fungi* medium have been presented in Fig. 4.17b, showing very less damage/loss of samples for pure *PCL* while severe weight loss has reported in nanocomposites, as expected from the

kinetics of biodegradation experiment (Fig. 4.16a). The thickness of pure PCL film decreases causing 40% weight loss in 75 days even though the dimension remains almost the same after biodegradation.

The changes in weight loss due to biodegradation in compost manure have been shown in Figure 4.18a. The rate of biodegradation is quite slow for pristine PCL ca. ~ 50 wt% degradation in 60 days. On the other hand, the biodegradation rate has enhanced significantly in nanocomposites in presence of nanoclays with 63 and 83 wt% loss observed in the same time for PCL-C18 and PCL-30B, respectively. Nonetheless, the biodegradation rate of matrix polymer has been tuned by dispersing suitable nanoclays into it. The higher degradation rate in presence of nanoclay has been attributed to the presence of hydroxyl groups on the edges of montmorillonite, which can catalyze faster hydrolytic degradation of the matrix polymer [64]. Comparatively higher biodegradation rate of PCL-30B nanocomposite is presumed to the greater interaction between PCL and 30B nanoclay, as evident from the lowest heat of fusion/crystallinity against PCL-C18 nanocomposite. In addition, the presence of excess hydroxyl ions present in the organic modifier of 30B nanoclay may accelerate the hydrolytic decomposition vis-a-vis C18 nanoclay, where methyl and octadecyl groups are at hand in the organic modifier.

The biodegradation rate has significantly been suppressed in bioreactive Ganges water at room temperature with the same dimension of samples $\sim (1 \times 1 \times 0.1 \text{ cm}^3)$ used in compost or other media. Only 20% weight loss has been observed after 200 days of exposure. Here again, the biodegradation rates of the nanocomposites have increased slightly with respect to pure PCL. In contrast, the overall biodegradation for all the samples were low as compared to the degradation rate in other media, primarily due to the lower abundance of specific microorganism necessary to hydrolyze PCL present in the Ganges water. It is worth mentioning here that the biodegradation experiment has also been performed in distilled water both for pure PCL and its nanocomposites. Surprisingly, weight loss has not occurred for pure PCL and its nanocomposites up to 200 days of exposure at 30°C . So, the presence of microorganism is a precondition for starting the hydrolysis and subsequent biodegradation. The PCLs have been shown to be degraded by enzymes secreted by a number of bacterias, yeasts and fungus [65–67]. The surface roughening due to biodegradation has been shown using SEM micrographs of pure PCL and its nanocomposites in compost manure (Fig. 4.18b) showing greater loss of samples from the surfaces as bigger pits are observed in nanocomposites as compared to pristine PCL. Furthermore, the pittings appeared in excess in PCL-30B than that of PCL-C18 nanocomposite for the same time of exposure (60 days). Both PCL and its nanocomposites do not exhibit any pitting before biodegradation. So, surface roughening occurs in greater extent in nanocomposites due to enhanced biodegradation with respect to pristine PCL. The globular pattern was observed for pure PCL while striations were seen in case of PCL-30B and PCL-C18 nanocomposites as revealed through AFM tapping mode micrographs (Fig. 4.18c). The striation pattern in the matrix has been generated by the disc-like layered silicates with aspect ratio ~ 250 . The surface morphology

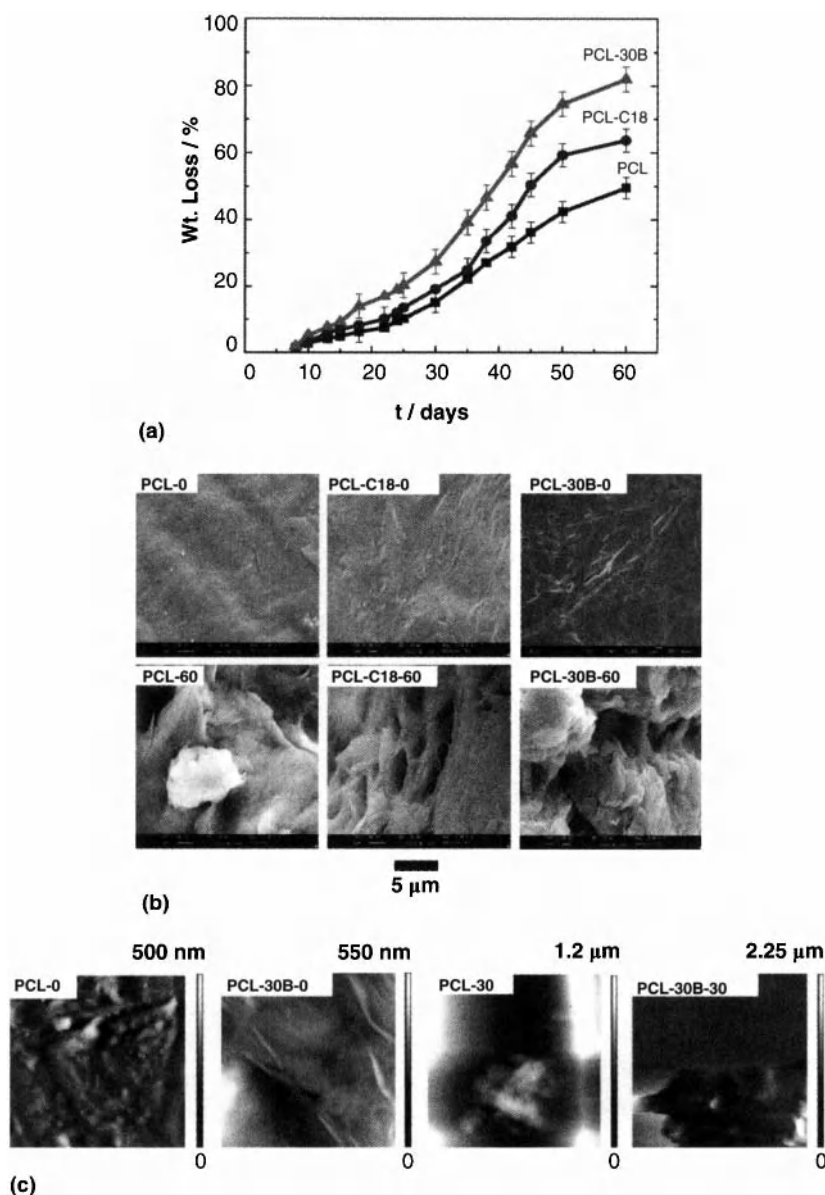


Figure 4.18 (a) Percentage weight loss of *PCL* and its indicated nanocomposites during biodegradation at room temperature in compost medium, (b) FE-SEM images of *PCL* and its indicated nanocomposites and (c) AFM images of *PCL* and its indicated nanocomposites thin film ($12 \mu\text{m} \times 12 \mu\text{m}$) before and after biodegradation obtained in tapping mode. Height profile is given after each image. The numbers after the specimens name represent the biodegradation time in days. The number "0" indicates the sample before starting biodegradation or pristine samples (35).

has changed significantly just after 20 days of biodegradation, exhibiting hill-ocks of globules in pure *PCL* as some parts of polymer surfaces have been eaten up by the microorganism. In contrast, the surface morphology becomes patchier in nanocomposite in the presence of layered silicates as evident from

the vertical scale (height profile) of ~ 2 mm against the value of ~ 1 mm in pure PCL. On the other hand, the surface roughness was around 500 nm both for pure PCL and nanocomposites before degradation. The greater irregularities of the surfaces of the nanocomposite as compared to pure PCL after biodegradation arise from the greater extent of biodegradation in nanocomposites, which we observed quantitatively in Fig. 4.18(a). However, significant loss of materials from its surfaces as evident from the pittings have clearly been shown in optical images, SEM, AFM and scanning confocal micrographs due to biodegradation, and the greater losses occurred for nanocomposites vis-a-vis pure PCL.

The controlled biodegradation rates of PCL have been presented with and without the presence of nanoclays. The regulated biodegradation is another topical interest. In other words, it is desirable to decrease or increase the rate of biodegradation of polymer apart from its natural degradation. The biodegradation rates of PCL and representative PCL/PCL-C18 nanocomposite samples quenched to room temperature and crystallized at 52°C up to full solidification (48 h crystallization) have been shown in Figure 4.19. The biodegradation rate has significantly been reduced for the samples crystallized at 52°C as compared to the quenched samples both for pure PCL and its nanocomposites. Nevertheless, the rate of biodegradation has always improved for nanocomposite as compared to neat PCL for a particular thermal history. The sluggish biodegradation rate of the samples crystallized at higher temperature is due to the higher amount of crystallinity as evident from the higher heats of fusion and bigger size of spherulites [68]. The biodegradation rate has decreased by ~ 25 – 35% with an increase in crystallization temperature from 30 – 52°C . Usually, the biodegradation starts in the amorphous region [69], and then it is reasonable to expect the rate of biodegradation to be more sluggish for samples with a greater amount of crystallinity and bigger spherulite dimension [70–72]. The molecular weight is almost the same before and after biodegradation both for

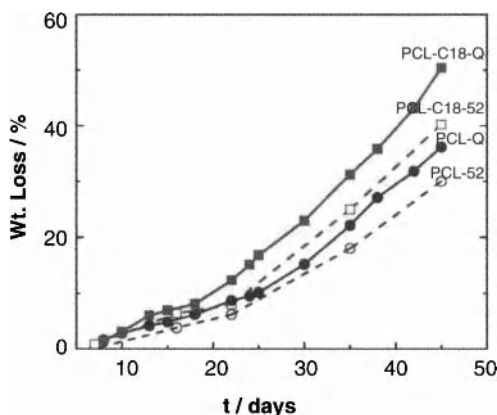


Figure 4.19 Percentage weight loss of PCL and its indicated nanocomposites during biodegradation at room temperature in the compost media. “Q” represents the samples quenched to room temperature and “52” represents the samples crystallized at 52°C (35).

pure PCL and its nanocomposites indicating that the biodegradation phenomena occurred initially in the amorphous region [73, 74]. Thereby, one can fine-tune the rate of biodegradation either to increase or to decrease with respect to their natural degradation rate either by suitably processing or dispersing nanoparticle in the matrix.

In order to explore the mechanism of biodegradation, fusion heats of samples have been shown at various stages of biodegradation (Figure 4.20). The ΔH is lower for the nanocomposites as compared to neat PCL before starting biodegradation as a result of enhanced interaction between PCL and nanoclay. High amorphous content along with the catalytic effect of clay, as mentioned earlier, enhances the biodegradation rate of nanocomposites as compared to pristine PCL. The heat of fusion increases sharply with biodegradation time for the nanocomposites while there is only slight increase for neat PCL after 6 weeks of biodegradation. The rapid increase in ΔH indicates that the amorphous part is first attacked/eaten up by the enzyme/microorganisms causing more crystalline materials to remain in the samples. As the biodegradation starts in the amorphous region, it is rational to expect the rate of biodegradation to be more sluggish for samples with a greater amount of crystallinity. Further, it is reported that the activity of enzymes (mostly depolymerase), secreted by microorganism, plays an active role in degrading the polymer. Murphy *et al.* showed higher depolymerase activity of the enzyme (PCL-depolymerase extracted from *Fusarium solani*) in slightly basic medium (pH $\sim$ 8.0) [75]. The measurement of pH of clay solutions (4 wt%) in water has shown 7.5 and 8.4 for C18 and 30B nanoclay, respectively. The depolymerase activity, calculated from optical density measurement of PCL colloidal solution using enzyme extracted from fungi (*Aspergillus fumigatus*), has been presented in Figure 4.21 as a function of pH.

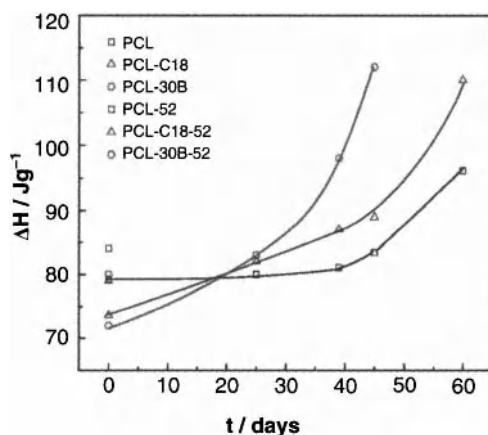


Figure 4.20 Heats of fusion as a function of biodegradation time for pure PCL and its indicated nanocomposites. The solid lines are guide to the eye. The solid symbols are for room temperature quenched samples and open symbols represent the samples crystallized at 52°C for 48 hrs (before starting biodegradation) (35).

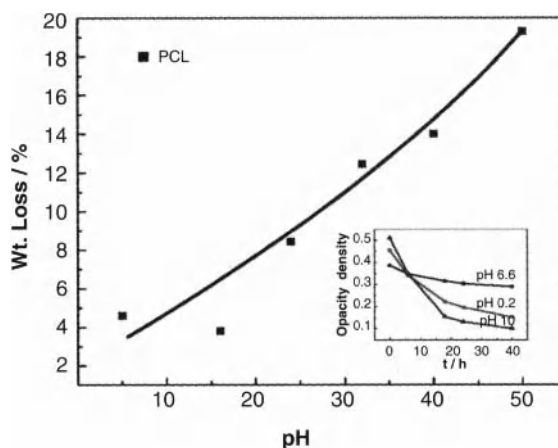


Figure 4.21 Effect of *pH* on *Aspergillus fumigatus* PCL-depolymerase activity. The inset figure represents the optical density of PCL suspension of varying *pH* as a function of time in hour. The depolymerase activity was calculated the initial slope of the curves (35).

Depolymerase activity increases with increasing *pH* of the solution indicating higher activity of the enzyme in alkaline media. Therefore, the greater enzyme (PCL-depolymerase) activity in slight alkaline nanocomposite caused higher biodegradation in the presence of nanoparticles. It is worth mentioning here that the *pH* of PCL solution was 7.0. Higher *pH* (8.4) in PCL-30B nanocomposite explains the higher biodegradation rate as compared to PCL-C18 nanocomposite (*pH*~7.5). Therefore, the enhanced enzymatic activity in nanocomposites is due to alkaline *pH* together with higher amorphous content. Moreover, the biodegradation has been restricted up to ~2 wt% only in alkaline buffer (*pH*~9, but in absence of any microorganism) even after 100 days of exposure both for pristine PCL and its nanocomposites. So, the presence of microorganism is a precondition for initiating the biodegradation, and the activity of enzyme gets enhanced in alkaline condition causing higher biodegradation rate in nanocomposites as compared to pure PCL. The biodegradation of PCL composites with sepiolite has also been studied [76]. Figure 4.22 shows PCL and PCL/SEPS9 before and after degradation in compost at 40°C.

Both the samples show a significant surface degradation with evidence of erosion after 35 days, indicating a large extent of degradation in the medium. The degradation was further assessed by optical microscopy (Figure 4.23), confirming a high surface degradation of the specimens against time [76]. This phenomenon was attributed to an enzymatic surface degradation mechanism for PCL in this medium [76–78], as well as possibly better contact of the compost in the most degraded areas and/or to an inhomogeneous growth of the microorganisms on the sample surface. Figure 4.24 shows the values of residual mass of PCL and PCL/SEPS9 against degradation time; both materials show

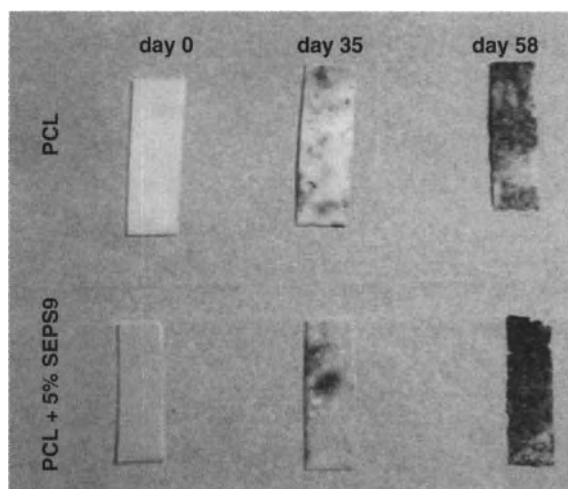


Figure 4.22 *PCL* and its composites with *SEPS9* before (day 0) and after 35 and 58 days of degradation in compost at 40°C (76).

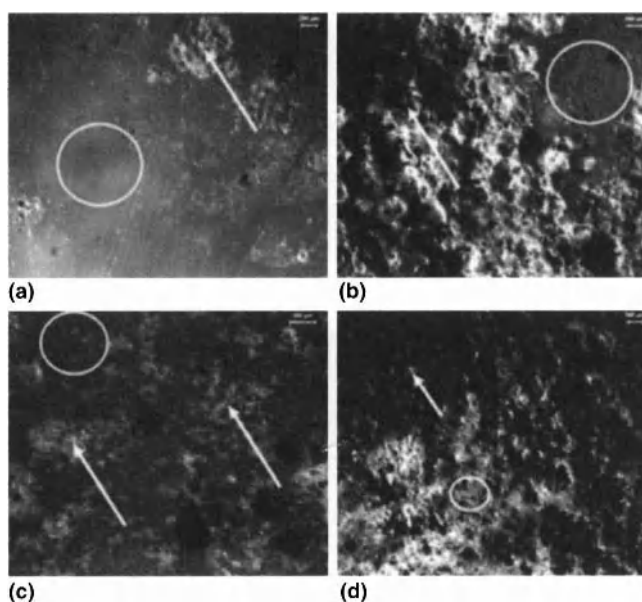


Figure 4.23 Optical micrographs of *PCL* after (a) 35 days and (b) 58 days; and *PCL* ρ *SEPS9* after (c) 35 days and (d) 58 days in compost (76)

considerable decreases of their weight against time with weight losses of ca. 50% after 100 days indicating high efficiency of microorganisms for compost towards *PCL* degradation. The incorporation of 5 wt% of sepiolite does not significantly affect the degradation rate of *PCL*. Size Exclusion Chromatography (SEC) data of *PCL* and *PCL/SEPS9* against degradation time are reported in Figure 4.25. Both materials show decrease of the molecular weight indicating

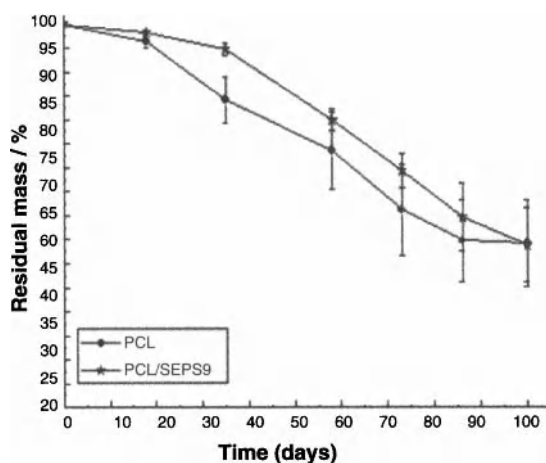


Figure 4.24 Residual mass for *PCL* and *PCL/SEPS9* composite as a function of degradation time in compost (76).

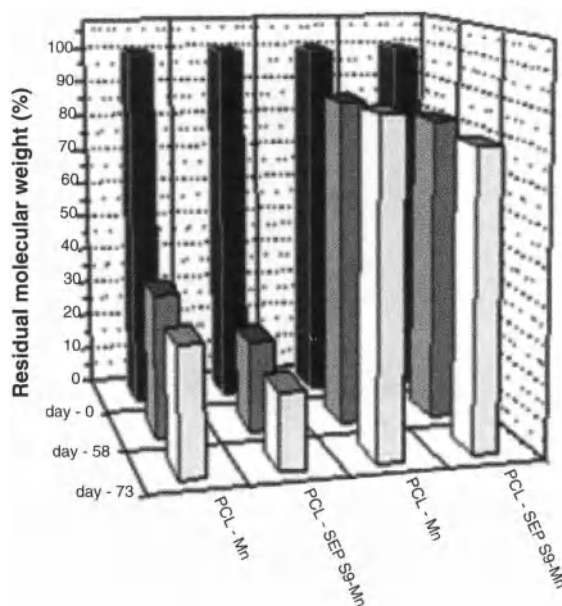


Figure 4.25 Residual average molecular weights of *PCL* and *PCL/SEPS9* composite before and after 58 and 73 days of degradation in compost (76).

their effective degradation. Recently, Neppalli *et al.* [79] Studied the tuned biodegradation behavior of PCL by addition of nylon fiber and polyvinylpyrrolidone (PVP) as filler. The cooperative effect of nylon and PVP fiber was able to obtain composites capable of degrading more quickly than the matrix or the samples containing either of the two fibers, while retaining a remarkable dimensional stability even after 20% loss of the total mass. Hala Zreiqat and his coworkers reported degradation of biphasic calcium phosphate scaffolds coated with hydroxyapatite-PCL composites [80].

4.5 Biocompatibility and Drug Delivery Application

Biodegradable polymers are classified as the second generation bio-implant materials due to their advantages over the first generation metallic and ceramic implants. This is because they do not leave undesired foreign elements in the body and do not need a second surgery to replace the implant from the body. One of the main applications of the biodegradable polymers is as temporary scaffolds in bone tissue engineering. Coombes *et al.* [81] investigated the preparation of fibroblast/composite constructs using cell culture as a first stage in mimicking the dermal/epidermal structure of skin. Fibroblasts were found to attach and proliferate on all the composites investigated reaching a maximum of $2 \times 10^5/\text{cm}^2$ on 1:20 collagen:PCL materials at day 8 with cell numbers declining thereafter. Keratinocyte growth rates were similar on all types of collagen:PCL materials investigated reaching a maximum of $6.6 \times 10^4/\text{cm}^2$ at day 6. The results revealed composite films of collagen and PCL as favorable substrates for growth of fibroblasts and keratinocytes and may find utility for skin repair. Sun and his coworkers [82] prepared the polycaprolactone/hydroxyapatite tissue scaffolds and studied mechanical property, structural integrity and *in vitro* cell scaffolds interaction using primary fetal bovine osteoblasts to assess the feasibility of scaffolds for bone tissue engineering application. The cell proliferation and differentiation were calculated by Alamar Blue assay and by determining alkaline phosphatase activity. The osteoblasts were able to migrate and proliferate over the cultured time for both PCL as well as PCL-HA scaffolds. The demonstration of the viability of the novel precision extrusion deposition (PED) process to fabricate PCL and PCL-HA composite scaffolds having necessary mechanical property, structural integrity, controlled pore size and pore interconnectivity desired for bone tissue engineering is unique. Drop on demand printing (DDP) is a solid freeform fabrication (SFF) technique capable of generating microscale physical features required for tissue engineering scaffolds [75]. Thermoplastic porogens were designed to generate PCL and PCL-CaP (calcium phosphate) composite scaffolds by injection molding of molten polymer. Porogens designed with 600, 400 and 250 μm pore sizes have been produced with PCL and PCL-CaP composite scaffolds (83). The views based on μCT analysis of 80:20 PCL-CaP composite scaffolds fabricated using a 600 μm porogen are shown in Figure 4.26. The cytocompatibility of PCL and PCL-CaP composite 3D scaffolds was assessed using the Alamar BlueTM assay for cell metabolic activity/cell proliferation. HEPM cells growing on the scaffolds were visualized by fluorescent staining of cell nuclei. In the case of 3D scaffolds of PCL and 80:20 PCL-CaP, HEPM cells were able to attach as evidenced by fluorescent nuclear staining with Hoechst 33258 (Figure 4.27). These images indicate attachment onto the struts of both PCL (Fig. 4.27a) and 80:20 PCL-CaP composite scaffolds (Fig. 4.27b). Based on the Alamar BlueTM data, the initial seeding efficacy was not significantly different for the materials used. This similar level of HEPM cell attachment to all materials used was probably due to the fact that all scaffolds were precoated with a mixture of

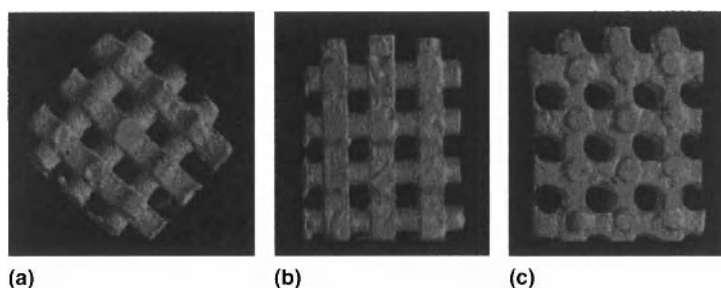


Figure 4.26 Microcomputed tomography analysis of 80:20 *PCL-CaP* composite scaffolds: (A) view of horizontal build plane (looking down the longest dimension), note the sharp square pores; (B) view of horizontal build plane (looking down shortest dimension), note the sharp square pores; (C) view of vertical build plane, note the rounded pores (83).

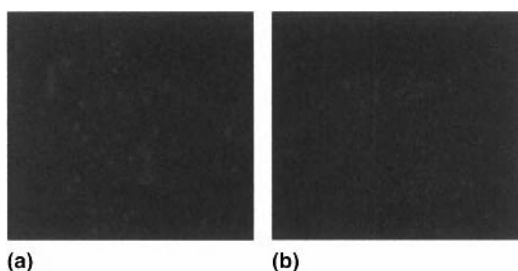


Figure 4.27 Bisbenzimidazole nuclear staining of adherent *HEPM* cells following 24 h of orbital shaker seeding on *PCL* (Panel A, 200 $\times$) and 80:20 *PCL-CaP* composite (Panel B, 100 $\times$) scaffolds, 600 μ m pore sizes. Images are captured by imaging the surface of a strut on the outside of the scaffold (83).

MatrigelTM, a reconstituted extracellular matrix, and collagen type I solution. Cellular attachment to the synthetic surfaces was minimal without this coating. *HEPM* cells proliferated on all types of 3D scaffolds, as assessed from the Alamar BlueTM fluorescence data (Figure 4.28) with

some differences between materials. The normalized cell proliferation data indicated an identical cell growth on pure *PCL* and 90:10 *PCL-CaP* scaffolds. By contrast, cell growth on the 80:20 scaffolds was significantly enhanced. Figures 4.27 and 4.29a,b were corroborated qualitatively with the observed increase in the density of Hoechst 33258-stained nuclei following 4 days of post-seeding culture *in vitro* on the various scaffolds (Fig. 4.29). At this time, cells were visibly growing both on the struts (Figs. 4.29a,c), as well as on the interior pore structures of all scaffolds (Figs. 4.29b,d). For further confirmation of cellular ingrowth into the scaffold center, the scaffolds were cut into segments using a scalpel. The presence of cells on all interior surfaces was visualized by nuclear staining (Fig. 4.29e). In summary, these cytocompatibility tests clearly indicate that all porogen-based scaffolds coated with suitable ECM proteins facilitate attachment and support proliferation of *HEPM* cells *in vitro*. In addition, the presence of *CaP* in the *PCL-CaP* composite enhances the proliferation of *HEPM* cells and reduces their spreading in favor of multilayer assembly. Santis *et al.* [84] prepared TiO_2 and TiO_2 glasses containing *PCL* up to 24% using the sol-gel

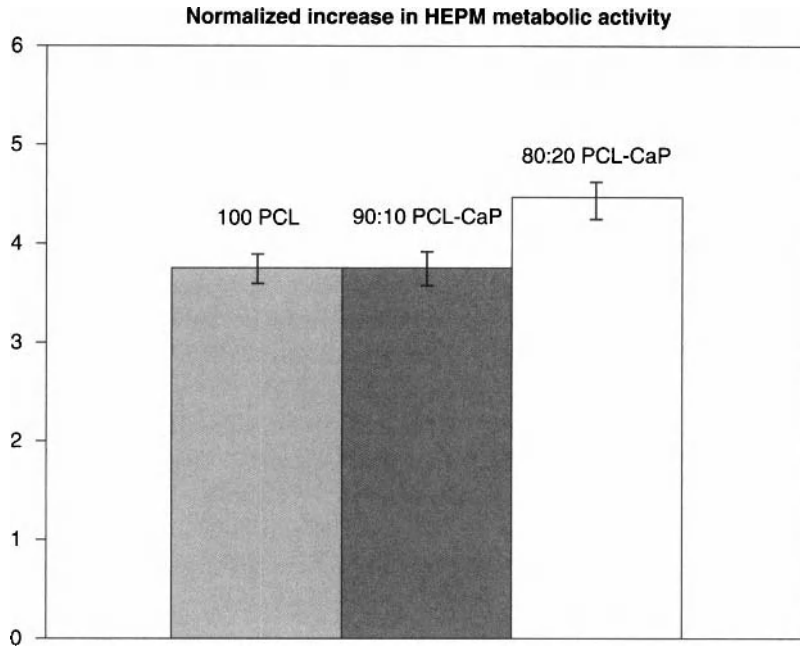


Figure 4.28 Normalized increase in Alamar Blue™ readings over the 4-day in-vitro culture period following the initial 24 h seeding period for 600 μm pore size pure *PCL*, 90: 10 and 80:20 *PCL-CaP* scaffolds. Metabolic activity as measured by Alamar Blue™ at 96 h post-seeding was normalized to the Alamar Blue™ readings taken immediately following the 24 h seeding period (83).

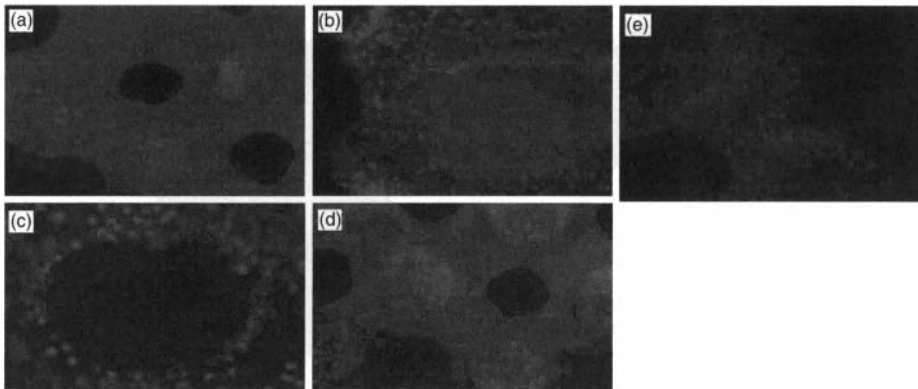


Figure 4.29 Bisbenzimidazole staining of human embryonic palatal mesenchymal HEPM cells cultured on *PCL* (Panels A and B) and 80:20 *PCL-CaP* composite (Panels C and D) scaffolds for 5 days: (A) HEPM cells on the surface struts of a 600 μm pore size *PCL* scaffold (50 $\times$); (B) HEPM cells growing round and into a pore on the same scaffold imaged in panel A (100 $\times$); (C) HEPM cells on the surface struts of a 600 μm pore size 80:20 *PCL-CaP* composite scaffold (50 $\times$), note the increased density of nuclear staining relative to the *PCL* scaffold; (D) HEPM cells colonizing a pore in the scaffold imaged in panel C (original magnification 200 $\times$); (E) HEPM cells growing on a strut from the scaffold centre (100 $\times$) (83).

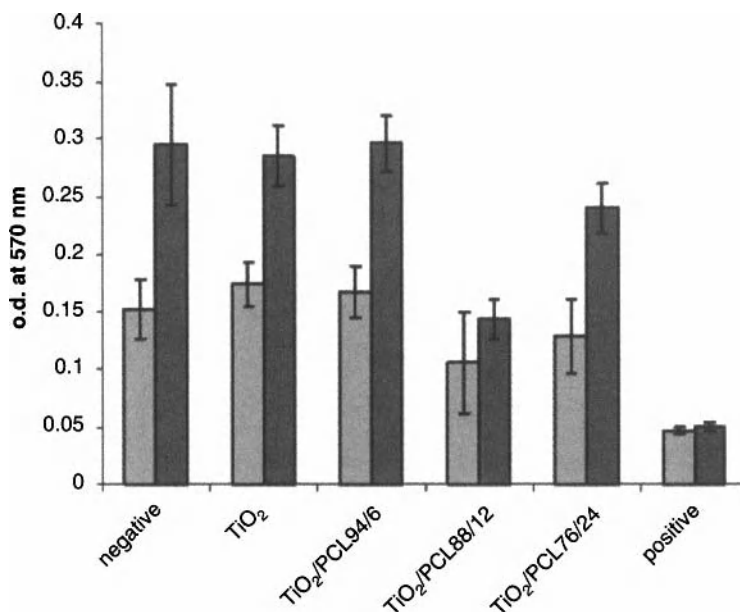


Figure 4.30 MTT indirect elution study: cells exposed for 48 h in 24 and 72 h eluants of materials sterilized by autoclave (20 min at 120°C) (84).

process. The mechanical properties of the composites are in between those of spongy and cortical bone and these materials can be suitable for several applications related to bone surgery. They tested the biocompatibility of organic/inorganic composites, carried out by using human cells using the MTT assay to determine viability. The results of MTT assay after 48 h of incubation are shown in Figure 4.30. Optical density values measured for cells exposed to the eluants for 24 and 72 h suggested no significant difference between control, TiO₂ and TiO₂ composite. The lower values measured for TiO₂-PCL 88/12 and TiO₂-PCL 76/24 are probably due to some leachable trapped solvent used to prepare the discs. However, the samples with a higher amount of PCL show an increase in cell metabolic activity after 72 h exposure (Fig. 4.30), and are consistent with light microscopic images (Figure 4.31).

The normal cell viability was restored and this was confirmed by the live/dead cell images reported in Figure 4.32. As expected, positive control supernatant resulted in severe cell death. Figs. 4.32i,j clearly show the toxic condition for the cells; the cellular response with rounded live cells and larger number of dead cells suggesting apparent cell death. Functionally graded nanocomposite scaffolds of PCL-β-TCP have been fabricated by using a hybrid twin-screw extrusion electrospinning process, and its potential for tissue engineering, and to bone-cartilage interface formation in particular, were demonstrated using mouse preosteoblast cells (MC3T3-E1) [85]. The typical scanning electron micrographs of tissue constructs from the functionally graded scaffolds after 1 and 4 weeks of culturing and fixed/dehydrated are shown in Figure 4.33. SEM characterization of the tissue reveals the period of cell attachment and division followed by the initiation of collagen synthesis in the extracellular matrix

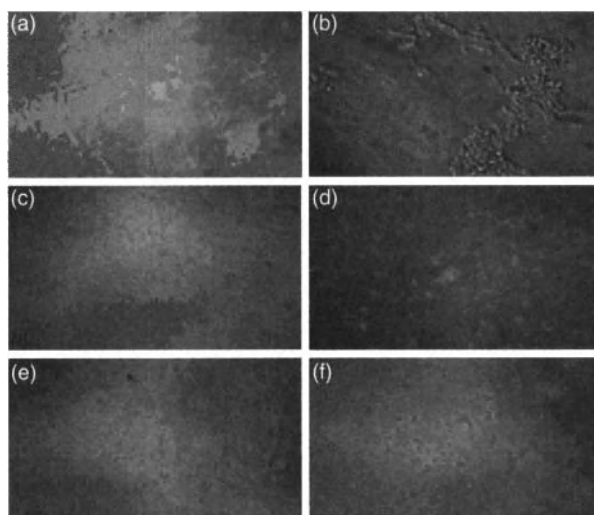


Figure 4.31 Light micrographs of cells incubated for 48 h in the 72 h eluant: (a) negative control tissue culture plastic; (b) positive control, cells incubated in 10% ethanol; (c) TiO_2 ; (d) TiO_2PCL (6%wt); (e) TiO_2PCL (12%wt); (f) TiO_2PCL (24%wt)

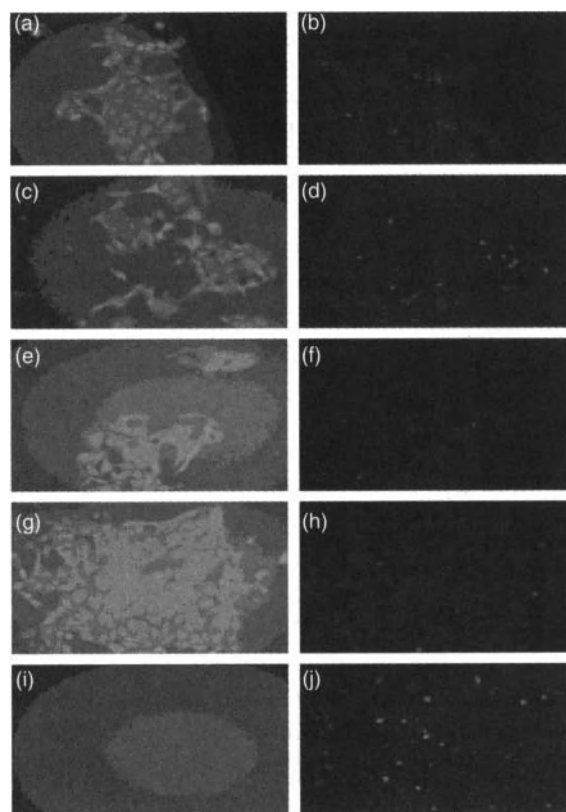


Figure 4.32 Live-dead assay: Olympus IX51 fluorescent microscope. Assay of cells incubated for 48 h in the 72 h eluant; the live cells were stained green and the dead cells red. (a and b) Negative control tissue culture plastic; (c and d) TiO_2 ; (e and f) TiO_2PCL (6 wt%); (g and h) TiO_2PCL (24 wt%); (i and j) positive control, cells incubated in 10% ethanol (84).

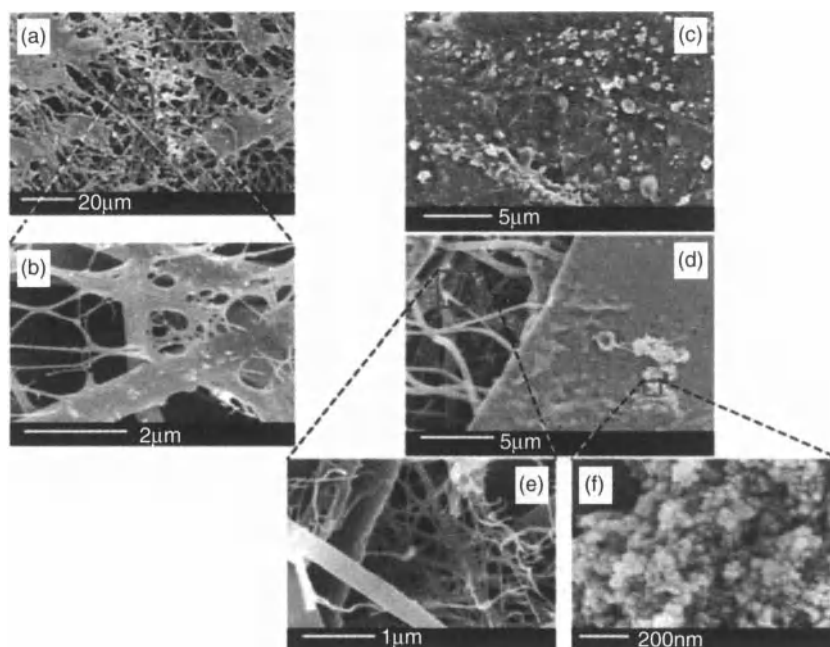


Figure 4.33 SEM image of MC3T3-E1 cells seeded on PCL- β -TCP scaffolds (a) Tissue construct after 1 week of cell culture. The surface of the scaffold is partially covered with osteoblast like cells. (b) Higher magnification of scaffold surface after 1 week. The spread of cells over the nanofibrous structure and initiation of both Ca deposition and collagen synthesis were observed. (c) Tissue construct after 4 weeks of cell culture. The surface of the scaffold is fully covered with cells. Considerable amount of Ca deposition is noticeable as an indicator of differentiation. (d) Formation of denser collagen bundles and Ca deposition in the bulk of the tissue construct. (e) Higher magnification of the bulk of the tissue with a focus on collagen bundles. (f) Higher magnification of Ca particles deposited by the cells (85).

after 1 week, and abundant mineralization in the form of Calcium deposition after 4 weeks, as shown in Fig. 4.33. The top surface of the functionally graded scaffold was partially covered with preosteoblast cells initiating collagen synthesis and calcification after one week of culture (Fig. 4.33a,b). Cells are well spread over the nanofibrous scaffold and the beginning of a multilayered cell structure was observed. The surface was fully covered with multilayers of cells and considerable amount of calcification took place in 4 weeks, (Fig. 4.33c). The osteoblastic differentiation process is more visible after 4 weeks, which was marked by the formation of denser collagen bundles with diameters of around 100 nm (Fig. 4.33e). Significant amounts of Ca deposition were also observed (Fig. 4.33f). The preosteoblastic MC3T3-E1 cells mostly differentiated into osteoblasts after a series of processes eventually converting the PCL- β -TCP nonwoven composite meshes into a bone tissue-like construct.

Lahiri *et al.* [86] prepared composites based on the biodegradable polylactide-polycaprolactone copolymer (PLC)/boron nitride nanotubes (BNNTs) for orthopedic scaffold application, and investigated the biocompatibility of composite materials in the context of viability of osteoblast cells. The viability ratio obtained through counting the cells under the fluorescence microscope showing a higher count for PLC-2BNNT and PLC-5BNNT composites with $90 \pm 2\%$

and $91 \pm 4\%$, respectively (Figure 4.34). The osteoblast viability for PLC film has been found to be as low as $59 \pm 4\%$ ($p < 0.05$). The fluorescent images present a representative picture of live and dead cells on the three PLC composite films (Figure 4.35). Fluorescence microscopy images after 2.5 days of growth show a typical lens shape characteristic of osteoblasts, suggesting the presence of normal cell behavior on PLC, PLC-2BNNT and PLC-5BNNT composite films with no significant difference in the cellular morphology. These results indicate that the addition of BNNTs improve the biocompatibility of the PLC copolymer in terms of osteoblast cell viability. In order to evaluate the effects of PLC-BNNT composites on the differentiation state of osteoblasts, the levels of expression of the transcription factor Runx2 have been assessed by semi-quantitative real time PCR. Runx2 (also known as Cbfa) is a master regulator of osteoblastogenesis and coordinates the integration of signalling events and other transcription factors involved in this process. Fourfold and sevenfold increases in the levels of expression of Runx2 in osteoblasts grown on PLC-2BNNT and PLC-5BNNT, respectively, have been observed when compared to the levels in the osteoblasts grown on PLC alone (Figure 4.36). The positive effect of the presence of BNNTs on accelerated osteoblast differentiation and growth could be due to the natural affinity of protein to BNNTs. The interactions of the osteoblasts and macrophages with bare BNNTs prove them to be non-cytotoxic. PLC-BNNT

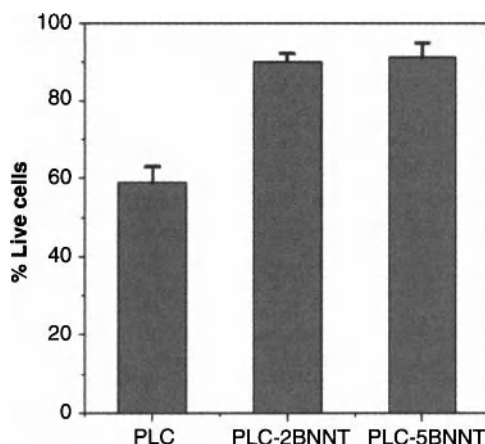


Figure 4.34 Viability (% live cells) count for osteoblast cells grown on PLC, PLC-2BNNT, PLC-5BNNT composite films, as studied through FDA-PI staining and fluorescence microscopy (86).

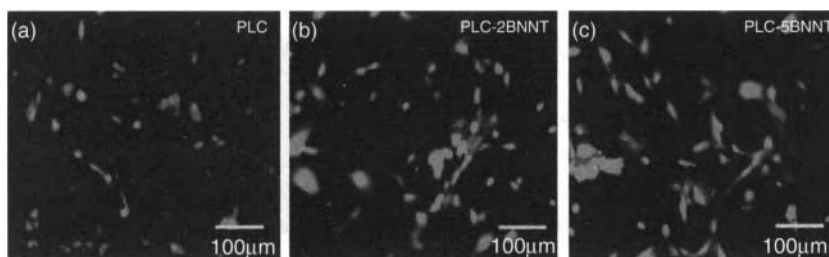


Figure 4.35 Fluorescent images of live (green) and dead (red) osteoblast cells obtained through FDA-PI staining after 2.5 days of growth on (a) PLC (b) PLC-2BNNT and (c) PLC-5BNNT films (86).

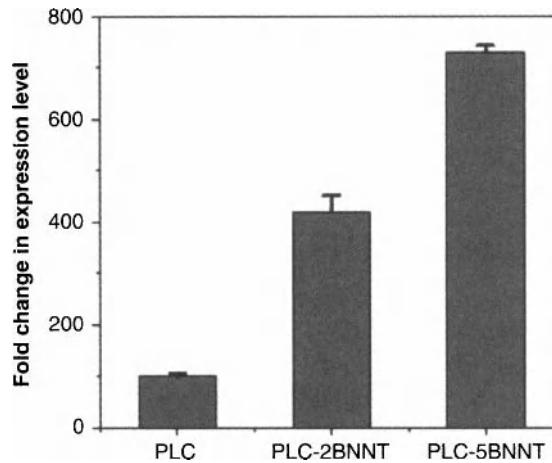


Figure 4.36 Runx2 (Cbfa1) expression level obtained in gene expression study of osteoblasts grown on PLC, PLC-2BNNT, PLC-5BNNT composite films (86).

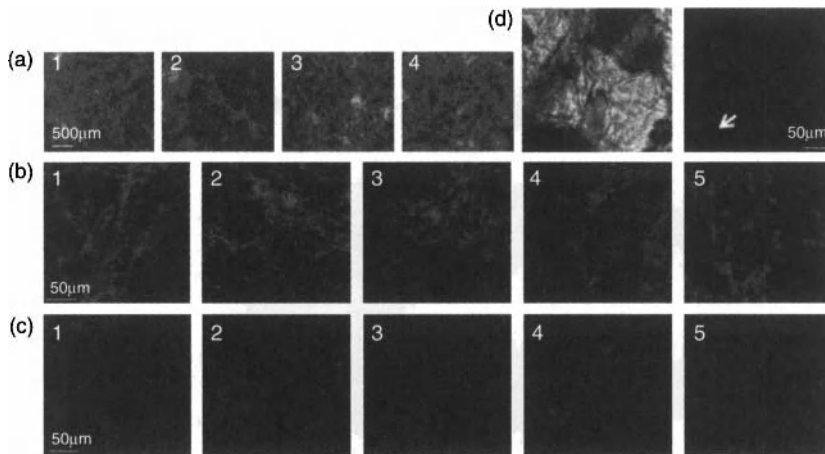


Figure 4.37 Attachment of human lens epithelial cells to composite materials in-vitro. (A) Attachment of SRA 01 /04 cells to composite material at 24 h following seeding; 6 wt.% pSi, as-prepared (1); 20 wt.% pSi, as-prepared (2); 6 wt.% pSi, oxidized (3) and 20 wt.% pSi, oxidized (4). Attachment assayed by Hoechst 33342 staining; (B) Attachment of SRA 01 /04 cells to composite material at 72 h following seeding. (C) Continued cell survival and adherence to composite material 7 days after seeding; 6 wt.% pSi, as-prepared (1), 20 wt.% pSi, as-prepared (2); 6 wt.% pSi, oxidized (3); 20 wt.% pSi oxidized (4) and PeL-only (5). Attachment and morphology visualized by Hoechst 33342 and Alexa Fluor 594 phalloidin staining. (D) Cells will attach to scaffold architecture in the vicinity of pSi nanoparticles (marked with arrow), suggesting that release of silicic acid does not reach levels inhibitory to cell attachment and growth (87).

composites displayed increased osteoblast cell viability as compared to the PLC matrix. The addition of BNNTs also resulted in an increase in the expression levels of the Runx2 gene, the main regulator of osteoblast differentiation indicating BNNT as a potential reinforcement for composites for orthopedic applications.

The suitability of porous silicon (pSi) encapsulated in microfibers of the biodegradable polymer (PCL) for ophthalmic applications has been investigated using both a cell attachment assay with epithelial cells and an *in vivo* assessment

of biocompatibility in rats [87]. Biocompatibility of the composite materials was initially probed by examining the attachment and response of the relevant HLE cell line, SRA 01/04. Attachment of SRA 01/04 cells to the fibers was evident within 24 h of seeding (Figure 4.37a). Cells have colonized the space between the fibers in each of the porous materials tested by 72 h of culture (Fig. 4.37b). Staining of the actin cytoskeleton shows a highly elongated and interconnected cell network. Contiguous cell sheets spanning the PCL fiber network were observed on the pSi-PCL scaffolds. Cells remained viable and attached to all composite materials tested after seven days of seeding (Fig. 4.37c). Viable cells were detected growing in the immediate vicinity of the pSi particles (Fig. 4.37d), suggesting that the silicic acid released during degradation did not reach required toxic levels during the culture period. Overall, the composite membranes tested were assessed to be conducive to human lens epithelial cell attachment and proliferation. Implants of the four composite materials were placed under the superior, temporal and inferior conjunctiva of rat eyes and were then monitored for 8–9 weeks under an operating microscope (Figure 4.38). The rats were sacrificed and sections of the enucleated eye were prepared for end-point histology after 8–9 weeks, (Fig. 4.38). There was no evidence of neutrophilic inflammation or morphologically significant neovascularization around any implant. Instead, the

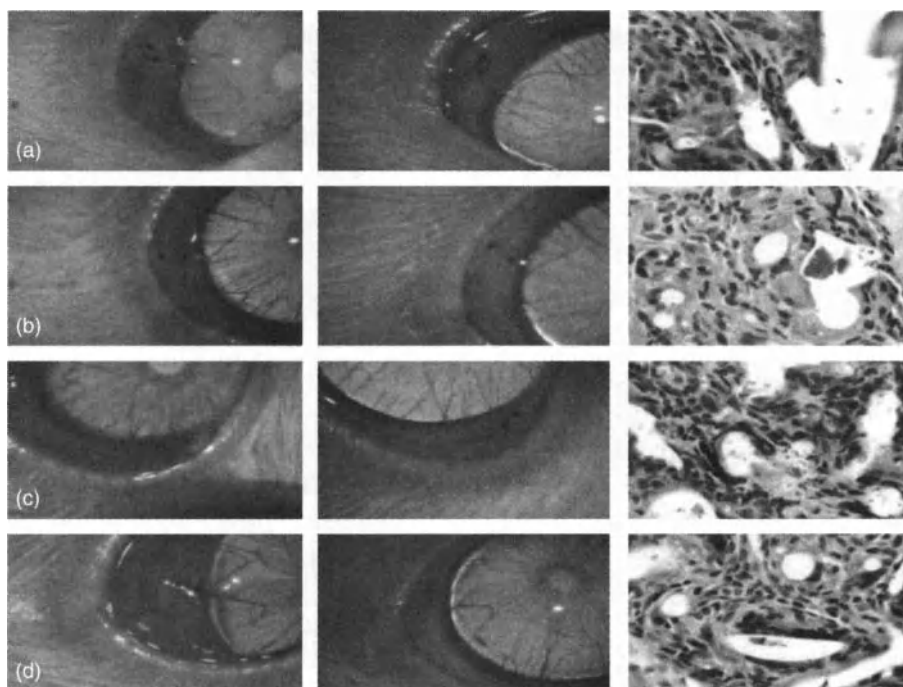


Figure 4.38 Compatibility of composite materials in the rat eye. (A) 20 wt% oxidized pSi in PCL at 1 week (left panel) and 8 weeks (middle panel) after implantation beneath the conjunctiva; end-point histology (right panel) (B) 6 wt.% oxidized pSi in PCL at 1 week (left panel) and 8 weeks (middle panel) after implantation beneath the conjunctiva; end-point histology (right panel). (C) 20 wt.% as-prepared pSi in PCL at 1 week (left panel) and 8 weeks (middle panel) after implantation beneath the conjunctiva; end-point histology (right panel); (D) 6 wt.% as-prepared pSi in PCL at 1 week (left panel) and 8 weeks (middle panel) after implantation beneath the conjunctiva end-point histology (right panel) (87).

appearances were those of a histiocytic foreign-body type granulomatous reaction, very similar to the tissue reaction that is commonly seen around sutures, including those used to hold the implant in place. Histologically, a thin fibrous capsule was observed around the implant. The surrounding tissue appeared quite normal. Composite fibers containing the surface-oxidized pSi particles appeared to perform better, as gauged by the lack of a measurable fibrous capsule in these samples. There was no significant difference in the tissue response to 6 and 20 wt% of pSi-loaded materials. However, PCL-based composites have enough potential for use in biomedical applications.

4.6 Conclusion

Poly(ϵ -caprolactone) nanocomposites with different types of nanoparticles have been presented. PCL matrix composites have the advantage of being very versatile, which can tailor various properties. The specific requirements of nanohybrids were discussed using a wide range of polymeric matrices based on PCL reinforcements and processing routes. Many of the works are still ongoing, and a definite conclusion has yet to be reached on the effect of nano-sized inclusions on polymer systems. This chapter presented material preparation, characterization, properties, processing and biomedical applications, taking into consideration the PCL and its nanohybrids with varying nanoparticles. The discussions suggest that the combination of biodegradable polymer like PCL with the appropriate nanostructures, open a new perspective in nanodevices for biomedical applications with tunable mechanical, thermal, morphological and biodegradation behavior.

Acknowledgement

The authors express their sincere thanks to Dr. Ashutosh Tiwari, Dr. Hisatoshi Kobayashi and Dr. Anthony PF Turner, the Editors of this book, for their kind invitation for this contribution. They are also grateful to Elsevier, Wiley and the American Chemical Society for giving permission to use the tables and figures from their publications used in this chapter. The authors acknowledge the receipt of research funding from Department of Biotechnology (DBT), New Delhi, Ministry of Science and Technology, Government of India (Project No. BT/PR-6929/BCE/08/433/2005) during the preparation of this chapter. NKS also acknowledges the receipt of funding for his fellowship from CSIR.

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Bone Substitute Materials in Trauma and Orthopedic Surgery – Properties and Use in Clinic

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Abstract

Large defects resulting from among other things, trauma, infection, or tumor resection, often do not heal spontaneously and require surgical intervention. Not only variations in size or location of the defect but also patient-related factors, such as age and disease status, determine the therapeutic approach. Herein, bone grafts provide support, fill voids, and enhance the biological repair of the defect. Autogenous bone, either cortical or cancellous, harvested from the patient's iliac crest is considered the gold-standard graft. However, as the cells do not necessarily survive transplantation, the clinical benefit is not guaranteed. Moreover, autografting is associated with complications and is not always possible. Alternative bone grafts like iso-, allo-, and xeno-transplants have been applied, but due to (major) disadvantages their use is discouraged.

An increasing number of graft alternatives is available; these contain (combinations of) tricalciumphosphate (TCP) and hydroxyapatite (HA), calcium sulphate (gypsum), type I collagen and nonbiological substrates like degradable polymers and bioactive glass. The products available differ in composition, characteristics, appearances, and delivery forms (e.g., pastes, solid matrices, or granules). Availability of an increasing number of products may seem attractive; however, without sufficient knowledge on their properties and behavior *in vivo* it will become more and more complicated to select the product that best mimics the bone to be replaced. Determining which product to use is based upon many factors including the size and location of the defect, as well as the handling properties and ability to deliver the material to the surgical site. This chapter provides an overview of currently available bone substitute products and their structure and biological and biomechanical behavior *in vitro* and *in vivo*. It also provides an overview of the current clinical use of these products.

Keywords: Bioactive glass, biomaterial, biomechanics, bone defect, bone substitute, calcium phosphate, calcium sulphate, review, clinical, trauma

5.1 Introduction

The treatment of fractures remains a challenge in trauma and orthopedic surgery. Trauma patients are generally young and, consequently, the medical and socioeconomic costs of fractures are high [1, 2]. The lifetime risk of fractures is one in two for males and one in three for females. These numbers are equivalent to the lifetime risk of coronary artery disease and demonstrate that fractures are a substantial public health problem [3].

Fracture healing is a complex and well-orchestrated process involving cell and tissue proliferation and differentiation. Many molecules and cells play an important role. The molecules include growth factors, inflammatory cytokines as well as hormones, antioxidants and other nutrients. Bone breakdown (osteoclast) and bone-building (osteoblast) cells also play a critical role. There are three distinct phases in fracture healing [4]:

1. Inflammation phase: A blood clot forms after fracture, and attracts inflammatory clean-up cells to the wound area. A subsequent cytokine attracts repair cells to the fracture gap, which differentiate into osteoblasts and chondroblasts. These cells lay down new bone matrix and cartilage. Finally, osteoclasts dissolve and recycle bone debris.
2. Reparative phase: From two weeks post-fracture onwards, proteins produced by the osteoblasts and chondroblasts start to consolidate into soft callus. Over a period of 6 to 12 weeks, this soft, new bone substance hardens into a hard callus.
3. Remodeling phase: During this phase, the callus matures and remodels itself. Osteoblasts and osteoclasts act together to remodel the callus into strong, lamellar bone.

The vast majority of fractures heal without complications, yet in a subset of 5–10% of patients, healing is impaired and requires additional treatment [2]. Significant bone defects or post-traumatic complications, such as delayed union or non-union, may require bone grafting to fill the defect. Filling a bone defect with a graft provides mechanic support and subsequently enhances the biological repair of the defect. Worldwide, over two million bone grafting procedures are performed each year, representing approximately 10% of all skeletal reconstructions [5–7]. For a bone defect to heal, four critical elements should be present: (i) osteogenic cells (e.g., osteoblasts or progenitor cells); (ii) osteoinductive signals provided by growth factors; (iii) an osteoconductive matrix; and, (iv) adequate blood and nutrient supply [8]. Osteogenesis, osteoinduction, and osteoconduction are the three most essential elements of bone regeneration along with the final bonding between the host and grafting material. The latter is called osteointegration.

- *Osteogenicity* is the presence of bone-forming cells within the bone graft. Osteoprogenitor cells living within the donor graft may

survive during transplantation, and could potentially proliferate and differentiate to osteoblasts and eventually to osteocytes. These cells represent the "osteogenic" potential of the graft [9, 10].

- *Osteoinduction* is the stimulation and activation of host mesenchymal stem cells from the surrounding tissue, which differentiate into bone-forming osteoblasts. This process is mediated by a cascade of signals and the activation of several extracellular and intracellular receptors, the most important of which belong to the TGF-beta superfamily [9,11].
- *Osteoconductivity* is the ability of the graft to function as a scaffold for ingrowth of new bone and sprouting capillaries [12]. It describes the facilitation and orientation of blood vessels and the creation of the new Haversian systems into the bone scaffold [13].
- *Osteointegration* describes the surface bonding between the host bone and the grafting material [13].

5.2 Types of Bone Grafts

Several types of bone grafts exist, each with their advantages and limitations.

5.2.1 Autologous Transplantation

Autologous transplantation uses autologous bone that is mostly harvested from the iliac crest. The iliac crest provides easy access to good quality and quantity autologous graft. This type of graft is considered the gold standard since it meets all criteria as mentioned above. It provides a scaffold for bone ingrowth and contains living bone cells that offer osteogenesis and growth factors that stimulate osteoinduction [9]. However, there are also limitations. The cellular elements do not necessarily survive the transplantation and may result in graft failure; therefore the clinical benefit is not guaranteed per se [14]. The graft harvesting procedure adds time to the surgical procedure. Perhaps even more importantly, the graft amount may be insufficient or the form is often inappropriate. Autograft harvesting is associated with an 8–39% risk of complications such as infection, hematoma, nerve injury, urethral injury, pelvic instability, cosmetic problems, and residual (sometimes even chronic) pain at the donor site [10, 13, 15–17]. Finally, autografting in elderly or pediatric patients or in patients with malignant or infectious diseases is discouraged.

5.2.2 Allotransplantation and Xenotransplantation

As an alternative, *allotransplantation* can be performed, in which a graft from another person is used. Allografts can be cancellous or cortical, and are prepared in fresh, frozen or freeze-dried forms. Bone allografts are usually distributed through regional tissue banks and may also be sent overseas. Allograft is the most frequently chosen bone substitute and is regarded as the surgeon's

second option [18]. The essential graft characteristics as described above are poorer in allografts than in autografts, and the clinical results are variable. Moreover, allografts carry the risk of transferring contaminants and pathologies such as viral (especially HIV and hepatitis B and C) and bacterial infections, malignancy, systemic disorders (autoimmune disease), or toxins. For this reason, fresh allografts are rarely used. In order to minimize disease transmission as much as possible, extensive processing of the graft (e.g., freezing or irradiation) is required. Processing lowers the risk of disease transmission, but at the same time it may significantly weaken the biological and mechanical properties that were present in the original bone tissue [10]. The processing procedures are generally associated with osteogenic cell death and growth factor washout, and may also change the structural properties of the graft. And still, disease transmission is not always preventable. Frozen and freeze-dried allografts are generally osteoconductive, but are considered to have weak osteoinductive capabilities compared with the fresh allografts. These changes result in a higher clinical failure rate. Consequently, these alternative transplantations generally have major biocompatibility disadvantages compared with autografting and their use is suboptimal at best [19, 20].

A modified approach would be to use a graft from another species, which is called *xenotransplantation*. Similarly as with allografts, xenografts also require extensive processing in order to prevent transmission of pathogens, viruses, or diseases. Xenografts have even more disadvantages than allografts and are only rarely used.

5.2.3 Alternative Bone Substitute Materials for Grafting

Due to the associated complications and limitations reported, *alternative bone substitutes* are needed. For a bone substitute to be a perfect graft, it should be osteoconductive, osteoinductive, biocompatible, and bioresorbable. It should induce minimal or no fibrotic reaction, and should have similar strengths to that of the bone being replaced. Finally, it should be available in sufficient amounts and at the time it is needed.

Since the mid-twentieth century, technological evolution and an improved understanding of bone-healing biology have resulted in the development of alternative (artificial) bone substitutes. At present, a wide variety of products is available. Monoproducts as well as mixtures of hydroxyapatite, tricalcium phosphate, dicalcium phosphate, calcium sulphate (plaster of Paris), bioactive glass, and other components are available. Over thirty companies have developed products that are currently available for use in trauma and orthopedic surgery. Although it may seem attractive that novel products are being put into the market every year, the downside is that for the majority of products only limited information is available. Since the exact composition of the product affects its properties, and thus its use, it becomes more and more challenging for a surgeon to use the best graft for a particular clinical indication. An evidence-based guideline may assist surgeons in their selection process. Unfortunately no such guideline is available yet.

5.3 Bone Substitute Materials

5.3.1 General Considerations

Architecture and strength in combination with chemical composition and application form determine how a bone substitute material will behave *in vivo*. Osteoconductive porous biomaterials provide a scaffold for the ingrowth of bone. Pores with sizes $< 5\mu\text{m}$ (i.e., microporosity) are necessary for the bioresorbability of the material [21], whereas pores larger than $100\mu\text{m}$ (i.e., macroporosity) contribute to the osteoconductive potential of the graft. At a size of $400\text{--}600\mu\text{m}$, pores facilitate infiltration by fibrovascular tissue and revascularization. The large differences in pore size and porous structure of the different bone substitute materials may explain the observed differences in conductivity and resorbability. There is consensus in literature that the optimal pore radius for bone ingrowth is $>50\mu\text{m}$ and perhaps as large as $150\mu\text{m}$ [22–25]. In addition to the pore volume, the pore connectivity determines the effectiveness of porosity for achieving osteoconduction [22, 26–33]. Interconnections provide the space for vascular tissue that is required for bone ingrowth [25, 27, 28]. White & Shors indicated that such pore interconnections must be larger than $100\mu\text{m}$ [29].

Although bone ingrowth can be faster if the graft has a more porous and interconnected structure, denser ceramics have better mechanical integrity [22, 25, 26]. In general, porosity and compression strength are negatively correlated [34]; an increase of the total porous volume from 10–20% can result in a fourfold decrease in mechanical strength [25, 35, 36]. The bone substitute materials described below represent the major classes of (artificial) bone grafts [37]. Although market approval differs between countries, most of the products listed are widely available. Detailed knowledge on the structure as well as the biological and biomechanical properties of individual graft materials is a prerequisite for identifying the applicability of a specific clinical situation. In the end, this clinical applicability is all that matters. Whereas defects within the long bones of the lower limbs may benefit more from a product with high compressive strength, small defects in the wrist or ulna might better be filled with a biodegradable product that has more tensile strength.

5.3.2 Calcium Phosphates

Calcium phosphate salts were introduced to the clinic in the 1980s. Overall, these products can be separated into hydroxyapatite (HA) and tricalcium phosphate (TCP) [38]. In addition to pure forms of TCP and HA, multiple combinations (which are called composite grafts) exist. Different application forms have been tested, including pastes, putties, solid matrices and granules. The production process of calcium phosphate grafts affects the structure of the end-product. The production process can result in a ceramic or a cement product. A ceramic is an inorganic, nonmetallic solid product. It is prepared by thermal treatment known as sintering and subsequent cooling [39]. During

sintering, volatile chemicals are removed. Sintering also increases the crystal size, which results in a solid but porous material. Cements, on the other hand, consist of a mixture of calcium phosphates and are usually packaged as powder, which should be mixed with a specified amount of water in the operating room. Careful mixing for a specified time results in a homogenous paste that can be applied and harden *in situ* due to precipitation reactions. The setting reaction leads to the formation of either precipitated HA or dicalcium phosphate dihydrate. Cements have a solid structure characterized by limited porosity and pore size [40].

Although the ceramics do not occur in nature, they have the capacity to induce a biological response similar to that of bone [40]. Calcium phosphates are osteoconductive, but may also be osteoinducted [41, 42]. Since no osteogenic cells are present, none of the products are osteogenic. The *in vivo* degradation (resorption) seems related in composition and structure of the graft. Due to a general lack of macroporosity, calcium phosphate cement degrades layer by layer from the outside to the inside. HA ceramics are rather inert, whereas the HA cement degrades by osteoclastic activity. Combining HA and β -TCP improves the porosity of HA cement paste following implantation, because macropores are introduced into the HA composite after passive resorption of the β -TCP component. Subsequently, active resorption by monocytes/macrophages and osteoclasts can take place.

Biomechanical strength is an important characteristic in addition to the biological properties, and plays a key role in the clinical applicability of the individual products. Calcium phosphates are generally rather brittle and have limited tensile strength. β -TCP has a compressive strength similar to that of cancellous bone [43], which may allow earlier weight bearing. TCPs are less brittle, and may be able to sustain more stress initially, than HA; however, their degradation results in subsequent loss of mechanical strength over time.

5.3.2.1 CaPO_4 : Hydroxyapatite Products

5.3.2.1.1 Cerabone

Cerabone® is HA ceramic made of purely anorganic material of bovine origin. It is available as a molding block in different sizes and as granules of differing dimensions. Cerabone has a pore size of 100–1500 μm and it is osteoconductive [44]. No data on biomechanical strength are available, although the supplier claims it has a compression strength of 4.2–5.6 MPa and a shear strength of 1.2–3.4 MPa. Cerabone has been used successfully in tibia plateau fractures [45].

5.3.2.1.2 Endobon

Endobon® is a HA ceramic of bovine origin. It has a high crystallinity, with grain sizes of $1.57 \pm 0.78 \mu\text{m}$ and a density of 0.35–1.25 g.cm^{-3} [26, 46]. The overall porosity is 60–80% [47], and pore sizes range from 390–1360 μm [26]. It is osteoconductive [47–57] but not osteoinductive [48, 57]. Also, it is rather inert [49–51, 57–60]. Endobon has an *in vitro* compression strength of 1–11 MPa [26] and an *in vivo* strength of 2–20 MPa [47, 52, 58]. In one study, the compressive strength preimplantation in rabbit femoral condyles was 2–9 MPa,

which increased *in vivo* by 195% to 6–11 MPa after five weeks [58]. The Young's modulus is between 20–1200 MPa [26, 47, 52]. The tensile strength and shear strength are currently unknown. In the clinic, Endobon can be used to fill bone defects of several fracture sites: specifically, proximal tibia [50, 53, 61–63], distal tibia [53], distal radius [54, 64, 65], and calcaneus [53].

5.3.2.1.3 Ostim

Ostim® is a ready-to-use, injectable HA cement that is available in paste form. It consists of 100% synthetic, nanoparticulate, phase-free hydroxylapatite. It has osteoconductive potential [44, 45, 66–72] and is resorbed by osteoclastic activity [44, 45, 66–69]. Although minimal resorption was seen in rabbits at 12 weeks [73], approximately 70% was resorbed at one year after implantation in minipigs [69]. There are no data on the biomechanical properties after implantation, but Ostim as pure product had an overall *in vitro* porosity of $52.66 \pm 10.14\%$ ($50.52 \pm 4.49\%$ open pores, and $0.43 \pm 0.324\%$ closed pores), with a mean pore size of $56.66 \pm 07.97 \mu\text{m}$ [34]. Overall, it has a high porosity with small pores, since 95% of pores were smaller than $85 \mu\text{m}$, and no pores $> 100 \mu\text{m}$ were found. The *in vitro* ultimate compression strength of Ostim was $0.24 \pm 0.05 \text{ MPa}$, with a Young's modulus of $6.17 \pm 2.95 \text{ MPa}$ [34]. Ostim has been used clinically in tibia plateau fractures, both as single product [70] and combined with Cerabone [45]. As sole product Ostim has also been used for the treatment of calcaneal fractures [70], distal radius fractures [70, 74, 75], and for filling periodontal interbony defects [76–78].

5.3.2.1.4 Pro Osteon 500

Pro Osteon 500® is a HA ceramic, derived from marine coral exoskeletons. It is available in blocks of different sizes as well as in a granular form. It has a pore size of 280–770 μm . It is osteoconductive [59, 79, 80] and undergoes limited to no resorption over time [81, 82]. Literature data on its biomechanical properties are not available. Pro Osteon 500 has mainly been used for filling defects after benign bone tumor resections at multiple sites such as the humerus, tibia, femur, calcaneus, fibula, and ulna [83]. It was also used in distal radius fractures [84], during acetabular reconstructions [82], and as bone graft extender in lumbar fusions [85].

5.3.2.2 CaPO_4 : Tricalcium Phosphate Products

5.3.2.2.1 ChronOS

ChronOS™ is a synthetic bone void filler manufactured from β -tricalcium phosphate granules and a resorbable polymer (poly(lactide co- ϵ -caprolactone)). ChronOS has a particle size of 1.4–2.8 mm [86], a porosity of 60–75% [87–89], and a pore size of 100–400 μm [87, 89]. It is osteoconductive [86, 88, 90–92] and between 6–24 months it is resorbed by osteoclastic activity and replaced by bone [88, 91, 93]. There are no data on the biomechanical properties of ChronOS. ChronOS has been successfully used for stabilizing vertebral fractures [88]. According to the product sheet, it is also suitable for use in fractures of the femur, tibia plateau, calcaneus, humerus, and distal radius.

5.3.2.2.2 Vitoss

Vitoss® is a β -TCP ceramic that is available as blocks that can be shaped with a scalpel and gently tamped into place, and as morsels that can be packed into irregularly shaped voids in the defect site. It has a porosity of 88–92% and a pore size of 1–1000 μm [34, 94]. Osteoconductivity has been shown [86, 95]. From 6–12 months the graft is resorbed by osteoclastic activity [86] and replaced with bone. There are no data on the *in vivo* biomechanical properties of Vitoss. Vitoss has been used for the treatment of fractures of the tibia plateau, distal tibia, calcaneus, and humerus [96]. The clinical efficacy established for Vitoss as a spinal graft extender, as well as for periodontal, dental and orthopedic tumor defects has been reviewed by Damron [97].

5.3.2.3 CaPO_4 : Composite Grafts

5.3.2.3.1 BoneSave

BoneSave® is a composite, ceramic graft that consists of 80% TCP and 20% HA. According to the supplier, it has a porosity of 50% and a pore size of 300–500 μm . There are no data to prove osteoconductivity or osteoinductivity. Also, it is not known to what extent the product is resorbed over time. Likewise, data on the biomechanical properties are lacking. No reports on clinical experience with the use of BoneSave were found.

BoneSave was successfully used in spinal fusion surgery [98]. Also, BoneSave alone or in combination with allograft was used in impaction grafting of the femur at revision total hip arthroplasty [99, 100].

Data of *in vitro* studies showed that a BoneSave-allograft mixture exhibits both much greater mechanical stability and reproducibility than the pure allograft ($p < 0.05$) at all tested loads (200–800 N) [100]. A mixture of 90% BoneSave and 10% allograft provided higher mechanical stability than a 50:50% BoneSave-allograft mixture [100].

5.3.2.3.2 BoneSource

BoneSource® is a composite graft, consisting of tetracalcium phosphatate and dicalcium phosphate anhydrous. BoneSource is a self-setting cement with a porosity of 46% [101, 102] and a pore size of 2–50 μm [34, 103]. In one study, a much lower porosity of the pure product was found *in vitro* (overall porosity 1.9%, with 1% open pores and 0.9% closed pores) [34]. Over 95% of pores were smaller than 60 μm , of which approximately half were $< 26.7 \mu\text{m}$. No pores $> 100 \mu\text{m}$ were found [34]. BoneSource is osteoconductive [101, 104–110, 111, 112] and osteoinductive [102]. It is biodegradable [104, 107, 108, 110, 111]. BoneSource has a compressive strength of 6.3–34 MPa [34, 113, 114], with a Young's modulus of 3.6–4.7 MPa [104, 115]. In one study, a Young's modulus of 476 MPa was found [34]. BoneSource can be used in the treatment of fractures of the femur, tibia, calcaneus, humerus, and distal radius [106, 116].

5.3.2.3.3 Calcibon

Calcibon® is a synthetic, composite graft, consisting of 62.5% α -TCP, 26.8% dicalcium phosphate anhydrous, 8.9% calcium carbonate, and 1.8% HA. It is a

cement that has a density of 1.84 g.cm^{-3} [117] and a porosity of 30–40% [117, 118]. Reported pore sizes range from $< 1 \text{ }\mu\text{m}$ [118] to $42 \text{ }\mu\text{m}$ [34]. In the latter study it was shown that 95% of pores were smaller than $125 \text{ }\mu\text{m}$. Calcibon is osteoconductive [118–122], but not osteoinductive [119] or osteogenic. Biodegradability has been shown [120, 123, 124]. Calcibon has a compressive strength of 34–55 MPa [34, 117, 125], with a Young's modulus of 790–3000 MPa [34, 117] and a tensile strength of 4.5 MPa [126]. Calcibon is used for stabilizing thoracolumbar fractures [120, 121, 127–129] and for filling defects resulting after tumor resection [130]. In addition, the manufacturer states that it is intended for the filling and reconstruction of aseptic, metaphyseal, cancellous bone defect, with well-trying indications being fractures of the tibia plateau, calcaneus, and distal radius.

5.3.2.3.4 Camceram

Camceram[®] is a composite graft consisting of 60% HA and 40% β -TCP. It is a ceramic. No data are available on its osteoconductivity, osteoinductivity, resorption and biomechanical characteristics. No reports on clinical experience with the use of Camceram were found.

5.3.2.3.5 ChronOS Inject

ChronOS[™] Inject is a composite graft consisting of 73% β -TCP 21% monocalcium phosphate monohydrate and 5% magnesium hydrogen phosphate trihydrate. It is a cement that was shown to be osteoconductive [131–133]. ChronOS Inject is almost completely resorbed by osteoclastic activity within 6 months [131–133]. At the time of implantation, ChronOS Inject has an overall *in vitro* porosity of $6.93 \pm 1.32\%$ ($2.86 \pm 0.92\%$ open pores, and $3.59 \pm 0.41\%$ closed pores). It contains pores up to $500 \text{ }\mu\text{m}$, with 95% of pores being smaller than $250 \text{ }\mu\text{m}$; the mean pore size is $97.43 \pm 27.24 \text{ }\mu\text{m}$ [34, 86, 89]. The ultimate compression strength *in vitro* was $0.81 \pm 0.32 \text{ MPa}$, with a Young's modulus of $53.5 \pm 20.4 \text{ MPa}$ [34]. ChronOS Inject provides an alternative treatment for benign bone cysts that are unlikely to heal spontaneously [134, 135]. No reports on clinical experience with the use of ChronOS Inject other than after tumor resection were found. However, the supplier states that it was designed for use in fractures of the femur, tibia plateau, calcaneus, and humerus.

5.3.2.3.6 HydroSet

HydroSet[™] is a composite graft, consisting of tetracalcium phosphate, dicalcium phosphate, and trisodium citrate. It is an injectable, sculptable, and self-setting cement that converts to hydroxyapatite after application. Due to its chemical structure, it is osteoconductive; however, there are no data on osteoinductivity or biodegradability available. At the time of implantation, HydroSet has an overall *in vitro* porosity of 2.8% (0.3% open pores, and 2.7% closed pores) and a mean pore size of $59 \text{ }\mu\text{m}$ [34]. A few pores up to $230 \text{ }\mu\text{m}$ were found, but 95% of pores were smaller than $125 \text{ }\mu\text{m}$ [34]. HydroSet was shown to have a compressive strength of 10–24 MPa [34, 136], a Young's modulus of 125–355 MPa [136] [34], and a tensile strength of 0.11–0.17 MPa [137]. No reports on clinical experience with the use of HydroSet were found; however, the product sheet states that it is indicated to fill bone voids or gaps (either

surgically created of osseous defects created from traumatic injury) that are not intrinsic to the stability of the bone structure.

5.3.2.3.7 Norian SRS

Norian SRS® (skeletal repair system) is a composite graft consisting of α -TCP, calcium carbonate, and monocalcium phosphate monohydrate. It is an injectable and moldable calcium phosphate cement, that sets at body temperature into carbonated apatite. It has a density of 1.3 g.cm⁻³ [138]. Norian SRS was shown to be osteoconductive [132, 139–143], and it is biodegraded by osteoclasts and replaced with bone [132, 139–141]. At the time of implantation, Norian SRS has an overall *in vitro* porosity of 0.5% (0.3% open pores, and 0.3% closed pores) [34]. It contains a few pores up to 230 μ m, however 95% were smaller than 125 μ m (mean pore size 47 μ m) [34]. Norian SRS has a compressive strength of 23–55 MPa [34, 138, 144, 145], a Young's modulus of 674 MPa [34], a tensile strength of 2.1 MPa [144, 145], and a shear strength of 0.85–1.3 MPa [146]. As augmentation compound, Norian SRS increases resistance of cortical screws to torque forces [147].

Of all the calcium phosphate cements, Norian SRS has been tested most extensively. In the upper extremity it has been used successfully for treating fractures of the humerus [148], and distal radius [143, 149–156] has been achieved. In the lower extremities, up to 95% success rates have been achieved in tibia plateau fractures [157–162]. Norian has also been successfully used in femoral fractures [163, 164] and calcaneal fractures [140, 165, 166]. Another study shows postoperative full weight bearing as early as 3 weeks in standard open reduction with internal fixation in calcaneal fractures [140]. According to its supplier, Norian SRS is indicated mainly for elderly/osteoporotic patients, in particular for fractures of the distal radius, the proximal and distal tibia, the calcaneus, the proximal and distal femur, the proximal humerus, and the acetabulum.

5.3.3 Calcium Sulphates

Calcium sulphates (CaSO₄) is also known as plaster of Paris. It was introduced into the clinic for filling a bone defect already in the late 1800s [167]. Heating gypsum results in a dry powder, which can be used in the clinic. Prior to its use, water should be added. This results in an exothermic reaction, which in the end leads to crystallization and hardening of the preparation. CaSO₄ products are available as powder, granules or blocks. Calcium sulphates act as an osteoconductive scaffold, but lack osteogenic and osteoinductive properties. Calcium sulphate is biocompatible, elicits little or no macrophagic reaction, and is fully dissolved within 6–12 weeks [168]. Of the available osteoconductive bone graft substitutes, calcium sulphate is the most rapidly resorbed. Because of its rapid resorption rate and low mechanical strength, calcium sulphate is recommended as a bone graft extender rather than as void filler. Calcium sulphate grafts have mainly been used for filling bony voids resulting after tumor resection surgery, but several papers also report on successful use in fracture healing.

5.3.3.1 *BonePlast*

BonePlast® is a calcium sulphate bone substitute that is available as powder. Upon mixing with an included liquid, a paste forms that can be injected into a defect area. Very limited information is available for BonePlast. Based upon its chemical structure it is expected to be osteoconductive, however there are no studies available to prove this. Moreover, there are no publications on the biomechanical properties. Surgical indications of BonePlast include pelvic, humerus, calcaneus and femoral bone after aspiration of recurrent aneurysmal bone cysts [169]. According to the manufacturer, BonePlast may be used in non-load bearing, bony voids or gaps anywhere in the skeletal system, even in the presence of infection.

5.3.3.2 *MIIG X3*

MIIG® X3 is an injectable calcium sulphate paste. MIIG X3 was found to be osteoconductive and it undergoes biodegradation and replacement by new bone over time [170–172]. At the time of implantation, MIIG X3 has an overall *in vitro* porosity of 0.6% (0.3% open pores, and 0.3% closed pores), with a mean pore size of 67 µm [34]. Prior to implantation, the ultimate compression strength was 21.8 MPa, with a Young's modulus of 665 MPa [34]. At 26 weeks after implantation in the humerus of a dog, the compressive strength of MIIG X3 was 0.6 MPa [173]. There are currently no data on the tensile strength and shear strength of MIIG X3. MIIG X3 has been used for grafting bone defects of the proximal or distal tibia, patella, calcaneus, ileum, femur, and humerus [170, 171, 173], as well as after bone tumor resection [170]. Recently, MIIG X3 HiVisc, which has a higher viscosity to provide additional work time, was successfully used as augmentation graft for an internal locking system for the minimally invasive treatment of osteoporotic proximal humeral fractures in elderly patients [174].

5.3.3.3 *OsteoSet*

OsteoSet® is a surgical grade calcium sulphate in a pure, uniform crystalline form. It has the same chemical composition as MIIG X3, but is available in granules or blocks. The blocks are available in different size, and need to be cut to the desired size and shape prior to use. OsteoSet was shown to be osteoconductive in several studies [168, 173, 175–177] but denied in two other studies [178, 179]. At 26 weeks after implantation in the humerus of a dog, the compressive strength of OsteoSet was 0.6–0.9 MPa [173, 176]. The Young's modulus of the same samples was 59 MPa [176]. Details on the tensile and shear strength were not found in literature. OsteoSet has mainly been used after tumor resection [180–184]. It has also been used for filling defects after fracture of the humerus, radius, ulna, femur, tibia, fibula and calcaneus [180–183, 185]. Based upon currently available data showing the occurrence of stress fractures after grafting [182, 184], it seems like OsteoSet may not provide sufficient biomechanical support when used in skeletal repair. A recently published modification is the use of Osteoset® T; this calcium sulphate void filler contains 4%

tobramycin sulphate, and the sulphate cement is used as an antibiotic carrier [186]. Clinical results in 12 patients were promising; however the antibiotic dosage may require adjustment.

5.3.3.4 *Stimulan*

Stimulan® is manufactured from medical grade calcium sulphate hemihydrate ($\text{CaSO}_4 \cdot 0.5 \text{H}_2\text{O}$) and is available as pellets. It is an osteoconductive bone substitute that completely reabsorbs and is replaced with new bone. There are no publications on the biomechanical properties and clinical use of Stimulan. The supplier propagates its use in filling defects after tumor resection. They state that Stimulan pellets are indicated only for bony voids or defects/gaps that are not intrinsic to the stability of the bony structure. According to the product sheet, it can safely be used at an infected site. The latter is supported by literature. Moxifloxacin, fusidic acid, and daptomycin may be eluted at high concentrations *in vitro* by using Stimulan as a carrier [187, 188]. In an experimental study, experimental osteomyelitis caused by methicillin-resistant *Staphylococcus aureus* was successfully treated with a synthetic carrier of calcium sulphate (Stimulan) releasing moxifloxacin [189].

5.3.4 Bioactive Glass

Bioactive glasses were introduced into the clinic in the 1980s [190]. They are hard and solid, nonporous products. The main component is silicon dioxide (silicate), but bioactive glass also contains sodium oxide, calcium oxide, and phosphorous [10]. Solubility and resorbability can be modified by varying the proportions of sodium oxide, calcium oxide and silicon dioxide [191]. Bioactive glass can be manufactured into microspheres, fibers and porous implants. Bioactive glass has osteoinductive and osteoconductive properties [10]. Bioactivity, i.e., bonding between the graft and the surrounding bone, depends on the relative amount of silicon dioxide, with 45–52 percent of silicon dioxide being the optimum [192]. Following contact with body fluids, a silicate-rich layer is formed which becomes covered with a layer of HA to direct new bone formation. Osteoprogenitor cells are attracted by the extracellular proteins and proliferate into matrix-producing osteoblasts [192, 193]. Due to the low porosity and high osteointegration, bioactive glass possesses a much better mechanical strength than calcium phosphate products [10]. Bioactive glass has been applied for craniofacial reconstructive surgery, dental surgery, and trauma or orthopedic surgery. However, the number of scientific publications that report on clinical applications of bioactive glass is still limited.

5.3.4.1 *Cortoss*

The best known bioactive glass bone graft is Cortoss®. Cortoss is an injectable polymer composite available as a paste. It is provided in a double-lumen cartridge with specially designed tips for mixing. No data concerning other structural properties of Cortoss, such as porosity and pore size, was found.

The supplier claims that the porosity is 1%. Cortoss has osteoconductive properties [194], but is not resorbed [195]. It does not induce cytotoxicity [196], and offers biocompatibility and reduced risk of thermal necrosis [197]. Cortoss has a unique porous structure [34]; at the time of implantation, it has an overall *in vitro* porosity of 1.5% (0.5% open pores, and 1.0% closed pores) and a mean pore size of 148 μm . A large range of pore sizes (25–300 μm) is equally present. Overall, 95% of pores had sizes up to 390 μm , although pores of 500 μm were also found [34]. Cortoss has a compressive strength of 47–179 MPa, a Young's modulus of 316 MPa and a shear strength of 8.4 MPa [34, 198, 199]. According to the manufacturer, Cortoss has a tensile strength of 52 MPa; however, this has not been confirmed in other studies. Reports on clinical applications of Cortoss are few. Cortoss has been successfully for screw augmentation in ankle fractures [200], for vertebroplasty and kyphoplasty of vertebral compression fractures [201–204], and for filling a defect after a distal radius fracture [195].

5.3.5 Miscellaneous Products

5.3.5.1 *Bio-Oss*

Bio-Oss® is isolated and purified from bovine bone and is available as cancellous and cortical bone void filling granules [205]. It has a Ca/P ratio of 1.47 ± 0.17 . The fill volume, which relates to the space available for conducting cellular ingrowth and new bone deposition, is $1.69 \pm 0.04 \text{ cm}^3.\text{g}^{-1}$. Approximately 65% of macropores are between 200 and 600 μm , and pores with a size of 0.3–0.6 μm represent 50–60% of all micropores (i.e., pores with a size of 0.1–1.0 μm). It was designed for filling defects in the oral cavity. No trauma surgery applications are known.

5.3.5.2 *Collagraft*

Collagraft® matrix is composed of purified Type 1 bovine dermal fibrillar collagen and approximately 65% hydroxyapatite and 35% tricalcium phosphate. It is available in strips that can be cut to the required size. In itself it has no biomechanical strength, but rather functions as a carrier for osteogenic cells or osteoinductive proteins. Collagraft can be mixed with autologous bone for achieving adequate bone ingrowth into the defect [206]. In a rabbit model, Collagraft was shown to resorb over time, during which it was replaced with new bone [79].

Because it lacks mechanical strength, Collagraft is indicated only for use in bony voids or gaps that are not intrinsic to the stability of the bony structure. The bony defects should always be internally or externally fixated to achieve biomechanical strength.

5.3.5.3 *CopiOs*

CopiOs® is a composite graft consisting of dibasic calcium phosphate and purified Type I bovine collagen. It is available as sponge or as paste compressed

powder disc. In itself it is an osteoconductive scaffold that due to the spongy structure does not provide any biomechanical strength. For achieving optimal bone growth, CopiOs should be mixed with autologous bone marrow aspirate (containing osteogenic cells and osteoinductive proteins) or bone mineral proteins. According to the supplier, CopiOs, in combination with autologous blood products, is intended for use only for filling bone voids or gaps of the extremities, pelvis, and spine (combined with appropriate stabilizing hardware) that are not intrinsic to the stability of the bone structure. Literature to support this is awaited.

5.3.5.4 *NuOss*

NuOss™ is isolated and purified from bovine bone and is also available as cancellous and cortical bone void filling granules. NuOss™ has properties that closely resemble those of Bio-Oss [205]. It has a Ca/P ratio of 1.58 ± 0.16 and a fill volume of $1.70 \pm 0.01 \text{ cm}^3\text{g}^{-1}$. Approximately 65% of macropores are between 200 and 600 μm , and, pores with a size of 0.3–0.6 μm represent 50–60% of all micropores (i.e., pores with a size of 0.1–1.0 μm). The clinical indications are unknown based on reading the literature, but since it has physical and chemical characteristics very similar to Bio-Oss it is to be expected that NuOss will perform equally as effective as Bio-Oss for bone void fill in oral surgical applications.

5.3.6 Future Directions

Calcium phosphate ceramics and polymers have proven poor strength-to-weight ratios and toughness under loading conditions [207, 208]. The currently available products provide a good range of properties, yet all have their advantages and disadvantages. With new information and technologies the search for novel products seems never-ending. One area that requires further exploration is the use of porous metal scaffolds, which can be made by novel techniques. Improvements in processing techniques have provided new porous materials with both increased porosity and interconnecting channels between the pores [209–211]. Porous metallic scaffolds gained interest because of their biomechanical strength and fatigue resistance over extended periods. Titanium (Ti) is widely proven to enhance bone contact and growth on its surface. Several metal implants have been reported on [212–214]. These have sufficient mechanical properties and biocompatibility for clinical use under load-bearing conditions [215]. Adding an osteoinductive stimulus to the graft might enhance healing.

Titanium foam produced by freeform fabrication techniques gained special interest during recent years since it allows optimization of the structural properties of the foam. Titanium foam produced using a rapid prototyping technique in combination with selective laser melting seems very promising. Three-dimensional images made of cancellous or cortical bone can be used as a template for production, which makes it possible to fully control the structure of the titanium foam.

Zardiackas reported porous tantalum with high porosity up to 80%, and a compression yield strength of around 50 MPa [214]. When this porous tantalum is coated with calcium phosphate, it also possesses good osteoconductivity and osteoinductivity *in vivo* [216, 217]. Li *et al.* reported a porous titanium alloy [Ti6Al4V] with a porosity of 80% and a compression strength of 10 MPa [218]. This porous titanium alloy can be successfully coated with calcium phosphate by the biomimetic method, and showed enhanced osteoconductivity and osteoinductivity *in vivo* [219]. Wen and colleagues [220, 221] developed a Ti foam using a powder metallurgical process to generate a bimodal pore distribution (macropores and micropores) resembling natural bone architecture. Assad *et al.* developed a porous NiTi with a high elasticity and low elastic modulus to prevent stress shielding. The product showed excellent biocompatibility, although its nickel content creates some concerns [212, 222]. Takemoto *et al.* reported a plasma-spray method for producing porous titanium implants with a unique interconnected porous structure and an ultimate compression strength of 281MPa [215]. The compression yield strength is approximately 85 MPa, which is close to cortical bone (80–120MPa). The low compressive modulus (4.7 GPa) of their porous bioactive titanium may be beneficial to reduce stress shielding under load-bearing conditions, compared to other load-bearing implants made of solid titanium (110GPa) or other metals [222, 223].

Given the excellent overall biocompatibility, commercial pure titanium (Ti) and alloys (Ti6Al4V) have been widely tested for joint replacement and spinal-fusion procedures [223–225]. One of the first *in vivo* studies on the use of Ti foam was recently published by Sargeant and colleagues [226]. They tested a hybrid implant material by assembling Ti6Al4V foam and peptide-amphiphile nanofiber matrix using a rat femora model. The histological examination at four weeks revealed new bone deposition from the cortical bone toward the implants and inside the pores, indicating that Ti foam promoted anchorage from the surrounding bone tissue to increase the mechanical stability of the implanted material. Good biocompatibility (i.e., osteointegration) has also been shown when using titanium sponge rods in a canine humeral osteotomy model [227] and a rabbit distal femoral fracture model [228]. The outcomes of the *in vivo* studies confirm the findings of *in vitro* studies [214, 223, 229] that Ti foam allows for bone ingrowth through interconnected pores. This group of products requires further exploration in other experimental situations.

5.4 Combinations with Osteogenic and Osteoinductive Materials

Although (ceramic) bone substitute biomaterials have been developed in the direction of materials with a structure that resembles natural bone matrix (i.e., they can be porous, dense, resorbable, bioactive, or bioinert), they are generally only osteoconductive. They provide a scaffold for the ingrowth of the

newly formed bone, but are not osteoinductive. Since they also do not contain bone precursor cells they are not osteogenic. Consequently, their clinical application is restricted merely to relatively small bone defects [230]. As a solution, osteoconductive bone substitute materials can be combined with osteogenic or osteoinductive materials. Multiple combinations have been tried, either in preclinical studies or even already in clinical situations. A full review of the osteogenic and osteoinductive substances that can be added to bone substitute materials is not the scope of this chapter; however, several promising options are summarized below.

5.4.1 Osteogenic Substances

Bone marrow is known to contain pluripotent stem cells. The osteogenic properties of bone marrow were described for the first time by Goujon in 1869 [231]. Upon transplantation into a bone defect, bone marrow induces primary bone formation. The initial bone is subsequently remodeled by invading host tissue [232]. This sequence of events equals the response observed after transplantation of a cancellous bone graft [233] or a fresh bone autograft [234, 235]. It has been shown that the osteogenic capacity can be due to several factors in the bone marrow. These include a marrow cell origin of osteoblasts [236–239] and osteoprogenitor cells that are derived from mesenchymal stem cells (MSCs) present in the bone marrow stroma [240, 241]. The osteogenic properties of marrow cells either alone or in combination with porous (ceramic) composites have been demonstrated in several studies [232, 242–250]. The bone substitute acts as a delivery vehicle for bone marrow. Its porous structure influences the neovascularization in a way that prevents cartilage formation. The bone substitute material can be soaked in marrow cells prior to implantations. The porosity determines the ability of the material to retain these marrow cells [242, 243, 251]. The degree of new bone formation depends upon the initial cell density of the cell suspension in which the substitute material is soaked before implantation. A suspension of less than 5×10^5 cells/mL showed insufficient osteogenesis, whereas a cell density of more than 5×10^6 cells/mL showed consistent osteogenesis [239, 242, 244, 246]. These cells can be obtained from an iliac crest puncture or by *in vitro* culture of red bone marrow.

Another cell type present in bone marrow that may contribute to fracture healing are endothelial progenitor cells (EPCs). EPCs are bone marrow-derived cells with the ability to differentiate into endothelial cells and to participate in the establishment of neovasculature [252]. They represent a population of precursor cells that enter the circulation in response to trauma and home to sites of tissue ischemia [253–255]. EPCs have the ability to differentiate into osteogenic cells *in vitro* and they are upregulated in response to orthopedic trauma in humans [254]. EPCs may contribute to neovascularization and thus new bone formation in fracture healing [256]. EPCs have been shown to augment fracture healing and local angiogenesis in segmental defect models in rat femurs [257, 258] and sheep tibias [259].

5.4.2 Osteoinductive Substances

Urist was the first to describe bone morphogenetic proteins (BMPs) as the active proteins responsible for ectopic bone formation after subcutaneous or intramuscular implantation of demineralized bone matrix [11]. The BMPs belong to the TGF- β (transforming growth factor) super family and have the ability to stimulate differentiation of MSCs into a chondroblastic and osteoblastic direction [260–263]. BMPs are released upon injury to the bone matrix. They evoke various mechanisms that contribute to bone formation, including angiogenesis and chemotaxis, and differentiation of mesenchymal cells. Two BMPs with osteoinductive capacities are BMP-2 and BMP-7 (also known as osteogenic protein-1, OP-1). These BMPs and their receptors have been demonstrated in fractures and in callus [264–268] and were shown to stimulate bone defect healing [269–283]. Combining porous calcium phosphate ceramics with osteoinductive BMPs seems to improve healing of bone defects [284–292].

Several other growth factors including parathyroid hormone (PTH), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor- β (TGF- β), and platelet-rich plasma (PRP) have been tested in preclinical and early clinical situations. These are effectively reviewed by Nauth *et al.* [293].

5.5 Discussion and Conclusion

The repair of large bony defects resulting from trauma or malignant bone disease remains a major problem in trauma and orthopedic surgery. Treatment options depend upon the size and location of the defect, but patient characteristics, like bone quality, age and co-morbidities may also affect the outcome. There is a continuous search for novel bone substitute materials, and the number of products available for use in trauma and orthopedic surgery is steadily increasing.

Critical characteristics that determine the clinical usefulness of bone grafts are their osteogenicity, osteoinductivity, and osteoconductivity. The current void fillers are osteoconductive, but only a limited number are also osteoinductive, and none are osteogenic. Calcium phosphates may possess osteoinductive properties under certain conditions [41, 42]. Osteoconductivity is dependent on the pore size, porosity and degradation potential of the bone substitute material. A macroporous structure of pores ranging from 150–500 μm in size is considered optimal for ingrowth of new bone [25]. A higher number of interconnected pores facilitate the ingrowth of new bone [25, 27, 28]. Availability of pores $< 5 \mu\text{m}$ is considered important for bioresorbability [21]. The resorption rate of the current products is highly variable and ranges from weeks to decades, depending on their chemical composition. HA are the most inert material, and remains *in situ* even after 10 years. TCP and calcium phosphate cement are degraded within approximately 2 years as a result of osteoclastic activity [40], and calcium sulphates generally dissolve within 2–3

months [294]. Faster resorption is not necessarily better or worse, it depends on the local needs at the defect side.

Besides optimal biological properties, bone substitutes should have similar biomechanical strengths as the bone being replaced. The biomechanical properties vary between locations; cortical and cancellous bone have different properties, but also weight-bearing and non-weight-bearing bones differ in strength. Human cortical bone has a compressive strength of 130–290 MPa and a tensile strength of 90–190 MPa, whereas the compressive strength of cancellous bone ranges between 2 and 38 MPa [295]. As the structure and concomitantly also the biomechanic properties of void fillers change *in vivo* due to osteointegration, bioresorption and ingrowth of new bone, the ultimate strength over the course of time may be difficult to predict. Grafting a defect with a bone substitute material that has a higher initial biomechanical strength than the surrounding bone may result in stress shielding and subsequent bone resorption at the bone-implant interface, or may lead to delayed fractures along it. Using a bone substitute material with a lower biomechanical strength than the surrounding bone may lead to delayed fractures due to the lack of biomechanical stability [37].

Of the currently available bone substitute products, none seems to be suitable for grafting of significant cortical bone defects without additive support. Additives include (mainly bone-marrow derived) osteogenic cells like MSCs and EPCs as well as osteoinductive stimuli such as bone morphogenetic proteins, VEGF, and PDGF [296, 297].

As mentioned above, the structural, biological and biomechanical properties of bone substitute materials are critical to their clinical success. The bone substitutes described in this chapter each have their individual characteristics, and provide orthopedic and trauma surgeons with a choice of products that varies largely in architecture and strength. Since the currently available products have been tested using different designs (e.g., different animals with different defect locations and sizes, and various *in vitro* models) and with different outcome measures and scoring systems, it is not possible to relate the results of a certain product in comparison to another [34, 37]. The data outlined here will assist surgeons in selecting the most suitable product for specific clinical indications. Further studies on their *in vivo* behavior are needed for developing clinical guidelines for use of alternative bone substitute materials in orthopedic trauma surgery.

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Surface Functionalized Hydrogel Nanoparticles

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Abstract

The controlled release of therapeutic agents to the specific site of action at the optimal rate and extent has been a major goal in designing the nanoparticulate systems as the efficient drug delivery devices. Nowadays, hydrogel nanoparticles having the unique properties of nano-range size as well as extreme hydrophilicity and high water content, are one of the most promising and attractive nanocarrier systems in the drug delivery field. Among the natural polymers, chitosan and alginate have been studied extensively for preparation of hydrogel nanoparticles and from synthetic group, hydrogel nanoparticles based on poly (vinyl alcohol), poly (ethylene oxide), poly (ethyleneimine), poly (vinyl pyrrolidone), and poly-N-isopropylacrylamide have been reported with different characteristics and features with respect to drug delivery. The hydrogel nanoparticles' surface acts as such an important bridge between the nanogel bulk and the outer environment, and many specific surface properties, such as wettability, paintability, adhesion susceptibility, biocompatibility and so on, must be incarnated through this layer. Conventional polymer surface functionalization techniques are always dependent on the chemical or physical methodologies including corona, chemical reaction, surface grafting, and surface coating or etching of finished nanoparticles. However, the complicated processes and the specialized equipment are always required for using these techniques. Thiol-functionalized hydrogel microparticles for oral and nasal drug delivery applications, N-Succinyl-chitosan nanoparticles, β -cyclodextrin modified magnetic chitosan nanoparticles, alginate-graft-poly (ethylene glycol) and α -cyclodextrin, Chitosan-g-poly(N-isopropylacrylamide)-based nanogels for tumor extracellular targeting, and Peptide-functionalized nanogels for targeted siRNA delivery are the some instances of surface modification of hydrogel nanoparticles.

Keywords: Hydrogel nanoparticles, Surface functionalization, Chitosan, Alginate, Poly (vinyl alcohol), Poly (ethylene oxide), Poly (ethyleneimine), Poly (vinyl pyrrolidone), Poly-N-isopropylacrylamide

6.1 Hydrogel Nanoparticles

Today, hydrogel nanoparticles, having unique properties of nano-range size as well as extreme hydrophilicity and high water content, are one of the most promising and attractive nanocarrier systems in the drug delivery field [1]. Both natural and synthetic polymers have been used for preparation of hydrogel nanoparticles, each having its own advantages and drawbacks. Chitosan and alginate are typical examples of natural polymers and poly(vinyl alcohol), and poly-N-isopropylacrylamide are among the most used synthetic polymers for preparation of hydrogel nanoparticles [1–3]. Hydrogels are polymeric networks with unique three-dimensional structures with high capacity for the absorption of water or biological fluids. Hydrophilic groups such as $-\text{OH}$, $-\text{CONH}-$, $-\text{CONH}_2$, and $-\text{SO}_3\text{H}$ in polymer chemical structure are responsible for the crosslink interaction between and or within the polymeric chains, thereby resulting in the formation of hydrogel [4]. In addition to the high water absorptivity of these polymeric networks, the soft and rubbery texture of the hydrogels along with their low interfacial tension with water or biological fluids, are the other common physical properties of the different types of hydrogels. Two main approaches are used routinely for crosslinking of the hydrogel matrices, including chemically-based and physically-based methods. Highly important in the context of drug delivery applications of hydrogel nanoparticles, is the swelling behavior of a hydrogel instead of being dissolved upon exposure to aqueous media which, in turn, results in different, while unique, drug loading and release characteristics of these nanocarriers [5–6].

The drug release mechanism from hydrogel nanoparticles is complex, mostly dependent on the main polymers forming the matrix as well as the chemical structure of the diffusant (e.g., the drug). Regarding the rate-limiting steps of the overall drug release from these nanoparticles, three distinct categories can be recognized, including: i) diffusion-controlled, ii) swelling-controlled, and iii) chemically-controlled. Of the above steps, the diffusion-controlled drug release from hydrogels is the dominant mechanism, described mainly by Fick's first law of diffusion. The mesh size within the matrix of the gel is the most important factor in drug diffusion out of a hydrogel matrix [1]. Several parameters determine the mesh sizes of a hydrogel, such as crosslink degree, monomers chemical structure, and, when applicable, type as well as intensity of the external stimuli. Other physical properties like mechanical strength, degradability, and diffusivity from a hydrogel network are closely associated with the mesh size. Typical mesh sizes reported for biomedical hydrogels range from 5–100 nm (in their swollen state) [7–8], which are much larger than most of the small-molecule drugs. Therefore, the diffusion of small molecules from this matrix would be very hard to control, while the macromolecules such as oligonucleotides, peptides, and proteins seem to be better candidates for time-controlled drug delivery using hydrogel structures including nanoparticles made of hydrogels [9–11]. In the case of the swelling-controlled release mechanism, when the apparent release rate of a drug is significantly faster than the one predicted by the Fickian diffusion model, swelling is considered most probable to be controlling the release behavior. Finally, the

occurrence and rate of a particular chemical reaction in the gel matrix is sometimes the release-controlling mechanism. This chemical reaction is mainly cleavage of the polymeric chain making the hydrogel crosslink via hydrolytic or enzymatic degradation and/or the reversible or irreversible reactions that are taking place between the polymer network and the releasing drug. In addition to the above-mentioned release mechanisms, under certain circumstances, surface or bulk erosion of hydrogels or the binding equilibrium among the drug-binding moieties incorporated within the hydrogels, are two different mechanisms reported as controlling the rate of drug release from hydrogels [12–14].

Hydrogel nanoparticulate materials show the interesting characteristics of hydrogels and nanoparticles at the same time. Hydrophilicity, flexibility, versatility, high water absorptivity, and biocompatibility are some beneficial properties brought about from the hydrogel nature of these nanoparticles. Other properties are a long lifespan in blood circulation and the possibility of being actively or passively targeted to the desired biophase (e.g., tumor sites come from the tiny sizes of these nanocarriers). In this chapter, some of the most often studied and attractive categories of hydrogel nanoparticles, both natural and synthetic types, are reviewed briefly with the main focus being on the recent experiences in the drug delivery era. Next, an overview will be presented of the most recent field of interest – the surface functionalization of these nanoparticles – as a highly promising scenario in the way to make novel, efficient applications from these nanostructures.

6.2 Hydrogel Nanoparticles Based on Chitosan

Chitosan, α (1-4)-2-amino-2-deoxy β -D-glucan, is a deacetylated form of chitin, an abundant polysaccharide presented in crustacean shells. This biopolymer is one of the most interesting materials in general biomedical applications, especially in drug delivery research [3, 15–17]. Several research groups around the world are working actively on the application of chitosan in the nanoparticulate delivery of drugs and other bioactive agents, including genes. Chitosan is a hydrophilic and positively charged polymer which enables this polymer to interact with negatively charged polymers, macromolecules, and even certain inorganic polyanion. These interactions, in turn, result in sol-gel transitional structures, mainly hydrogels with high potential for nano-encapsulation purposes. Another interesting property of chitosan which is the reason for the use of this polymer in mucosal drug delivery is its high affinity for adherence to the mucosal surfaces within the body. More recent studies have shown that chitosan has this capacity because of its ability to open the tight junctions between epithelial cells [18–23]. Chitosan has a remarkable biocompatibility and biosafety profile, therefore, many articles on the potential of chitosan for pharmaceutical applications have already been published [24]. Therefore, the main scope of the current review is to focus on the specific features and applications of the chitosan-based nanoparticulate systems prepared and characterized to date for delivery of macromolecular compounds such as peptides, proteins, antigens, oligonucleotides, and genes [25].

Hydrogel nanoparticles based on chitosan are routinely prepared using a method referred to as ionotropic gelation which is based on the electrostatic interactions between the polycation chitosan and an inorganic polyanion, typically tripolyphosphate [26–31]. The nanoparticles prepared by this method have diameters in the generally accepted range of less than 200 nm along with a high degree of reproducibility and low polydispersity indices. The main problem, however, with these nanoparticles is the high degree of burst release in initial time periods which, in turn, necessitates equipping them with some additional physical or chemical barriers to retard the drug release out of the particles. The biodegradable crosslinked chitosan nanoparticles might be useful and have been attempted for delivery of small molecules, proteins and peptides, and genetic materials [15, 32–33].

6.3 Hydrogel Nanoparticles Based on Alginate

Alginic acid is an anionic biopolymer consisting of linear chains of α -L-glucuronic acid and β -D-mannuronic acid. Alginic acid has a high degree of solubility in aqueous media; high capacity of gelation in proper conditions with high porosity of the resulting gels, biocompatibility, and a well-documented biosafety profile. Due to the addition of counter-ions to alginate, it can be used as hydrogel structured drug delivery carriers such as micro- and nanoparticles. The sequence of the reaction can be initiated by any kind of cationic species, but calcium chloride is the most used one by researchers. The preparation methods can be optimized by the control of gelification events, thus resulting in different particle size ranges [1]. The size range obtained is affected by various factors such as alginate concentration and viscosity, counter-ion concentration, the speed of adding counter-ion solution onto the alginate solution, etc. In 1993, Rajaonarivony *et al.* presented a novel alginate-based drug carrier system with a wide range of particle sizes of 250–850 nm. In this study, calcium chloride was added to the sodium alginate solution followed by the addition of poly-L-lysine. In order to form a gel, in this study, the concentration of the polymer and counter-ion solutions were lower than those used regularly. Doxorubicin was used as a model drug in this study and it was reported that the loading capacity could be reached at more than 50 mg of drug per 100 mg of alginate. From the late 1990s up until now, studies based on the alginate nanoparticulate system continue to grow. Different therapeutic agents such as insulin, and antitubercular and antifungal drugs have tried to be loaded by the alginate-based nanocarriers, and have even shown promising results in the field of gene delivery. While the order of counterion addition to the alginate solution can control the size range of alginate nanoparticles, some scientists demand the advantages of the polyelectrolyte addition in this complexation steps. One of the examples is the insulin-loaded alginate-chitosan nanoparticles prepared by Sarmiento *et al.* where chitosan was used as polyelectrolyte complexing agent. In this study, particles with size range of a nanometer and loading capacity of 14.3% were optimized. In another investigation, insulin-loaded nanoparticles

were prepared by alginate using dextran as complexing agents by the use of nanoemulsion dispersion method. The resulting size range of 267 nm to 2.76 μm was achieved. The unimodal size distribution and insulin encapsulation efficiency of 82.5% were achieved. The modified release drug delivery systems based on alginate have also been used for preparation of a novel delivery system for the treatment of mycobacterial infections. On the basis of this investigation a study was designed to assess the pharmacokinetic and tissue distribution of free and NP-encapsulated antitubercular drugs in different doses; alginate nanoparticles containing isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) were orally administered to mice [34–35]. The average particle size of 235.5 nm was observed for these particles and the encapsulation efficiencies of 70–90%, 80–90%, and 88–95% for INH, RIF, and EMB, was reported respectively. It was shown in this study that all the encapsulated drugs have significantly higher bioavailability than the equidose free drug. Furthermore Ahmad *et al.* prepared inhalable alginate-based nanoparticles for the same drugs, with the exception of EMB, and reported the same results of capacity and size. Not long ago, the same research group published another study on alginate-based nanoparticles loaded by the azol antifungal and antitubercular agents [36].

Alginate-based hydrogel matrices were used by Dong-Hwan Kim *et al.* to incorporate dexamethasone-loaded PLGA nanoparticles. The release of this anti-inflammatory agent was investigated from this particulate system. Solvent evaporation technique was used to incorporate dexamethasone in PLGA nanoparticles. The nanoparticles were characterized for size, drug loading and *in vitro* release. The crosslinking density of the hydrogel was studied and its correlations with the kinetics of drug release were investigated. The typical particle size was 400–600 nm and the *in vitro* release of DEX from nanoparticles entrapped in the hydrogel showed that 90% of the drug was released over 2 weeks [37].

6.4 Hydrogel Nanoparticles Based on Poly(vinyl Alcohol)

The free radical polymerization of vinyl acetate and the following hydrolysis of acetate group to hydroxyl result in the formation of poly(vinyl alcohol), generally referred to as PVA, with a wide molecular weight distribution. One of the important properties of PVA is the critical role of the molecular weight of the polymer in the characteristics of the polymer, which, in turn, has a determining effect on crystallizability, adhesion, mechanical strength, and diffusivity of the polymer [38]. Among the synthetic polymers which are used in hydrogel studies, PVA is the most promising one. In the late 1990s, PVA nanoparticles were prepared as a delivery system for peptides/proteins using water-in-oil emulsion/cyclic freezing-thawing method. The average particle size of the PVA nanoparticles obtained was 675.5 ± 42.7 nm with a skewed or log-normalized size distribution. In this study, bovine serum albumin was used as a model protein drug for loading in hydrogel nanoparticles. The reported loading efficiency

was $96.2 \pm 3.8\%$ and a diffusion-controlled release was observed [39]. The PVA hydrogel nanoparticles have an affinity for water absorption and swelling behavior. With increasing temperature, the swelling degree will increase. The release mechanism of BSA from PVA hydrogel nanoparticles was shown to be diffusion-controlled, which is shown to be capable of prolonged release of the drug up to 30 h. Significantly, both the number of freezing-thawing cycles and the release temperature were shown to influence the BSA release from nanoparticles. With a decrease in freezing-thawing cycles or an increase in the release temperature, the drug release became faster [38–40]. In another study, Galindo-Rodriguez *et al.* used three separate production methods including salting-out, emulsification-diffusion, and nanoprecipitation, as a comparative scale-up production evaluation to reach PVA-ibuprofen-loaded nanoparticles. The pilot-scale stirring rates of 790–2000 rpm led to mean sizes ranging from 174–557 nm for salting-out, and from 230–565 nm for emulsification-diffusion. Heterogeneously structured composites involving PVA have been of interest in the field of hydrogel nanoparticles [40]. Short poly(lactone) chains grafted to PVA or charge-modified sulfobutyl-PVA (SB-PVA) were prepared as biodegradable polyesters with potential to be used as a new class of comb-like polyesters with water solubility property. These polymers have the property of being self-assembled and produce nanoparticles that form stable complexes with a number of proteins such as human serum albumin, tetanous toxoid and cytochrom C [41]. Therefore, nanoparticles prepared using these copolymers did not use any solvents or surfactants [42–43].

Dmitri A. Ossipov and Jöns Hilborn presented a novel functionalized PVA with pendent acetylene and azide groups. Carbonyldiimidazole (CDI)-mediated couplings of the amines terminated with functional groups, 1-azido-2-aminoethane, propargylamine, or N-methylpropargylamine, to PVA is performed in this investigation. In order to maintain the solubility of PVA in water the same method should be performed in a low degree, between 1–5%. Azide-modified PVA and alkyne-modified PVA components were crosslinked by mixing their solutions together with Cu(I) catalyst, a type of Huisgen's 1,3-dipolar azide-alkyne cycloaddition, recently defined as a powerful "click" chemistry. In order to prepare a hydrogel nanoparticle, the multiple triazoles crosslink is formed by the reaction of two different polymers, thus resulting in a coupling which is chemoselective between alkynyl and azido functional groups. Synonymous with this study, another PVA-based hydrogel was prepared by crosslinking of alkyne-modified PVA with the telechelic bifunctional poly(ethylene glycol)-diazide crosslinker. By using the viscoelastic properties of hydrogels in their swollen state and their soluble fraction, their equilibrium swelling state in water were characterized. Based on these studies, the indicative point is higher gelation capacity of polyfunctional PVA crosslinkers than the bifunctional PEG crosslinker which influenced the network properties of these polymers. In this study, another chemical hydrogel preparation technique was presented which utilizes bifunctional low-molecular-weight crosslinkers [38, 43–44].

6.5 Hydrogel Nanoparticles Based on Poly(ethylene Oxide) and Poly(ethyleneimine)

A novel hydrogel nanoparticulate material is presented by the crosslinking of poly(ethylene oxide) (PEO) and poly(ethyleneimine) (PEI), PEO-cl-PEI which results in formation of nanocomposite material by the interaction of anionic/amphiphilic molecules or oligonucleotides with PEO-cl-PEI [45]. In nanocomposite structures the hydrophobic region of polyion complexes are connected with the hydrophilic chain of PEO. The collapse of dispersed gel particles occurs as a result of polyion complex formation. Due to the stabilizing effect of the PEO chain, the complexes form stable aqueous dispersion. The negatively charged biologically active compounds such as retinoic acid, indomethacin, and oligonucleotides (bound to polycation chains) or hydrophobic molecules (incorporated into nonpolar regions of polyion-surfactant complexes) have been immobilized in this system [46–47]. To intensify the receptor-mediated delivery, hydrogel nanoparticles can be used as a carrier for biologically active compounds that have been modified with polypeptide ligands. There are reports on the cellular uptake and intracellular release of oligonucleotides incorporated in PEO-cl-PEI hydrogel nanoparticles. The results have indicated the enhanced antisense activity of oligonucleotides in a cell model as a consequence of oligonucleotides-hydrogel nanoparticles association. Besides, this delivery system also shows the enhanced oral and brain bioavailability of oligonucleotides using polarized epithelial and brain microvessels endothelial cell monolayers, as a cell model. Hydrogel nanoparticles composed of PEO-cl-PEI are synthesized by crosslinking of branched PEI with bis-functional PEO molecules. Transparent hydrogels can form in only 3–5 min via a reaction between the amino groups of PEI and imidazolylcarbonyl ends of activated PEO in a homogenous aqueous solution. As expected, these bulk hydrogels are susceptible to preserve a large quantity of water, approximately 50-fold by weight, compared to the dried substance. When the molar ratio of PEO/PEI is minimal (about 6 or higher) a rigid hydrogel might be produced. In order to achieve a finely dispersed system, the modified solvent emulsification/evaporation method is used to carry out the crosslinking reaction. Based on this method, the emulsification of activated PEO solution in dichloromethane in the aqueous solution of PEI is done by using sonication. A clear suspension is obtained after removing the organic solvent by vacuum. The fractionation of the hydrogel nanoparticles prepared by this method is difficult because of their very low density. Therefore, separation of hydrogel nanoparticles from crude suspensions can be made by gel-permeation chromatography [48].

In another study, Se Yong An *et al.* have used liposome as a template to prepare size-controlled and monodispersed poly(ethylene glycol) (PEG) hydrogel nanoparticles. In this study, PEG nanoparticles are encapsulated into the cavity of the liposomes using the photopolymerizable PEG hydrogel solution which is polymerized by UV irradiation after extrusion of sample through a specific pore size membrane. The size distributions of the prepared particles

were $1.32 \pm 0.16 \mu\text{m}$ (12%), $450 \pm 62 \text{ nm}$ (14%), and $94 \pm 12 \text{ nm}$ (13%) after extrusion through membrane filters with pore sizes of $1 \mu\text{m}$, 400 nm , and 100 nm , respectively. This approach makes possible the surface modification of nanoparticles using only a one-step procedure. To functionalize the surface of a PEG nanoparticle, methoxy poly(ethylene glycol)-aldehyde is added as copolymer to the hydrogel-forming components and aldehyde-functionalized PEG nanoparticles could be obtained easily by UV-induced photopolymerization, followed by conjugation with poly-l-lysine-FITC through amine-aldehyde coupling. Using confocal microscopy has shown strong fluorescence from FITC on the edge of the prepared PEG particles. This delivery system allowed the loading of biomaterials such as β -galactosidases in hydrogel nanoparticles without any additional procedures during the hydration step of liposome preparation process [49].

6.6 Hydrogel Nanoparticles Based on Poly(vinyl Pyrrolidone)

Another hydrophilic, biocompatible and non-antigenic polymer which is FDA approved and is safe for biological experiments is Poly(vinyl pyrrolidone), PVP. Baharali *et al.* have reported the use of nanoreactors which are formed by the aqueous cores of reverse micellar droplets as a procedure for the preparation of hydrogel nanoparticles based on PVP with final size diameter of less than 100 nm [50]. Because of the monodispersed reverse micellar droplets and well-controllable droplet sizes, the nanoparticles prepared using reverse micellar medium are ideally monodispersed with narrow size distribution. Furthermore, the particle size can be controlled by the modulation in the reverse micellar droplet size. Guowie *et al.* used PVA as a crosslinker to form PVP-based hydrogel nanoparticles to, ultimately, synthesize a magnetic macromolecular delivery system. The PVP hydrogel magnetic nanospheres exhibited passive drug release that could be exploited to enhance therapeutic efficacy. Magnetic nanospheres based on PVP-hydrogels demonstrated a promising carrier for magnetically guided chemotherapeutic drug delivery [51–52].

6.7 Hydrogel Nanoparticles Based on Poly-N-Isopropylacrylamide

Poly-N-isopropylacrylamide (PNIPAM) is probably the most famous member of the responsive polymer class. PNIPAM exhibits a low critical solution temperature. This very sharp transition is attributed to the disruption of hydrogen bonding of water molecules around the amide group of the polymer side chains. G. Huang *et al.* have described a hydrogel nanoparticle network containing dextran. In their study, using covalent crosslinking, PNIPAM-co-allylamine nanoparticulate networks and PNIPAM-co-acrylic acid nanoparticulate networks are formed [53]. In this study, monodispersed nanoparticles

of poly-N-isopropylacrylamide-co-allylamine (PNIPAM-co-allylamine) and PNIPAM-co-acrylic acid (PNIPAM-co-AA) have been synthesized. The close-packed PNIPAM-co-allylamine and PNIPAM-co-AA nanoparticles were converted to three-dimensional gel networks by covalently crosslinking neighboring particles at room temperature and neutral pH using glutaric dialdehyde and adipic acid dihydrazide, respectively. Controlled release studies were conducted using dextran markers of various molecular weights as model macromolecular drugs. Release was quantified under various physical conditions, including a range of temperatures and dextran molecular weights. Dextran, entrapped in cavities in the nanoparticle network, was released with a rate regulated by their molecular weights and cavity size. No release from a conventional bulk PNIPAM gel, with high crosslinking density, was observed. The rate of release from the PNIPAM-co-allylamine network was temperature-dependant, being much faster at room temperature than the human body temperature. In contrast, release of low molecular weight dextrans from the PNIPAM-co-AA network showed a temperature-independent profile. These nanoparticle networks have several advantages over conventional bulk gels for controlling the release of high molecular weight biomolecules [53–54].

Moreover, Gan and Lyon, using seeding and feeding precipitation polymerization method, have synthesized thermoresponsive core-shell PNIPAM nanoparticles [54].

6.8 Smart Hydrogel Nanoparticles

Responsive hydrogel systems are one of the most focused upon areas in drug delivery research in regards to their potential applications in different drug targeting strategies. These systems undergo a physicochemical change in response to external stimuli, most importantly pH and temperature. In a typical study, pH- and temperature -sensitive hydrogel nanoparticles have been fabricated using a copolymer of vinyl pyrrolidone (VP) and acrylic acid (AA), crosslinked by N,N-methylene-bis-acrylamide (MBA), as described by Sahoo *et al.* [55]. In this study particle sizes up to 50 nm were obtained which were loaded with the marker compound FITC-dextran. In acidic pH the release of FITC-dextran was slow, while by increasing the pH of the release medium, the release of the loaded drug became faster. Moreover, the release rate increased as the temperature increased. In another study, magnetically-responsive hydrogel nanoparticles that were also responsive to the temperature have been presented. These systems have a great potential for being a micro- or nanoscale carrier for the delivery of pharmaceutically active compounds and are expected to be of great interest in various biomedical applications. In this context, nanocomposite hydrogel systems based on the temperature-sensitive N-isopropylacrylamide hydrogels crosslinked with ethylene glycol dimethacrylate, tetraethylene glycol dimethacrylate, and poly(ethylene glycol) 400 dimethacrylate (PEG400DMA) have been synthesized and characterized. The incorporation of iron oxide magnetic nanoparticles into the hydrogel system

using polymerization of nanoparticles and monomer solution mixture has been reported [56]. The swelling response of these composite systems to different crosslinking molecular weights and temperature, and the effect of the presence of the magnetic nanoparticles were examined. Another drug delivery carrier is based on pullulan hydrogel nanoparticles which are self-assembled hydrogel nanoparticles made of cholesterol-bearing pullulan [57]. Kazunari *et al.* have presented a nanoscale (20–30 nm) carrier system for the first time for evaluation of the insulin complexation and stability. They were convinced that upon the complexation process the spontaneous dissociation of insulin from the complex and thermal denaturation/aggregation, were effectively put down. Synonymous with this study, Gupta *et al.* reported pullulan-based hydrogel nanoparticles incorporated by nucleic acid molecules for delivery into the cells. In this study, the aqueous droplets of w/o microemulsion caught pullulan nanoparticles that already carried plasmids. The results from transmission electron microscopy (TEM) images of these particles demonstrated a nanoscale size (45 ± 0.80 nm) and spherical particles [1].

In another study, thermally-initiated free radical polymerization method was used to prepare poly(methacrylic acid-grafted-poly(ethylene glycol)) (P(MA-g-PEG)) hydrogel nanoparticles. The swelling behavior of these hydrogel nanoparticles was shown to be pH-sensitive and strongly affected by the amount of crosslinker used during the procedure [58].

6.9 Self-assembled Hydrogel Nanoparticles

Self-assembled nanoparticles can be described as nanoparticles prepared via the intrinsic affinity of some compounds to form thermodynamically stable nanoparticles in a definite condition. Kim *et al.* described self-assembled hydrogel nanoparticles composed of dextran and PEG macromers prepared from glycidyl methacrylate dextran (GMD) and dimethyl methacrylate poly(ethylene glycol) (DMP) via radical polymerization. The synthesis of GMD glycidyl methacrylate was coupled to dextran in presence of 4-(N,N-dimethylamino)pyridine (DMAP) using dimethylsulfoxide (DMSO) as an aprotic solvent. DMP was synthesized from poly(ethylene glycol) and methacryloyl chloride. In order to investigate the self-assembly of these hydrogel nanoparticles in water a fluorescence probe technique was used with pyrene as a hydrophobic probe (58). Furthermore, stable polymeric nanoparticles prepared via inverse emulsion polymerization using PEG and poloxamer 407 (Pluronic® F127) made possible the successful encapsulation of doxorubicin (loading efficiency = 8.7%) [58].

Self-assembled hydrogel nanoparticles made from carboxymethylated (CM)-curdlan were introduced by Kun Na *et al.* Sulfonylurea (SU) was used as a hydrophobic moiety for substitution of carboxymethylated (CM)-curdlan as a force to make it self-assembling. The degree of SU substitution was 2.4, 5.6, or 7.2 SU groups per hundred anhydroglucose units of curdlan. Dynamic light scattering, transmission electron microscopy, and

fluorescence spectroscopy were used for characterizing these self-assembled hydrogel nanoparticles (DS 2.4, DS 5.6, and DS 7.2) in aqueous media. The mean diameter of all samples was less than 300 nm with a unimodal size distribution. The loading and release of all-trans retinoic acid (ATRA) was studied in this self-assembly. As the degree of substitution increased the ATRA loading efficiencies in CM-curdlan/SU nanoparticles increased. Also, the ATRA release rate was controlled by the degree of substitution and drug loading [59].

Other approaches for preparation of self-assembled hydrogel nanoparticles are using polymeric amphiphiles. In one study Jae Hyung Park *et al.* presented self-assembled nanoparticles based on polymeric amphiphiles, capable of being accumulated in solid tumors by the enhanced permeability and retention effect, following intravenous administration. In this study, a hydrophobically modified glycol chitosan carrier was prepared by chemical conjugation of fluorescein isothiocyanate or doxorubicin to the backbone of glycol chitosan, thus forming a nano-sized self-assembled particle. Tissue samples obtained from tumor-bearing mice were used to investigate the biodistribution of self-assembled nanoparticles (300 nm in diameter). These particles were self-aggregated when administered systematically via the tail vein. As the blood circulation time increased, the distribution of self-aggregates to the tumor site was enhanced. The toxicity of self-aggregated nanoparticles loaded with doxorubicin was much lower than the free doxorubicin when administered into the tumor-bearing mice via the tail vein. The results of this study revealed that the self-aggregates on the basis of glycol chitosan has the potential to be a carrier for hydrophobic antitumor agents [60].

The local acidic pH in various diseases such as tumors, ischemia and inflammation has been used as a targeting strategy for pH-sensitive hydrogel nanoparticles composed of pullulan acetate/oligo-sulfadimethoxine (PA/OSDM) conjugates. PA/OSDM nanoparticles prepared by dialysis at pH 9.5 had a spherical shape with a size range of 50–140 nm and low critical aggregation concentration (CAC) ($< 12 \pm 3$ mg/ml), dependent on the degree of substitution of OSDM (DS). The CAC gradually decreased as the dialysis pH decreased, because of the increasing hydrophobic nature of OSDM [61].

6.10 Surface Functionalization

The controlled release of therapeutic agents to the specific site of action at the optimal rate and extent has been a major goal in designing the nanoparticulate systems as the efficient drug delivery devices. To obtain long circulating and specific site targeting nanoparticles, surface modifications as the proper strategies have been utilized in many studies.

Various pharmaceutical nanocarriers, such as nanospheres, nanocapsules, liposomes, micelles, cell ghosts and lipoproteins are widely used for experimental delivery of therapeutic and diagnostic agents [62–64]. These carriers can play different roles and functions according to their structures,

physicochemical characteristics, and the mode of their use by the researchers (e.g., route of administration to the host body). As a main strategy to control the function(s) made by a nanoparticle *in vivo*, the surface modification on these entities has been of interest in recent years. The mostly attractive benefits expected from such modifications are increasing the resident time (mainly, life span in circulation), changing the biodistribution pattern of the particle, achievement of a site-specific drug distribution toward the desired site(s) of action, induction of stimuli-sensitivity (e.g., pH or temperature) for the drug release from the nanoparticles, and the possibility of the particle tracing within the body during the biomedical reaches on these carriers.

Surface modifiers investigated more frequently include soluble synthetic polymers for achieving longevity by carriers; specific ligands such as antibodies, peptides, folate, transferrin and sugar moieties for targeting effects; pH- or temperature-sensitive lipids or polymers for giving stimuli-sensitivity property to the carriers; chelating compounds, such as ethylene diamine tetra acetic acid (EDTA), diethylene triamine penta acetic acid (DTPA) or deferoxamine for giving a contrast moiety onto a drug carrier. It is obvious that the selection of a combination of different surface modifiers to be used on the surface of a particulate carrier is based on the intended ultimate application of the drug delivery system in preparation. For example, the achievement of properties such as longevity and site-targeting ability, targeting and stimuli-sensitivity, or longevity, targeting, and contrast properties can be regarded as the possible goals of such studies [65]. A general scheme of the strategies used in the development of pharmaceutical nanocarriers is illustrated in Figure 6.1.

Surface modification of nanoparticles in order to increase their resident time in blood systemic circulation, has been attempted mainly by surface coating with hydrophilic polymers (e.g., polyethylene glycol (PEG) and polyethylene oxide (PEO)) or surfactants (e.g., poloxamer, poloxamine, polysorbates, and lauryl ethers); and development of biodegradable copolymers with hydrophilic segments. The surface shell of nanoparticles may be modified with different strategies such as conjugation (e.g., covalent binding) of specific ligands, coating with polymers, graft polymerization, and a series of other methods. Coating of nanoparticles with hydrophilic polymers such as polyethylene glycol, heparin, or dextran, is a helpful approach to protect particles from being uptaken by macrophages, hepatic kupffer cells, or other types of reticuloendothelial system (RES) cells [63]. RES is an important part of the natural defense mechanism of the human body mainly responsible for the uptake and clearance of the foreign particulate materials from the blood circulation. These modifications are based on changing the surface charge (zeta potential), surface hydrophilicity, particle *in vitro* or *in vivo* stability, mucoadhesive properties, and protein adsorption onto the particle surfaces [63, 66].

One of the hydrophilic polymers used extensively for this purpose is polyethylene glycol. The application of polymeric micelles formed via the multimolecular assembly of block copolymer as the novel core-shell typed colloidal carriers for drug and gene targeting was comprehensively described in many studies. Specially, new approaches for the formation of functionalized

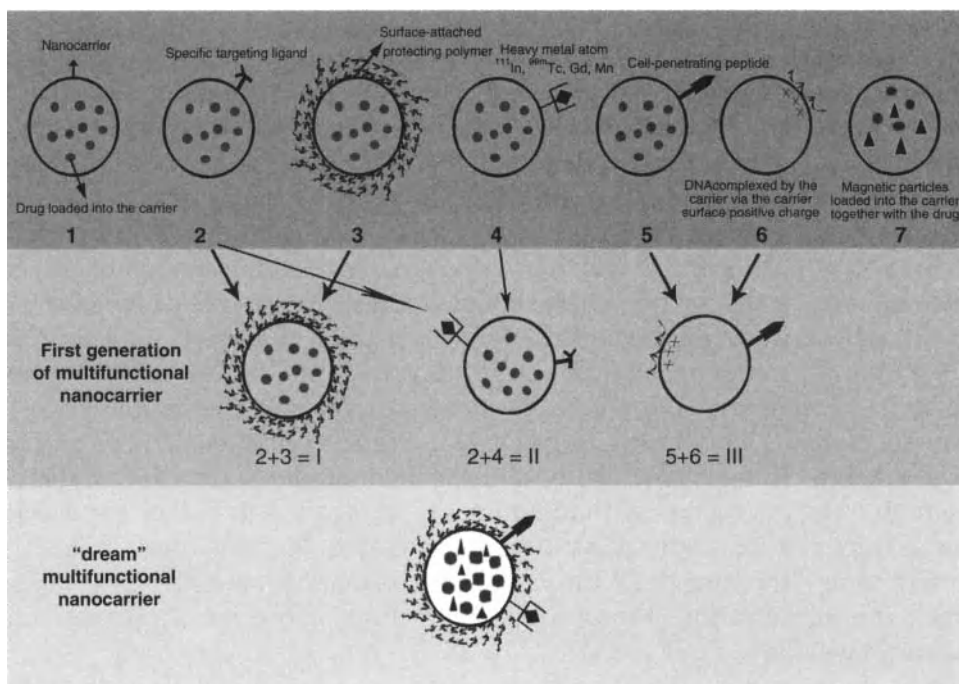


Figure 6.1 Typical representatives of monofunctional pharmaceutical nanocarriers: (1) classic nanocarrier; (2) targeted nanocarrier; (3) long-circulating nanocarrier; (4) contrast nanocarrier for imaging purposes; (5) cell-penetrating nanocarrier; (6) DNA-carrying nanocarrier; (7) magnetic nanocarrier. First-generation multifunctional pharmaceutical nanocarriers may include different combinations of individual functions – see examples I, II and III. Hypothetical “dream” multifunctional pharmaceutical nanocarrier combines the properties of all monofunctional carriers (65).

poly(ethylene glycol) (PEG) layers as hydrophilic outer shell were focused on to attain receptor-mediated drug and gene delivery through PEG-conjugated ligands with a minimal nonspecific interaction with other proteins. Surface organization of block copolymer micelles with crosslinking core was also described from a standpoint of the preparation of a new functional surface coating with a unique macromolecular architecture. The micelle-attached surface and the thin hydrogel layer made by layered micelles exhibited nonfouling properties and worked as the reservoir for hydrophobic reagents. In addition, the potential of multimolecular assembly derived from hetero-bifunctional PEGs and block copolymers were explored to systematically modify the properties of metal and semiconductor nanostructures (e.g., gold nanoparticles and quantum dots) by controlling their structure and their surface properties, making them extremely attractive for use in biological and biomedical applications [7, 67]. Carbohydrates, particularly polysaccharides, are another group of polymers used for the same purpose. Because of the mucoadhesive properties of polysaccharides they can play an important role in tissue dressing and transport mechanisms. Moreover, some of these polymers, such as chitosan,

have a mucoadhesive property [66]. As a typical example for emphasizing the latter advantages of coating as a surface modification strategy, poly(isobutyl cyanoacrylate) nanoparticles coated by chitosan or other thiolated chitosan derivatives have shown a good mucoadhesive property compared to the non-coated particles of the same type [68]. Different coating materials may induce different characteristics on a certain particulate system. For example, in 1995 Florence *et al.* reported that poloxamer coated on the surface of polystyrene particles can block particle uptake by the small intestinal regions of the gut, while tomato lectins coated on the surface of the same particles increased their uptake by the same epithelial cells [66]. Generally, polymers with bioadhesive property present nonspecific interactions with mucosal layers throughout the body. In contrast, lectins/thiol groups show specific interactions with the mucosal layer which, in turn, improves the particle adherence to the mucosal layer. Lectins can bind specifically to the glycoproteins of the mucus, thereby promoting the interaction with the mucosal surface. On the other hand, addition of thiol groups to the polymers, which results in compounds frequently referred to as "thiomers," accompanies the formation of disulphide linkage which can significantly improve the interaction of the main polymer with mucosal layers [69].

One of the major strategies to produce multifunctional pharmaceutical nanocarriers is the chemical conjugation of proteins, peptides, polymers and other molecules to the surface of the carriers, a process that may result in controlled properties of the nanostructure. This procedure can be carried out covalently or noncovalently, i.e., via the hydrophobic adsorption of certain intrinsic or specially inserted hydrophobic groups in the ligands. By this modification, hydrophobically-modified proteins or amphiphilic polymers can be attracted by the hydrophobic surface of nanoparticles [70] or can be loaded into the bilayer of liposomes [71] or hydrophobic core of micelles [65, 72–73].

Another strategy for surface modification is graft polymerization. Graft copolymers are obtained by copolymerizing macromonomers and other monomers. In other words, a new polymeric structure is obtained by grafting a side chain to a polymeric backbone. Due to the amphiphilic characteristics, graft copolymers, containing hydrophobic and hydrophilic components, exhibit surfactant behavior and can form micelles with a core-shell structure. The hydrophobic inner core can act as a drug-incorporation site, in which the hydrophobic drugs are entrapped through hydrophobic interactions. In such a structure, particular properties can be obtained suitable for different applications, such as: improved cellular adhesion and spreading, enhanced surface wettability, improved material biocompatibility, and formation of nonfouling coatings intended for surface functionalization and molecular immobilization. Graft polymerization, by making a protective coating, can also be used to specify surface areas [74]. In fact, hydrophobic polymeric nanoparticle surface modification can be achieved by physical attachment of a protecting polymer on the surface of the particles or by making chemical graft of a polymeric chain onto the particles. As an example for this surface modification, the adsorption of a series of polyethylene oxide and polypropylene oxide copolymers on the

surface of polystyrene latex particles via hydrophobic interaction mechanism has been reported [75]. These polymer-coated nanoparticles can protect the incorporated drug from RES uptake after intravenous injection. This approach not only decreases the uptake of particles by RES, but also the modification with some specific copolymers can direct the injected nanoparticles to other organs [76]. For example, poloxamer 407 used for coating of 60-nm polystyrene latex can result in increased particle accumulation in bone marrow [65].

Biodegradable polymers are a group of polymers used extensively for the fabrication of biomedically-interested nanoparticles, having the properties such as sustained drug action on the site, reduced systemic side effects, and controlled and targeted delivery of drugs. One of the mostly interested FDA-approved biodegradable polymers is poly(lactide-co-glycolide) (PLGA) which is used as a vehicle for hydrophobic as well as hydrophilic drugs. This carrier is also studied extensively for tumor targeting and imaging. Surface functionalization of this carrier with a hydrophilic layer of polyethylene glycol can stabilize the particles and minimize their RES uptake, thus the blood circulation time can be prolonged. On the other hand, this surface layer can be functionalized with different biological moieties for tumor-specific targeting. Chitosan and heparin have been used to functionalize the surface of the PLGA nanoparticle. This modification can improve the cellular uptake and consequently it can improve the accumulation of the particles in tumor tissue. Surface functionalization of PLGA nanoparticles with chitosan and heparin has been proven as an influential factor for *in vivo* tumor targeting [77]. The other groups of carriers attempted for surface modification are dendrimers and their derivatives. The surface of these nanostructures provide a high capacity for coupling with different high affinity molecules [78]. A simple one-step synthesis to convert each symmetrical end group of G4-PAMAM dendrimers into two reactive, distinct orthogonal and chemoselective groups is described by Navath and co-workers [79]. They have developed the amino acid-functionalized dendrimers with heterobifunctional chemoselective peripheral groups for drug delivery applications. The heterobifunctional groups at the dendrimer periphery could be chemoselectively conjugated to multiple moieties such as drugs (indomethacin and dexamethasone) and drugs and imaging agents (dexamethasone and FITC). The choice of amino acids as versatile linkers would enable biocompatible dendrimer scaffolds for use in drug delivery [79].

6.11 Surface Functionalized Hydrogel Nanoparticles

The hydrogel nanoparticles' surface acts as such an important bridge between the nanogel bulk and the outer environment, and many specific surface properties, such as wettability, paintability, adhesion susceptibility, biocompatibility and so on, must be incarnated through this layer [9–10]. These properties often demand special molecular structures which cannot just be satisfied only by a single homopolymer material. Accordingly, the surface modification has been developed as an effective way to obtain the desirable surface for the nanogels

to broaden their application. Conventional polymer surface functionalization techniques are always dependent on the chemical or physical methodologies including corona, chemical reaction, surface grafting, and surface coating or etching of finished nanoparticles, and so on. However, the complicated processes and the specialized equipment are always required for using these techniques. Especially, for the highly water-absorbed nanogels, integrate and uniform functional surface cannot be easily achieved. In addition, the sophisticated post-treatment methods have also largely faced the application of these methods with difficulties. Hence, much attention has been focused on the development of new simple, well defined, and reliable methods for the polymer surface functionalization in this kind of nanostructure [11, 80].

With the aim of chitosan functionalization, Sajeesh *et al.* have developed novel thiol-functionalized hydrogel microparticles for oral drug delivery applications [69]. In this study, poly(methacrylic acid)–chitosan–poly(ethylene glycol) (PCP) microparticles were prepared using modified ionic gelation process in aqueous medium. Surface modification of carboxylic acid group of PCP microparticles was carried out by thiol using Ellman's method. In this method, the carboxylic groups of PCP microparticles were coupled to L-cysteine with a water soluble carbodiimide. Dynamic light scattering was used to measure the average particle size. The average size of the PCP microparticles was increased after thiol modification. Thiolated microparticles significantly improved the paracellular permeability of FD4 across Caco 2 cell monolayers, with no sign of toxicity. However, the efficacy of thiolated system remained low when permeation experiments were carried out across excised intestinal membrane. This occurred because of the high adhesion of the thiolated particles on the gut mucosa. Finally, this investigation demonstrated that surface thiol functionalization can be an interesting strategy to improve paracellular permeability of hydrophilic macromolecules [81]. As a model for hydrophilic macromolecules, insulin was investigated using this system in order to prepare an oral delivery system. Cystein was grafted to the activated surface carboxyl groups of PCP hydrogels (Cys-PCP) to make the thiol-functionalization microparticles. Compared to the unmodified PCP microparticles, the thiolated microparticles resulted in less swelling and lower insulin encapsulation efficiency. However, thiolation was an effective strategy to improve insulin absorption across Caco-2 cell monolayers. These studies revealed that thiolation can be regarded as an strategy to improve oral delivery of proteins/peptides loaded in hydrogel microparticles [69].

Development of thiolated chitosan nanoparticles for the nasal administration of leuprolide is also described by Shahnaz *et al.* [82]. Leuprolide-loaded thiolated chitosan-thioglycolic acid (chitosan-TGA) and leuprolide-loaded unmodified chitosan nanoparticles were developed via ionic gelation with triphosphosphate (TPP). Sustained release of leuprolide from thiolated nanoparticles was demonstrated over 6 h, which might be attributed to inter- and/or intramolecular disulfide formation within the nanoparticles network. Ciliary beat frequency (CBF) study demonstrated that thiolated nanoparticles can be considered as suitable additives for nasal drug delivery systems. Compared to leuprolide solution, unmodified nanoparticles and thiolated nanoparticles

provoked increased leuprolide transport through porcine nasal mucosa by 2.0 and 5.2 folds, respectively. The results of a pharmacokinetic study in male Sprague-Dawley rats showed improved transport of leuprolide from thiolated nanoparticles as compared to leuprolide solution. Thiolated nanoparticles had a 6.9-fold increase in area under the curve, more than 4-fold increase in elimination half-life, and a ~3.8-fold increase in maximum plasma concentration compared to nasal solution alone. The relative nasal bioavailability (versus s.c. injection) of leuprolide thiolated nanoparticles calculated on the basis of AUC ((0-6)) was about 19.6% as compared to leuprolide solution 2.8%. The enhanced bioavailability of leuprolide is likely due to facilitated transport by thiolated nanoparticles rather than improved release.

Luo and co-workers have presented N-Succinyl-chitosan nanoparticles (NSCNP) induced mitochondria-dependent apoptosis in K562 [83]. By western blot they revealed that apoptosis induced by NSCNP was associated with accumulation of cytochrome C in cytosol and elevating expression of apoptosis-promoted protein Bax while depressing expression of apoptosis-restrained protein Bcl-2 in time- and dose-dependence. Cell cycle blocking in G2/M phase was evidenced by kinetic cell cycle analysis. The activations of caspase-9 and 3, not caspase-8 were observed by fluorescent and colorimetric assays. Their data suggested that the apoptotic signals triggered by NSCNP were mediated mainly through the intrinsic mitochondria-dependent pathway.

Moreover, the adsorption characteristics of hydroquinol from aqueous solutions onto the β -cyclodextrin modified magnetic chitosan nanoparticles (CMCN) have been investigated by Fan *et al.* [84]. The appropriate process conditions for the adsorption of hydroquinol have been obtained. Equilibrium experiments fitted well with the Freundlich isotherm model, and the maximum adsorption capacity of the CMCN at 303K was determined to be 1.75 mmol/g for hydroquinol at the concentration of 9.0 mmol/L, much higher than some conventional adsorbents. The CMCN was stable and easily recovered. Moreover, the adsorption capacity was about 90% of the initial saturation adsorption capacity after being used four times.

With the aim of alginate functionalization, Meng *et al.* have developed the hollow nanospheres based on the self-assembly of alginate-graft-poly(ethylene glycol) and α -cyclodextrin [85]. In these Alg-g-PEG/ α -CD complexes, the α -CDs are stacked along the PEG side chains to form a rod block, and alginate main chains act as a coil block. More rod-like blocks in Alg-g-PEG/ α -CD favor the formation of small assemblies. The assemblies of Alg-g-PEG/ α -CD show a dependence on concentration, temperature, pH, and salt concentration. At low concentration (below 0.125%) or high temperature (above 32°C), Alg-g-PEG/ α -CD particles were unstable and disrupted. Increasing the salt or decreasing the pH resulted in the aggregation of Alg-g-mPEG/ α -CD particles, as detected by the increase in the recorded hydrodynamic diameter.

In addition, multifunctional alginate microspheres for biosensing, drug delivery and magnetic resonance imaging have been described by Joshi *et al.* [86]. This research aimed to develop and investigate a multifunctional implantable system capable of biosensing, drug delivery and magnetic resonance imaging

(MRI) for continuous monitoring, controlled anti-inflammatory drug delivery and imaging, respectively. A glucose biosensor, diclofenac sodium (Diclo) and magnetic nanoparticles (MNP) were used as the biosensor component, anti-inflammatory agent and MRI contrast agent, respectively. MNP were synthesized by the co-precipitation technique and loaded with the sensor and drug components into alginate microspheres using a commercial droplet generator. The particles showed an excellent biocompatibility (> 80%) for all combinations of formulations. The system shows a great potential for biosensing with concurrent drug delivery and visualization for biomedical applications.

Surface modified electrospun poly(vinyl alcohol) membranes for extracting nanoparticles from water, is an example of poly(vinyl alcohol) functionalization described by Mahanta and Valiyaveetil [87]. Contamination of water from nanomaterials will be an emerging problem in the future due to incorporation of nanomaterials in many commercial products and improper disposal of waste materials. In this report, electrospun polyvinyl alcohol nanofibers (PVA NFs) with diameters ranging between 300 and 500 nm were used for the extraction of nanosized contaminants from the aqueous environment. To obtain the best extraction efficiency, surface hydroxyl groups of PVA NFs were chemically modified with functional groups, such as thiols and amines. Two model nanoparticles (silver and gold) dissolved in water were used for adsorption studies. Depending on the nature of the surface functionalities, the fibers showed unique ability to adsorb nanoparticles. The extraction studies revealed that the amine and thiol modified PVA NFs showed 90% extraction efficiency for both silver and gold nanoparticles. The thiol and amine functionalized PVA NFs showed maximum adsorption capacities towards Au NPs, which were around 79–84 mg g⁽⁻¹⁾. Similarly for Ag NP extraction, amine functionalized PVA NFs showed a value for at 56 mg g⁽⁻¹⁾. Their results highlight that functionalized nanofibers have high extraction efficiency for dissolved nanoparticles in water and can be used for removal of the nanocontaminants from the aqueous environment [87–88].

Chitosan-g-poly(N-isopropylacrylamide)-based nanogels for tumor extracellular targeting have been developed by Duan *et al.* [89]. The principle objective of this research was to develop and characterize pH-responsive and biocompatible nanogels as a tumor-targeting drug delivery system. The nanogels were self-assembled from chitosan-based copolymers, chitosan-graft-poly(N-isopropylacrylamide) (CS-g-PNIPAm). Based on MTT assay and cellular morphological analysis, the antitumor activity of these oridonin (ORI)-loaded nanogels was higher at pH 6.5 than at pH 7.4. In conclusion, the obtained nanogels appeared to be of great promise in tumor extracellular pH targeting for ORI.

Peptide-functionalized nanogels for targeted siRNA delivery have been presented by Blachburn *et al.* [90]. They described the synthesis of core/shell hydrogel nanoparticles (nanogels) with surface-localized peptides that specifically target ovarian carcinoma cell lines possessing high expression levels of the Eph2A receptor. These nanogels are also demonstrated to be highly effective in the noncovalent encapsulation of siRNA and enable cell-specific delivery of

the oligonucleotides in serum-containing medium. Cell toxicity and viability assays reveal that the nanogel construct is nontoxic under the conditions studied, as no toxicity or decrease in cell proliferation is observed following delivery. Importantly, a preliminary investigation of gene silencing illustrates that nanogel-mediated delivery of siRNA targeted to the EGF receptor results in knockdown of that receptor. Excellent protection of siRNA during endosomal uptake and endosomal escape of the nanogels is suggested by these results since siRNA activity in the cytosol is required for gene silencing.

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PART II

DIAGNOSTIC DEVICES

Utility and Potential Application of Nanomaterials in Medicine

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Abstract

The field of nanomedicine involves the application of nanotechnology to medicine. In a broader sense, nanomedicine is the process of diagnosing, treating, curing, preventing diseases and also dealing with traumatic injury, relieving pain, and preserving/improving human health by using molecular tools and molecular knowledge of the human body. However, one of the current problems facing nanomedicine is the need to resolve issues pertaining to the toxicity and environmental impact of nanoscale materials. Nanobiomaterial is a multidisciplinary scientific domain with roots in life science, material science and nanotechnology. Basic and applied nanobiomaterials have been a very hot topic in nanobiomedicine and nanobiotechnology research materials, quickly leading to an interest in biomedical implant and intervention medicine, tissue engineering and regenerative medicine, and drug/gene delivery and bioanalysis system, in an attempt to explore their possible applications in clinical practice. We will focus on the application of nanomaterials in nanomedicine. A comprehensive range of nanomaterials/nanobiomaterials will be considered including tissue regeneration (for orthopedics, cartilage, vascular, dental, nervous system, cardio vascular, etc.), drug delivery, ophthalmological applications, cancer treatment, dermatological applications, etc.

Keywords: Nanomaterials, nanomedicine, diagnostics, therapeutic, drug delivery, nanotechnology, nanoparticles, medical imaging, gene therapy, nanofiber, toxicity

7.1 Introduction

Nanomedicine is “ensuring the comprehensive monitoring, control, construction, repair, defence and improvement of all human biological systems, working from the molecular level using engineered devices and nanostructures,

ultimately to achieve medical benefit.” Nanomedicine is the process of diagnosing, treating, preventing disease and traumatic injury, relieving pain, and preserving and improving human health, using molecular tools and techniques for the benefit of humankind. Literally, nanomedicine is the application of nanotechnology to medicine. Nanomedicine has a highly interdisciplinary scientific crew, including biologists, physicians, mathematicians, engineers and computer scientists [1–4].

Living organisms are made up of cells with a size of 10 μm . The cell organelles are the much smaller submicron size, followed by the smallest which are macromolecules which include proteins, receptors, antibodies, enzymes, nucleic acid, etc. (2–5 nm). These are comparable with the dimensions of the smallest manmade nanoparticles. Nanoparticles are used as very small probes to study the cellular machinery of biological processes on the nanoscale level. The applications of nanomaterials to biology or medicine have potential for use in fluorescent biological labels, drug and gene delivery, biodetection of pathogens, detection of proteins, probing of DNA structure, tissue engineering, tumor destruction via heating (hyperthermia), separation and purification of biological molecules/cells, MRI contrast enhancement, and phagokinetic studies. The nanoparticles exist in the same size as proteins which makes nanomaterials utilized for biotagging or labelling. In order for nanoparticles to interact with a biological target, there needs to be a biological or molecular coating or layer acting as a bioinorganic interface. Biological coatings may include antibodies, biopolymers like collagen, or monolayers of small molecules which make the nanoparticles biocompatible [5–11].

Nanomedicine uses nanotechnology for the treatment, diagnosis, monitoring, and control of biological systems by the delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents involving precise targets (phagocytes, dendritic, endothelial, cancers cells and receptors) with specific clinical conditions and choice of the desired nanocarriers in order to achieve responses with no side effects. Nanomedicine is a wide area that uses a variety of nanobiomaterials, including DNA scaffolds, polymeric nanoconstructs as biomaterials, (e.g., molecular self-assembly and nanofibers of peptides and peptide-amphiphiles for tissue engineering, shape-memory polymers as molecular switches, nanoporous membranes), and nanoscale microfabrication-based devices (e.g., silicon microchips for drug release and micromachined hollow needles and two-dimensional needle arrays from single crystal silicon), sensors and laboratory diagnostics. The delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents via intravenous and interstitial routes of administration with nanosized particles in nanomedicine are very immense, including the advantage of the nanometer-scale size range, biological behavior, and safety profile. Nanoparticles have a much larger surface area per unit mass as compared with larger particles. The increase in the surface-to-volume ratio results in the increase of the particle surface energy (due to quantum effect) causing more reactive behavior which includes antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers for drug delivery, but also toxicity, induction of oxidative stress or of cellular dysfunction, or a

mix of both. Nanoparticles of silver and gold, display antimicrobial properties, which help to prevent infection and wound healing. Nanocrystal silver burn cream, nanocrystal shoes and athletic equipment are becoming popular. Sunscreens containing nanoparticles of zinc oxide or titanium dioxide is currently in use. Colloidal gold/Ag/Cu, zinc oxide, iron oxide crystals, and semiconductor quantum dots (QDs) or nanocrystals in the size range of 1–20 nm, have diagnostic applications in biology and medicine. Gold nanoparticles have application as quenchers in fluorescence resonance energy transfer measurement studies. For example, the distance-dependent optical property of gold nanoparticles has provided opportunities for evaluation of the binding of DNA-conjugated gold nanoparticles to a complementary RNA sequence. Iron oxide nanocrystals with superparamagnetic properties are used as contrast agents in magnetic resonance imaging (MRI), as they cause changes in the spin-spin relaxation times of neighboring water molecules, to monitor gene expression or detect pathologies such as cancer, brain inflammation, arthritis, or atherosclerotic plaques. QDs can label biological systems for detection by optical or electrical means *in vitro* and to some extent *in vivo*. The fluorescence emission wavelength (from the UV to the near-IR) of QDs can be tuned by altering the particle size, thus these nanosystems have the potential to revolutionize cell, receptor, antigen, and enzyme imaging. Their large surface-area-to-volume ratio offers potential for designing multifunctional nanosystems.

Nanoparticle forms the core of nanobiomaterial, nanovesicle surrounded by a membrane or a layer. The shape is more often spherical but cylindrical, plate-like and other shapes are possible. The size and size distribution might be important in some cases, for example, if penetration through a pore structure of a cellular membrane is required. The size and size distribution are becoming extremely critical when quantum-sized effects are used to control material properties. A tight control of the average particle size and a narrow distribution of sizes allow the creation of very efficient fluorescent probes that emit narrow light in a very wide range of wavelengths. This helps with creating biomarkers with many well distinguished colors. The core itself might have several layers and be multifunctional. For example, by combining magnetic and luminescent layers one can both detect and manipulate the particles. The core particle is often protected by several monolayers of inert material, for example silica. Organic molecules that are adsorbed or chemisorbed on the surface of the particle are also used for this purpose. The same layer might act as a biocompatible material. However, more often an additional layer of linker molecules is required to proceed with further functionalization. This linear linker molecule has reactive groups at both ends. One group is aimed at attaching the linker to the nanoparticle surface and the other is used to bind various moieties like biocompatibles (dextran), antibodies, fluorophores etc., depending on the function required by the application [12–20].

In tissue engineering, when the surface of an artificial bone implant is smooth, the body would reject it, because that smooth surface causes production of a fibrous tissue covering the surface of the implant and reduces the bone implant contact by loosening the implant and causing inflammation. However, when creating nano-sized features on the surface of the hip or knee prosthesis,

one can reduce the chances of rejection and also stimulate the production of osteoblasts. Titanium is widely used in orthopedics and dentistry as a bone repairing material because it has high fracture resistance and ductility but lack of bioactivity, cell adhesion and growth. Thus, apatite coatings on titanium with stable porous structure are required for a good bone repairing material with all the desired features by biomimetic approach. The layer of apatite possess a stable nanoporous structure and bioactivity due to biomimetic approach reported by Ma *et al.* [21]. A natural bone is a nanocomposite material, composed of hydroxyapatite crystallites in the organic matrix known as collagen. That is why bone is mechanically tough and plastic, to check mechanical damage. Isla *et al.* have prepared an artificial hybrid material with ceramic nanoparticles and poly(methyl methacrylate) copolymer (15–18 nm) using the tribology approach. Hybrid material was used as a coating on the tooth surface, to enhance scratch resistance and have the healing behavior similar to natural tooth [22]. To avoid this side effect, the hydrophobic version of the dye molecule was enclosed inside a porous nanoparticle [28]. The dye stayed trapped inside the Ormosil nanoparticle and did not spread to the other parts of the body. At the same time, its oxygen generating ability was not affected and the pore size of about 1 nm freely allowed for the oxygen to diffuse out.

Nanomedicines offer advantages over older medicines, including an effective targeting of specific sites, enhanced solubility, bioavailability, and minimized adverse effects. Nanocarriers used in drug delivery, nanosuspensions for enhanced drug solubility, and nanoparticles for bioimaging are the main sources of nanomedicines. However, the inherent properties of nanoparticles such as their size, shape, agglomeration/aggregation nature, and surface chemistry adversely affect the safety and quality of nanomedicines. As we know, there are no consistent regulatory guidelines established so far because of our inadequate knowledge about the systematic behavior of nanoparticles; its standardized nomenclature, test methods, well-defined characterization of products, lack of skilled personnel, safety protocols, and ineffective control of good manufacturing practices of nanomedicines. Thus, the urgent need for regulatory framework for controlling the manufacturing processes, product quality, and safety of nanomedicines is very great. Colloidal silver is widely used in antimicrobial formulations and dressings. The high reactivity of titania nanoparticles, either on their own or when illuminated with UV light, is also used for bactericidal purposes in filters. Enhanced catalytic properties of surfaces of nanoceramics, or those of noble metals like platinum, are used to destroy dangerous toxins and other hazardous organic material.

7.2 Nanoparticle Coatings

Song *et al.* have developed a new class of plasmonic vesicular nanostructures assembled from amphiphilic gold nanocrystals with mixed polymer brush coatings. The multifunctional vesicles containing stimuli-responsive polymers could enable their broader applications in biosensing, multimodality imaging,

and theragnostic nanomedicine [24]. Metal-based glyconanoparticles (GNPs) are biofunctional nanomaterials with good water solubility, biocompatibility and stability as targeting properties, which opens up the possibility to employ metallic GNPs in diagnostics and/or therapy. Addressing a major concern regarding nanotoxicity, it is expected that the sugar shell of GNPs will lower the intrinsic toxicity of metal nanoclusters better than other non-natural coatings [25]. The increasing use of zinc oxide nanoparticles (ZnONPs) in coatings, paints, and personal care products were reportedly enhanced by exposure to the body. Accurate and quantitative profiling on the tissue distribution and body clearance of ZnONPs, is needed to clarify the acute and chronic safety concerns of ZnONPs. Chen *et al.* have reported on the radioactive zinc oxide nanoparticles (R-ZnONPs) for *in vivo* biodistribution studies using mice as the animal mode [26]. Nanomedicine offers solutions for improving stent coating using nanomaterials with tailored and controllable properties that utilize human platelets response towards carbon-based nanocoatings via atomic force microscope (AFM), which facilitate the comprehensive analysis of platelets behavior onto stent nanocoatings and enable the study of thrombogenicity. Altering the surface topography of nanocoatings, the activation of platelets affected due to high surface roughness of carbon nanocoatings were found to be less thrombogenic in terms of platelets adhesion [27]. Magnetic nanoparticles were used for biological and biomedical applications such as protein separation, targeted drug delivery, hyperthermia treatment, contrast agents of magnetic resonance imaging, biosensing, and for magnetic fluids environmental remediation. However, different polymeric coatings are applied to provide solubility and stability in aqueous solution and additional functional groups for attachment [28]. Ventilator-associated pneumonia (VAP) is a serious and costly clinical problem today. VAP is especially difficult to diagnose in the pediatric population and causes high morbidity, mortality, and medical costs. The use of nanomodified coatings, with selenium and iron oxide have checked the bacterial adhesion inside ventilator surface [29].

Nanoparticles interact with proteins, membranes, cells, DNA and organelles and establish a series of nanoparticle/biological interfaces that depend on colloidal forces as well as dynamic biophysicochemical interactions. These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have biocompatible or bioadverse outcomes. The biomolecules may induce phase transformations, free energy releases, and restructuring and dissolution at the nanomaterial surface. Thus, the relationships between structure and activity are determined by nanomaterial properties such as size, shape, surface chemistry, roughness and surface coatings. This knowledge is important for the safe use of nanomaterials [30]. Nanosilver particles are smaller than 100 nm. At nanoscale level, silver exhibits remarkably unusual physical, chemical and biological properties. Due to its strong antibacterial activity, nanosilver is used in textiles, implants, contraceptives, water disinfectants, room sprays, and for the treatment of wounds and burns. Thus, use of nanosilver is becoming more and more widespread in medicine and related applications. Due to increasing exposure to

nanosilver, toxicological and environmental issues need to be addressed such as biodistribution, organ accumulation, and degradation, etc. [31]. Modalities to be employed towards medical diagnostics and therapeutics include: (i) real-time cellular interrogation technology, which enables real-time investigation, or examination of cellular signal transduction and gene expression as a response to environmental conditions; (ii) diagnostic devices, which include the fabrication of micro/nano sensors for the targeting of molecules indicative of cellular disorders; and (iii) therapeutics, or the engineering of membrane mimetics, or biomolecule-functionalized thin films as strategies for synthetic biological regenerative medicine, as well as therapeutically-active polymers as biocompatible coatings for implants. These approaches collectively represent a universally-applicable and comprehensive diagnostic-treatment strategy whereby cardiovascular disorders, or medical diseases in general, can be diagnosed, monitored, and fundamentally understood on unprecedented levels. In turn, these conditions can be therapeutically addressed using drug-releasing nanostructured polymers with minimal intrusion upon normal cellular behavior, or by biologically-active hybrid membrane devices based upon the interface of proteins with robust biomimetic polymers [32]. Magnetic nanoparticles with functionalized surface coatings are used clinically for various biomedical applications, such as magnetic resonance imaging, hyperthermia, drug delivery, tissue repair, cell and tissue targeting and transfection because of their nontoxic and biocompatible nature (cobalt, nickel, iron oxide-based materials), binding affinity to drugs, and their external magnetic field for attaching the targeting molecules, such as proteins or antibodies, at particle surfaces. A variety of polymers/biomolecules are currently used for nanoparticle coating to stabilize the suspensions of magnetic nanoparticles under *in vitro* and *in vivo* situations. These magnetic nanoparticles are utilized in the early detection of life style diseases like cancer, diabetes and atherosclerosis [33].

7.3 Cyclic Peptides

The ability to selectively deliver compounds into atherosclerotic plaques would aid in the detection and treatment of atherosclerotic disease using a 9-amino acid cyclic peptide, LyP-1. LyP-1 was identified as a tumor-homing peptide that specifically recognizes tumor cells, tumor lymphatics, and tumor-associated macrophages. LyP-1 was capable of delivering intravenously injected nanoparticles to plaques and the accumulation of LyP-1-coated superparamagnetic iron oxide nanoparticles was observed in the plaque interior. It is a promising agent for the targeting of atherosclerotic lesions [34]. Yang *et al.* have developed multifunctional and water-soluble superparamagnetic iron oxide (SPIO) nanocarriers for targeted drug delivery and positron emission tomography/magnetic resonance imaging (PET/MRI) dual-modality imaging of tumors with integrin $\alpha(v)\beta_3$ expression. An anticancer drug was conjugated onto the PEGylated SPIO nanocarriers via pH-sensitive bonds. Tumor-targeting ligands, cyclo(Arg-Gly-Asp-d-Phe-Cys) (c(RGDfC)) peptides, and PET ^{64}Cu chelators,

macrocyclic 1,4,7-triazacyclononane-N, N', N''-triacetic acid (NOTA), were conjugated onto the distal ends of the PEG arms. The effectiveness of the SPIO nanocarriers as an MRI contrast agent was evaluated via an *in vitro* r_2 MRI relaxivity measurement. cRGD-conjugated SPIO nanocarriers exhibited a higher level of cellular uptake than cRGD-free ones *in vitro*. Moreover, cRGD-conjugated SPIO nanocarriers showed a much higher level of tumor accumulation than cRGD-free ones according to noninvasive and quantitative PET imaging and *ex vivo* biodistribution studies. SPIO nanocarriers have demonstrated promising properties for combined targeted anticancer drug delivery and PET/MRI dual-modality imaging of tumors [35]. Ye *et al.* [36] has reported on a disulfide-based cyclic RGD peptide called iRGD, that is, c(CRGDKGPDC), that interacts with both integrin and neuropilin-1 receptors for cellular and deep tissue penetration to improve imaging sensitivity and therapeutic efficacy. The targeted delivery of nanoparticles to solid tumors is one of the most important and challenging problems in onconanomedicine. Huang *et al.* have reported quantitative tumor uptake studies for a class of elongated gold nanocrystals (called nanorods) that are covalently conjugated to tumor-targeting peptides. The results suggested that for photothermal cancer therapy, the preferred route of gold nanorod administration is intratumoral injection instead of intravenous injection [37]. Solid lipid nanoparticles (SLNs) have been used for carrying different therapeutic agents because they improve absorption and bioavailability [38]. Pathological retinal angiogenesis (neovascularization) is one of the most feared complications among retinal diseases, leading to visual impairment and irreversible blindness. Recent findings made by us on therapeutic applications of biologically synthesized silver nanoparticles (AgNPs) against VEGF-induced retinal endothelial cells, elucidates the effectual inhibitory activities of AgNPs over the downstream signaling pathways (Src and AKT/PI3K) leading to retinal angiogenesis [39]. Urisu *et al.* have developed two basic technologies for fabrication of supported planar lipid bilayer membrane ion channel biosensors: a defect-free lipid bilayer formation on the substrate surface with electrode pores and a patterning technique for the hydrophobic self-assembled monolayer to form the guard ring that reduces the lipid bilayer edge-leak current. The importance of the supported-membrane structure to achieve low noise and high-speed performance is suggested on the basis of the observed relation between the single-ion-channel current noise and the pore size [40].

7.4 Dendrimers

Dendrimers are highly branched macromolecules with controlled near monodisperse three-dimensional structure. Polymer growth starts from a central core molecule and growth occurs in an outward direction by a series of polymerization reactions. Hence, precise control over size can be achieved by the extent of polymerization, starting from a few nanometers. Cavities in the core structure and folding of the branches create cages and channels. The surface groups of dendrimers are amenable to modification and can be tailored for specific

applications. Therapeutic and diagnostic agents are usually attached to surface groups on dendrimers by chemical modification [41]. Drug delivery is an interdisciplinary field of research using pharmaceutical researchers, medical doctors and industry. A safe and targeted drug delivery could improve the performance of some classic medicines already on the market, and moreover, will have implications for the development and success of new therapeutic strategies such as anticancer drug delivery, peptide and protein delivery and gene therapy. Nanoparticles (NPs) have been developed as an important strategy to deliver conventional drugs, recombinant proteins, vaccines and more recently, nucleotides. NPs modify the kinetics, body distribution and drug release of nanoparticulate drugs using polymeric NPs, ceramic NPs, magnetic NPs, polymeric micelles and dendrimers as well as their applications in therapeutics, diagnostics and imaging. The bow-and-arrow approach is very important, where the drugs are the arrows and the delivery vehicles are the bows. Polymers, dendrimers, micelles, vesicles, and nanoparticles have been used as delivery vehicles. Mechanically interlocked molecules (MIMs) can be designed in such a way that they either change shape or shed some of their parts in response to a specific stimulus, such as changes in redox potential, alterations in pH, irradiation with light, or the application of an oscillating magnetic field, allowing a theranostic payload to be released from the nanopores to a precise location at the appropriate time. *Chlamydia trachomatis* is a bacterial pathogen responsible for genital infections, as well as inflammatory arthritis. Chlamydiae is treated with antibiotics such as azithromycin (AZ). Neutral generation-4 polyamidoamine (PAMAM) dendrimers are used as intracellular drug-delivery vehicles into chlamydial inclusions, conjugated with Azithromycin [42]. Ortega *et al.* have synthesized dendrimers with terminal amine groups by hydrosilylation of allyldimethylamine and suggested the antibacterial properties of the ammonium-terminated hyperbranched polycarbosilanes and dendrimers act as potent biocides against Gram-positive and Gram-negative bacterial strains. Polyethylene glycol (PEG) surface modification can make nanomaterials highly hydrophilic, reducing their sequestration in the reticuloendothelial system; using polyamidoamine (PAMAM) dendrimers bearing gadolinium (Gd) chelates, shows paramagnetic and pharmacokinetic properties. Various nanocarriers (liposomes, micelles, polymer-conjugates, polymerosomes, dendrimers, aptamers and carbon nanotubes) were reported [43]. Nanoconstructs such as liposomes are widely used in clinics, while polymer micelles are in advanced phases of clinical trials in several countries. Currently, the field of nanomedicine is generating a new wave of nanoscale drug delivery strategies, embracing trends that involve the functionalization of these constructs with moieties that enhance site-specific delivery and tailored release using established nanoparticle technologies such as liposomes, polymer micelles, and dendrimers regarding tumor targeting and controlled release strategies. These strategies are being incorporated into their design with the hope of generating a more robust and efficacious nanotherapeutic modality [44]. A variety of organic and inorganic nanomaterials are used for cancer therapeutic and diagnostic applications due to their unique characteristics of passive tumor targeting. A wide range of

nanomedicine platforms such as polymeric micelles, liposomes, dendrimers, and polymeric nanoparticles have been established for targeted delivery of anticancer agents, because they can accumulate in the solid tumor site via leaky tumor vascular structures, thereby selectively delivering therapeutic payloads into the desired tumor tissue. In addition, nanoscale delivery vehicles for small interfering RNA (siRNA) have also been developed as effective therapeutic approaches to treat cancer [45]. Anticancer agents can be either encapsulated in or conjugated to dendrimer, and can be delivered to the tumor via enhanced permeability and retention (EPR) effect of the nanoparticle and/or with the help of a targeting moiety such as antibody, peptides, vitamins, and hormones. Imaging agents including MRI contrast agents, radionuclide probes, computed tomography contrast agents, and fluorescent dyes are combined with the multifunctional nanomedicine for targeted therapy with simultaneous cancer diagnosis with the long-term viability and biocompatibility of the nanotherapeutics. The biocompatibility aspects of dendrimers such as nanotoxicity, long-term circulation, and degradation are a major cause of concern [46]. The development of nanoparticles-based drugs seems to be effective providing low side effects and targeted action on cancer cells. The nano drugs are targeted selectively and specifically towards tumors resulting in better treatment. The important molecules used for preparation nano drugs are cisplatin, carboplatin, bleomycin, 5-fluorouracil, doxorubicin, dactinomycin, 6-mercaptopurine, paclitaxel, topotecan, vinblastin, etoposide, etc. The most commonly used materials for preparing nanoparticles carriers are dendrimers, polymer, liposome, micelles, inorganic, organic nanoparticles, etc. However, the commonly used nano drugs to date are liposome dendrimer and some other materials-based nanoparticles [47]. Kesharwani *et al.* have developed the cancer targeting potential of folate, dextran and galactose functionalized polypropyleneimine (PPI) dendrimers and evaluated for *ex vivo* cytotoxicity on HeLa and SiHa cell lines using flow cytometry and MTT assay. The outcome of the MTT assay and flow cytometry suggested the order of targeting potential of various ligands as folate > dextran > galactose [48]. Zaman *et al.* have developed a synthetic peptide subunit vaccine based on polyacrylate dendritic polymer. Polyacrylate dendrimers offer a unique approach to a nasally administered vaccine for rheumatic fever and rheumatic heart disease, using the delivery of the J14 peptide, a C-terminal derivative of M-protein in group A Streptococcus [49]. The ability of Superparamagnetic Iron Oxide (SPIO) nanoparticles and Poly(propyleneimine) generation 5 dendrimers (PPI G5) to cooperatively provoke siRNA complexation was investigated in order to develop a targeted, multifunctional siRNA delivery system for cancer therapy. Poly(ethylene glycol) (PEG) coating and cancer specific targeting moiety (LHRH peptide) have been incorporated into SPIO-PPI G5-siRNA complexes to enhance serum stability and selective internalization by cancer cells. Such a modification of siRNA nanoparticles enhanced its internalization into cancer cells and increased the efficiency of targeted gene suppression *in vitro*. Moreover, the developed siRNA delivery system was capable of sufficiently enhancing *in vivo* antitumor activity of an anticancer drug (Cisplatin). The proposed approach demonstrates potential for the

creation of targeted multifunctional nanomedicine platforms with the ability to deliver therapeutic siRNA specifically to cancer cells in order to prevent severe adverse side effects on healthy tissues and *in situ* monitoring of the therapeutic outcome using clinically relevant imaging techniques [50]. Due to the challenging nature of treatment and prevention of HIV disease, the use of nanocarriers, such as nanoparticles (polymeric, inorganic, and solid lipid), liposomes, polymeric micelles, dendrimers, cyclodextrins, and cell-based nanoformulations have been established for delivery of antiretroviral drugs for HIV prevention or therapy. The application of nanocarrier systems to the delivery of anti-HIV drugs are effective at specific sites in the body with the least drug concentrations for the required period of time, providing a means to overcome cellular and anatomical barriers to drug delivery for combating this pandemic disease [51]. Enhanced optical breakdown of KB tumor cells labeled folate-targeted with silver-dendrimer composite nanodevices have been developed using topical laser irradiation. A human epidermoid cancer cell line was targeted with folated silver-dendrimer composite nanodevices and the labeled cancer cells were subsequently destroyed by the microbubbles generated due to the enhanced energy uptake of the silver nanoparticles from the laser irradiation, as compared to unlabeled cells [53]. Breast cancer is one of the world's most devastating diseases. Various nanocarriers have been introduced to improve the therapeutic efficacy of anticancer drugs, including liposomes, polymeric micelles, quantum dots, nanoparticles, and dendrimers. Targeted drug delivery systems for antitumor drugs have great potential to lower cytotoxicity and increase therapeutic effects [53]. The advent of nanoparticles, including nanostructured lipid particles and solid lipid nanoparticles, dendrimers, and quantum dots, have already many cosmetic applications as well as great potential in dermatologic applications [54]. The immune system alters due to nano-drug allergy reactions. Some of the most versatile nanostructured dendrimers-based drugs pose problems related to the modulation of the immune response. Nanoparticles offer unique advantages with which to exploit new properties and for materials to play a major role in new diagnostic techniques and therapies.

Fullerene-C60 and multivalent functionalized gold nanoparticles of various sizes have led to new tools and opened up new ways to study and interact with the immune system for diagnostic tests of allergic reactions [55]. A specific ion-pair approach can be involved in precise control of the size of gold nanodots. The dendrimers with terminal amine and hydroxyl groups were a hydrophilic and hydrophobic microcavity-template, respectively, for trapping gold salts. This facile strategy can significantly enhance the quantum yield of gold nanodots from 20% to 62% after microwave irradiation [56]. Being inspired by physiologically existing nanomachines, nanoparticles are designed to safely reach their target and specifically release their cargo at the site of the disease, and in this way increase the drug's tissue bioavailability. Nanoparticles have the advantage of targeting cancer by simply being accumulated and entrapped in tumors. Inorganic nanoparticles such as gold and iron oxide are usually coupled to the drug, PEG and the targeting ligand. It

appears that the PEG coating and ligand decoration are common constituents in most types of nanoparticles for cancer [57]. DNA is a versatile structural nanoscale material known for its functionality to help bridge the gap between materials science and biological systems to translate the specific therapeutic and diagnostic strategies within nanomedicine [58]. The physicochemical and biological interactions of nanocarriers within the biological environments are observed by atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), magnetic resonance imaging (MRI), superconducting quantum interference devices, combined techniques such as AFM-CLSM, correlative light and electron microscopy (CLEM), and SEM spectroscopy. The description of these techniques help to elucidate certain physicochemical and biological activities of nanocarriers such as dendrimers, polymers, liposomes, and polymeric/inorganic nanoparticles, thus providing a toolbox for bioengineers, pharmaceutical scientists, biologists, and research clinicians [59]. Among the various polymeric architectures, dendritic polymers have experienced an exponential development due to their highly branched, multifunctional, and well-defined structures. Dendritic polyglycerols (PGs) exhibit good chemical stability and inertness under biological conditions and are highly biocompatible. Oligoglycerols and their fatty acid esters are being used in a variety of consumer applications, e.g., cosmetics and toiletries, food industries, cleaning and softening agents, pharmaceuticals, polymers and polymer additives, printing photographing materials, and electronics [60].

Nanomaterials for biopharmaceutical applications are emerging, using liposomes, niosomes, micelles, nanoemulsions, dendrimers, nanocells, cell ghosts, lipoproteins to solid nanoparticles, and quantum dots. The pharmaceutical nanoparticulate carriers have been established as devices for enhancing the therapeutic index of clinically approved and experimental drugs, and for their utilization for imaging and monitoring biological functions. The ability to track and image the fate of any nanomedicine starting from the systemic down to the subcellular level becomes essential [61]. The use of dendrimers as nano-sized excipients/vectors in biological and pharmaceutical systems is dependent on the investigation of their toxicological profiles in biological media [62]. Application of nanomaterials in various diagnostic and therapeutic areas shows great promise. To translate nanotechnology into nanomedicine, it is important to understand any potential mechanisms of toxicity caused by nanomaterials, and to design approaches to mitigate any detrimental effects [63]. Carbon nanotubes hold great promise for their use as a platform in nanomedicine, especially in drug delivery, medical imaging, and cancer targeting and therapeutics. Shi *et al.* have presented a facile approach to modifying carbon nanotubes with multifunctional poly(amidoamine) (PAMAM) dendrimers for cancer cell targeting and imaging. In this approach, fluorescein isothiocyanate (FI)- and folic acid (FA)-modified amine-terminated generation 5 (G5) PAMAM dendrimers (G5-NH₂-FI-FA) were covalently linked to acid-treated multiwalled carbon nanotubes (MWCNTs), followed by acetylation of the remaining primary amine groups of the dendrimers. The MWCNT/G5.NHAc-FI-FA composites

are water-dispersible, stable, and biocompatible and thereby provide many possibilities for various applications in biomedical sensing, diagnosis, and therapeutics [64]. The field of nanomedicine-based contrast agents offers a great opportunity to develop highly sophisticated devices that can overcome many traditional hurdles of contrast agents including solubility, cell-specific targeting, toxicities, and immunological responses. These nanomedicine-based contrast agents including liposomes, micelles, dendrimers, multifunctional magnetic polymeric nanohybrids, fullerenes, and nanotubes, represent improvements over their traditional counterparts, which can significantly advance the field of molecular imaging [65]. Nanomedicine has the potential to increase the specific treatment of cancer cells while leaving healthy cells intact through the use of novel nanoparticles to seek and treat cancer in the human body. Various nanocarriers such as nanoshells, nanocrystals, nanopolymers, quantum dots, and dendrimers, have a role in early cancer detection and treatment [66]. Advances in nanomedicine can be made by engineering biodegradable nanoparticles such as magnetic iron oxide nanoparticles, polymers, dendrimers and liposomes that are capable of targeted delivery of both imaging agents and anticancer drugs. Increasing evidence suggests that the nanoparticles, whose surface contains a targeting molecule that binds to receptors highly expressed in tumor cells, can serve as cancer image contrast agents to increase sensitivity and specificity in tumor detection. In comparison with other small molecule contrast agents, the advantage of using nanoparticles is their large surface area and the possibility of surface modifications for further conjugation or encapsulation of large amounts of therapeutic agents. Targeted nanoparticles ferry large doses of therapeutic agents into malignant cells while sparing the normal healthy cells. Such multifunctional nanodevices hold the promise of significant improvement of current clinical management of cancer patients [67]. Targeted drug delivery of various drugs for the treatment of cancer, AIDS and brain disorders is the primary research area in which nanomedicines have a major role. However, the significant toxicity issues associated with these nanomedicines to be explored [68]. Dendritic molecules are able to interact with nucleic acids and deliver them into cells. Binding and delivery of genetic material into cells *in vivo* holds out the prospect of gene therapy [69]. The advances in dendrimer-based contrast agents for molecular imaging focusing mainly on the chemical design as applied to optical, magnetic resonance, computer tomography, radionuclide, and multi-modality imaging, have been reported by Longmire *et al.* [70]. Ligand exchanged gold quantum dots (GQDs) conjugated with cell-penetrating peptides are a new class of photoluminescent probes for nuclear targeting and intracellular imaging [71]. The potential applications of biochips, nanosensors, bioreactors, neural stem cells, immune nanoparticles, biodegradable polymers, and convection-enhanced drug delivery in the diagnostics and treatment of diseases is a hot topic of the future of nanomedicine. Numerous novel medicinal forms are polymeric nanoparticles, nanotubes, micelles, liposomes, dendrimers, fullerenes, and hydrogels [72].

Most ocular diseases are treated by topical drug application in the form of solutions, suspensions and ointment. These conventional dosage forms suffer

from the problems of poor ocular bioavailability, because of various anatomical and pathophysiological barriers prevalent in the eye. Applications of various nanoparticulate systems like microemulsions, nanosuspensions, nanoparticles, liposomes, niosomes, dendrimers and cyclodextrins in the field of ocular drug delivery can be utilized to explore the frontiers of ocular drug delivery and therapy [73]. Generation 4 PAMAM-NH₂ (G4NH₂) dendrimer labeled with tritium to measure the rate of uptake and permeability in Caco-2 cells was established by Kichens *et al.* using endocytosis inhibitors brefeldin A, colchicine, filipin, and sucrose and suggested that G4NH₂ internalization and transport involves an endocytosis pathway [74]. Dendrimers with precise control of size, shape and terminal group functionality has been established for a wide range of pharmaceutical applications [75]. There is growing interest in developing tissue-specific multifunctional drug delivery systems with the ability to diagnose or treat several diseases. Nanosized Au metal-dendrimer composite nanodevices (CNDs) consist of poly(amidoamine) dendrimers (in various sizes, surface substituents, and net charges) and inorganic nanoparticles, properties of both of which can be individually modified and optimized in mouse tumor models [76]. The mesoporous silica nanomaterials for controlled release drug, gene and neurotransmitter delivery applications have been established. The high surface area, tunable pore diameter (2–20 nm) and uniform mesoporous structure (hexagonal channels or cubic pores) of the mesoporous silicas offer a unique advantage for loading and releasing large quantities of biomedical agents. The strategy of using various removable capping moieties, such as photo- or redox-responsive organic groups, inorganic nanoparticles, dendrimers and polymers, to encapsulate guest biomolecules inside the porous matrices further enables the utilization of these surface-functionalized mesoporous silica nanomaterials for stimuli-responsive controlled release *in vitro* and *in vivo* [77]. Nanocarriers, such as nanoparticles, liposomes and dendrimers, are used to enhance ocular drug delivery. In combination with a controlled drug delivery, it should be possible to develop ocular formulations that provide therapeutic concentrations for a long period of time at the site of action, thereby reducing the dose administered as well as the instillation frequency [78].

7.5 Fullerenes/Carbon Nanotubes/Graphene

Soluble derivatives of fullerenes such as C₆₀, a soccer-ball-shaped arrangement of 60 carbon atoms per molecule, show great promise as pharmaceutical agents, with good biocompatibility and low toxicity even at relatively high dosages. They may be used as antiviral agents, antibacterial agents, photodynamic antitumor and anticancer therapies, antioxidants and antiapoptosis agents as treatments for amyotrophic lateral sclerosis and Parkinson's disease. Carbon nanotubes belong to the family of fullerenes and consist of graphite sheets rolled up into a tubular form. CNTs structures have obtained either as single- (a single graphene sheet) or multi-walled (several concentric graphene

sheets) nanotubes. The diameter and the length of single-walled nanotubes may vary between 0.5–3.0 nm and 20–1000 nm, respectively. Carbon nanotubes have been made water soluble by surface functionalization. Carbon nanotubes (CNTs) are a man-made form of carbon, they exist in many different forms and can be chemically modified and/or functionalized with biomolecules. Pristine single-walled CNTs (SWNT) are extremely hydrophobic tubes of hexagonal carbon (graphene) with diameters as small as 0.4 nm and lengths up to micrometers. Multiwalled CNTs (MWCNT) consist of several concentric grapheme tubes and diameters of up to 100 nm. Due to their unique chemical, physical, optical, and magnetic properties, carbon nanotubes have found many uses in industrial products and in the field of nanotechnology, including in nanomedicine. From the rapidly emerging applications of CNTs arises the urgent need for toxicological studies on CNTs. Exposure to CNTs is associated with effects on DNA, pulmonary toxicity, fibrotic lesions and skin toxicity. Molecular and ionic migration are possible through carbon nanotubes and offer opportunities for fabrication of molecular sensors and electronic nucleic acid sequencing. Carbon nanotubes may cross the cell membrane as nanoneedles without perturbing or disrupting the membrane and localize into cytosol and mitochondria, but the mechanisms are not yet understood clearly. A number of carbon nanotubes derivatives, such as trismalonic acid derivative of the fullerene C_{60} , express superoxide dismutase mimetic properties and are protective in cell culture and animal models of injury, including degeneration of dopaminergic neurons in Parkinson's disease and nervous system ischemia. Much is not known with respect to the toxicity of functionalized carbon nanotubes in terms of pharmacokinetic and toxicological studies, but it is presumed that the benefits of carbon nanotubes in drug, antigen, and gene delivery remain hyped [79–82].

Graphene has its interesting electrical, optical, mechanical and chemical properties, with potential applications in a wide range of areas, including biomedicine e.g., drug delivery, cancer therapies and biosensing [83]. Carbon nanotubes (CNTs) consist of carbon atoms arranged in sheets of graphene rolled up into cylindrical shapes with extraordinary properties, such as high electrical and thermal conductivity. In addition, CNT functionalization chemistry has led to an enhanced dispersibility in aqueous physiological media which indeed broadens their potential biological applications including gene delivery using cationic polymer-grafted multiwalled carbon nanotubes (MWNTs) (polymer-g-MWNTs) to efficiently complex and transfer plasmid DNA (pCMV- β Gal) *in vitro* without promoting cytotoxicity [84]. In biological media, SWNTs have unique near-infrared intrinsic fluorescence, inherent Raman spectroscopy and photoacoustic signal, which makes them ideal for noninvasive and high sensitivity detection. Functionalized SWNTs with significantly reduced toxicity have been employed as carriers to deliver various anticancer drugs, proteins and nucleic acids specifically to the diseased tissues, and maximize the bioavailability of the drugs by improving solubility and increasing circulation time [85]. The processability of giant (macro)molecules into ultrapure and highly ordered structures at surfaces is of fundamental

importance for studying chemical, physical and biological phenomena, as well as for their exploitation as active units in the fabrication of hybrid devices. It provides a new general route to process at surfaces extraordinarily large molecules, that is, synthetic nanographenes, into ultrapure crystalline architectures. Therefore it can be of interest for technological applications in the fields of electronics, (bio)catalysis and nanomedicine [86].

7.6 Functional Drug Carriers

There are numerous engineered constructs, assemblies, architectures, and particulate systems, whose unifying feature is the nanometer scale size range (from a few to 250 nm). These include polymeric micelles, dendrimers, polymeric and ceramic nanoparticles, protein cage architectures, viral-derived capsid nanoparticles, polyplexes, and liposomes. Therapeutic and diagnostic agents can be encapsulated, covalently attached, or adsorbed onto such nanocarriers. The reduced particle size entails high surface area and hence a strategy for faster drug release. Some of the carriers can be engineered in such a way that they can be activated by changes in the environmental pH, chemical stimuli, by the application of a rapidly oscillating magnetic field, or by application of an external heat source. Multifunctional NPs and micellar encapsulation of QDs have significant applications in biological environments. Lin *et al.* have evaluated ferritin nanocages as candidate nanoplatforms for multifunctional loading. Ferritin nanocages can be either genetically or chemically modified to impart functionalities to their surfaces, and metal cations can be encapsulated in their interiors by association with metal binding sites. Moreover, different types of ferritin nanocages can be disassembled under acidic condition and reassembled at pH of 7.4, providing a facile way to achieve function hybridization. Ferritin particles are a powerful nanoplatform in the era of nanomedicine [87]. Efficient and site-specific delivery of therapeutic drugs is a critical challenge in clinical treatment of cancer. Enhancement can potentially be achieved by conjugation of targeting ligands onto nanocarriers to achieve selective delivery to the tumor cell or the tumor vasculature. A variety of ligands have been investigated including folate, transferrin, antibodies, peptides and aptamers. Multiple functionalities can be incorporated into the design of nanoparticles, e.g., to enable imaging and triggered intracellular drug release [88]. The grafting of drugs to the single-walled carbon nanotube (SWCNT) was attained by the initial conversion of carboxylic groups in SWCNT to corresponding acyl chlorides. The active acyl chlorides in SWCNT were subsequently mixed with chemotherapeutic agents having NH , NH_2 , and OH functional groups to afford the formation of relevant amide and ester, respectively. The covalently grafted drugs to SWCNT were identified by infrared and UV-visible spectroscopy and transmission electron microscopy methods. From a clinical aspect, the grafting of drugs to the SWCNT can be used as a new tool and useful method for potential drug delivery in patients [89]. Mesoporous silicon particles show great promise for use in drug delivery and imaging applications as

carriers for second-stage nanoparticles and higher order particles or therapeutics. Modulation of particle geometry, surface chemistry, and porosity allows silicon particles to be optimized for specific applications such as vascular targeting and avoidance of biological barriers commonly found between the site of drug injection and the final destination. Multifunctional nanocarriers is a concept in which different particles are able to perform specific tasks in order to deliver single- or multi-component payloads to specific sub-cellular compartments [90]. Mesoporous silica nanoparticles (MSNs) are very important for drug delivery development. Relatively straightforward inside-out tuning of the vehicles, high flexibility, and potential for sophisticated release mechanisms make these nanostructures promising candidates for targeted drug delivery such as “smart” cancer therapies [91].

Block ionomer complexes (BIC) of poly(ethylene oxide)-b-poly(methacrylic acid) (PEO-b-PMA) and divalent metal cations (Ca^{2+}) were utilized as templates. Disulfide bonds were introduced into the ionic cores by using cystamine as a biodegradable crosslinker. The resulting crosslinked micelles with disulfide bonds represented soft, hydrogel-like nanospheres and demonstrated a time-dependent degradation in the conditions, mimicking the intracellular reducing environment. Doxorubicin (DOX)-loaded degradable crosslinked micelles exhibited more potent cytotoxicity against human A2780 ovarian carcinoma cells as compared to micellar formulations without disulfide linkages. The novel biodegradable crosslinked micelles are expected to be attractive candidates for delivery of anticancer drugs [92].

Multifunctional superparamagnetic nanoparticles have been developed for a wide range of applications in nanomedicine, such as serving as tumor-targeted drug carriers and molecular imaging agents. Sun *et al.* have reported a PEG-mediated synthesis process to produce well-dispersed, ultrafine, and highly stable iron oxide nanoparticles for *in vivo* applications. Utilizing a biocompatible PEG coating bearing amine functional groups, the produced nanoparticles serve as an effective platform with the ability to incorporate a variety of targeting, therapeutic, or imaging ligands, and demonstrated tumor-specific accumulation of these nanoparticles through both magnetic resonance and optical imaging after conjugation with chlorotoxin, a peptide with high affinity toward tumors of the neuroectodermal origin, and Cy5.5, a near-infrared fluorescent dye [93]. Cheng *et al.* have described a technique to create nanoparticulate cellular patches that remain attached to the membrane of cells. NeutrAvidin-coated nanoparticles were anchored on cells possessing biotinylated plasma membrane. Human bone marrow derived mesenchymal stem cells with nanoparticulate patches retained their inherent tumorigenic properties using a tumor model in a 3D extracellular matrix. Additionally, human umbilical vein endothelial cells with nanoparticulate patches were able to retain their functional properties and form multicellular structures as rapidly as unmodified endothelial cells. These results provide a novel strategy to actively deliver nanostructures and therapeutics to tumors utilizing stem cells as carriers and also suggest that nanoparticulate cellular patches may have applications in tissue regeneration [94].

Stem cells have been shown to selectively target injured brain and spinal cord tissue and improve functional recovery. Development of biologically compatible nanofiber scaffolds that mimic the structure of the extracellular matrix can serve as a permissive bridge for axonal regeneration or as a drug-delivery system. The incorporation of biologically active epitopes and/or the utilization of these scaffolds as stem cell carriers may further enhance their therapeutic efficacy [95]. Kim *et al.* have prepared a series of G3 poly-amidoamine (PAMAM) dendrimers derivatized with Alexa Fluor 488, varying numbers of PEG(550)/PEG(750)/PEG(2000), and nucleoside moieties derived from the A(2A) adenosine receptor (AR) agonist CGS21680(2-[4-(2-carboxylethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine) demonstrated the feasibility of using short PEG chains in the design of carriers that target ligand-receptor interactions [96].

Surface-functionalized mesoporous silica nanoparticles (MSNP) can be used as an efficient and safe carrier for bioactive molecules. Xia *et al.* have demonstrated that the enhanced cellular uptake of the nontoxic cationic MSNP enhances the delivery of the hydrophobic anticancer drug, paclitaxel, to pancreatic cancer cells. This novel use of a cationic MSNP extends its therapeutic use potential [97]. Well-ordered SrTiO₃ nanotube arrays capable of Sr release at a slow rate and for a long time were successfully fabricated on titanium by simple hydrothermal treatment of anodized titania nanotubes by Xin *et al.* Ti-based implant with SrTiO(3) nanotube arrays is an ideal candidate for osteoporotic bone implants. The proposed method can also be extended to load other biologically useful elements such as Mg and Zn [98]. Layered double hydroxides (LDHs) have now been known for their application in nanomedicine. LDH nanoparticles have been used in gene and drug delivery and proposed for the internalization of LDH nanoparticles into cells along with the intracellular fate of the particles and their cargo [99]. Cirstoiu-Hapca *et al.* have developed Paclitaxel (Tx)-loaded anti-HER2 immunonanoparticles (NPs-Tx-HER) by the covalent coupling of humanized monoclonal anti-HER2 antibodies (trastuzumab, Herceptin) to Tx-loaded poly (dl-lactic acid) nanoparticles (NPs-Tx) for the active targeting of tumor cells that overexpress HER2 receptors [100]. The synthesis of protein dendrimers using a strong but noncovalent interaction between a peptide and complementary protein is proposed as an efficient strategy to arrive at dendrimers fully functionalized with protein domains. The noncovalent synthetic strategy provides access to well-defined, dynamic, semisynthetic protein assemblies in high yield and is therefore of interest to the field of nanomedicine as well as biomaterials [101].

Multifunctional nanomedicine is emerging as a highly integrated platform that allows for molecular diagnosis, targeted drug delivery, and simultaneous monitoring and treatment of cancer. Several nanoscopic therapeutic and diagnostic systems based on nanocomposite materials are reported and also demonstrated the synergy of the multifunctional nanomedicine design [102]. Liu and Liu have developed novel carboxyl group-decorated crosslinked polystyrene nanoparticles via the soapless emulsion polymerization of styrene and divinyl benzene with oleic acid as functional comonomer. The functional

nanoparticles are proposed as carriers for biomolecules or drugs [103]. Zhang *et al.* have reported the engineering of a novel lipid-polymer hybrid nanoparticle (NP) as a robust drug delivery platform, with high drug encapsulation yield, tunable and sustained drug release profile, excellent serum stability, and potential for differential targeting of cells or tissues. The NP comprises three distinct functional components: (i) a hydrophobic polymeric core where poorly water-soluble drugs can be encapsulated; (ii) a hydrophilic polymeric shell with antibiofouling properties to enhance NP stability and systemic circulation half-life; and (iii) a lipid monolayer at the interface of the core and the shell that acts as a molecular fence to promote drug retention inside the polymeric core, thereby enhancing drug encapsulation efficiency, increasing drug loading yield, and controlling drug release [104]. Polymersomes (mesoscopic polymer vesicles) have large hydrophilic reservoir, and its thick hydrophobic lamellar membrane provides significant storage capacity for both water soluble and insoluble substances (such as drugs and imaging probes). In addition, the brush-like architecture of the polymersome outer shell can potentially increase biocompatibility and blood circulation times. The ability to conjugate biologically active ligands to the brush surface has provided targeted therapy and imaging [105].

Pluronic block copolymers cause various functional alterations in cells. The key attribute for the biological activity of Pluronics is their ability to incorporate into membranes followed by their subsequent translocation into the cells thereby affecting various cellular functions, such as mitochondrial respiration, ATP synthesis, activity of drug efflux transporters, apoptotic signal transduction, and gene expression. As a result, Pluronics cause drastic sensitization of MDR tumors to various anticancer agents, enhance drug transport across the blood brain and intestinal barriers, and cause transcriptional activation of gene expression both *in vitro* and *in vivo*, resulting in a remarkable impact on the emergent field of nanomedicine [106].

Carbon nanotubes (CNTs) have been used as multipurpose innovative carriers for drug delivery and diagnostic applications. CNTs can be functionalized with different functional groups to simultaneously carry several moieties for targeting, imaging, and therapy. CNT constructs carrying a fluorescein probe together with the antifungal drug amphotericin B or fluorescein and the anti-tumor agent methotrexate have been reported. Ammonium-functionalized CNTs can also be considered very promising vectors for gene-encoding nucleic acids. Stable complexes between cationic CNTs and plasmid DNA have also demonstrated the enhancement of the gene therapeutic capacity in comparison to DNA alone. Therefore, CNTs are playing a bigger and more important role in the emerging field of nanomedicine [107].

The nanoscale dendritic RGD clusters have great potential for tissue engineering and drug delivery applications. The arginine-glycine-aspartate (RGD) peptides at the nanoscale significantly affects cell responses. Dendrimers have a unique, highly branched, nearly spherical and symmetrical structure with low polydispersity, nanoscale size, and high functionality. Therefore, dendrimers are a class of ideal scaffold for construction of nanoscale dendritic

RGD clusters to trigger more favorable cellular responses [108]. Micellar nanoparticles made up of surfactants and polymers have gained wide attention in the materials and biomedical community for controlled drug delivery, molecular imaging, and sensing. They are a new class of robust, ultrafine silica core-shell nanoparticles formed from silica crosslinked with individual block copolymer micelles with improved stability that do not break down during dilution. Because of their unique structures and properties, these novel core-shell nanoparticles could potentially provide a new nanomedicine platform for imaging, detection, and treatment, as well as novel colloidal particles and building blocks for multifunctional materials [109].

7.7 MRI Scanning Nanoparticles

Yang *et al.* have developed multifunctional water-soluble superparamagnetic iron oxide (SPIO) nanocarriers for targeted drug delivery using positron emission tomography/magnetic resonance imaging (PET/MRI) dual-modality imaging of tumors with integrin $\alpha(v)\beta_3$ expression. It was suggested that they have promising properties for combined targeted anticancer drug delivery and PET/MRI dual-modality imaging of tumors [110]. Dendrimers and dendrimer-based therapeutics are beneficial for use as anticancer agents, either encapsulated in or conjugated to dendrimer, and delivered to the tumor via enhanced permeability and retention (EPR) effect of the nanoparticle and/or with the help of a targeting moiety such as antibody, peptides, vitamins, and hormones. Dendrimer-based therapeutics have long-term viability and biocompatibility for use as nanotherapeutics for cancer diagnosis and therapy with nanotoxicity, long-term circulation, and degradation [111].

Atherosclerosis is an inflammatory disease with high morbidity and mortality rates in the world. Lobatoo *et al.* developed enhanced anti-inflammatory action and reduced adverse effects of glucocorticoids (PLP), a nanomedicinal liposomal formulation, and they were intravenously applied at a dose of 15 mg/kg PLP to a rabbit model of atherosclerosis [112]. Zhang *et al.* have reviewed *in vivo* imaging modalities for detecting lymphatic vessels, lymphatic drainage, and lymphatic nodes. These include conventional lymphatic imaging techniques such as dyes and radionuclide scintigraphy as well as novel techniques for lymphatic imaging such as optical imaging, computed tomography, magnetic resonance imaging, ultrasound, positron emission tomography using lymphatic biomarkers, photoacoustic imaging, and combinations of multiple modalities [113].

Tumor hypoxia is the aggressiveness of a tumor. A number of invasive and noninvasive techniques have been exploited to measure tumor hypoxia, including polarographic needle electrodes, immunohistochemical staining, radionuclide imaging (positron emission tomography [PET] and single-photon emission computed tomography [SPECT]), magnetic resonance imaging (MRI), optical imaging (bioluminescence and fluorescence), etc. [114]. The nanoscale materials with dimensions less than 100 nm are significantly altered relative to

the corresponding bulk materials. They exhibit size-dependent behavior such as quantum size effects (depending on bulk Bohr radius), optical absorption and emission, coulomb staircase behavior (electrical transport), superparamagnetism and various unique properties. They are active components of ferrofluids, recording tape, and flexible disk recording media, and have potential for future applications in spintronics. Spintronics is a new paradigm of electronics utilizing intrinsic charge and spin of electrons for ultra-high-density data storage and quantum computing. They are used in biomedical applications such as bioseparation of biological entities, therapeutic drugs and gene delivery, radiofrequency-induced destruction of cells and tumors (hyperthermia), and as contrast-enhancement agents for magnetic resonance imaging (MRI). The nanoparticles have optimizable, controllable sizes as compared to cells (10–100 μm), viruses (20–250 nm), proteins (3–50 nm), and genes (10–100 nm) [115].

Magnetic resonance spectroscopic imaging (MRSI) is a noninvasive imaging technique that provides metabolic information on brain tumors. The role of MRSI as a molecular imaging technique is to provide biochemical information on human brain tumors [116]. Polymeric micelles are used in cancer targeting, drug delivery and tumor imaging applications. Guthi *et al.* have described a multifunctional micelle (MFM) system that is encoded with a lung cancer-targeting peptide (LCP), and encapsulated with superparamagnetic iron oxide (SPIO) and doxorubicin (Doxo) for MR imaging and therapeutic delivery, respectively. They suggested that the integrated diagnostic and therapeutic design of MFM nanomedicine potentially allows for image-guided, target-specific treatment of lung cancer [117]. Talelli *et al.* have investigated the ability of biodegradable thermosensitive polymeric micelles to stably encapsulate hydrophobic oleic-acid-coated superparamagnetic iron oxide nanoparticles (diameter 5–10 nm) [118]. The biodegradable polymer PLGA is an extremely versatile nano- and micro-carrier for several imaging contrast agents with the possibility of targeting diseased states at a molecular level [119]. The dendrimer-based contrast agents are ideal for the molecular imaging of organs and other target-specific locations. The advances in dendrimer-based contrast agents for molecular imaging focus mainly on the chemical design as applied to optical, magnetic resonance, computer tomography, radionuclide, and multimodality imaging [120]. Molecular imaging always requires accumulation of contrast agent in the target site, and is often achieved most efficiently by steering nanoparticles containing contrast agent into the target. This entails accessing target molecules hidden behind tissue barriers, necessitating the use of targeting groups.

Nanoparticles with multiple bioadhesive sites for target recognition and binding will be larger than 20 nm diameter. They share functionalities with many subcellular organelles such as ribosomes, proteasomes, ion channels, and transport vesicles, and materials used to synthesize nanoparticles that include natural proteins and polymers, artificial polymers, dendrimers, fullerenes and other carbon-based structures, lipid-water micelles, viral capsids, metals, metal oxides, and ceramics; whereas signal generators incorporated into nanoparticles include iron oxide, gadolinium, fluorine, iodine,

bismuth, radionuclides, quantum dots, and metal nanoclusters [121]. Yang *et al.* have adapted a tumor-targeting nanoimmunoliposome platform technology (scL) to encapsulate and deliver SPIO (scL-SPIO) *in vitro* and *in vivo* without chemical modification. They suggested that SPIO encapsulation in the scL complex results in an approximately 11-fold increase in SPIO uptake in human cancer cells *in vitro*, with distribution to cytoplasm and nucleus. Moreover, the scL nanocomplex specifically and efficiently delivered SPIO into tumor cells after systemic administration, demonstrating the potential of this approach to enhance local tumor concentration and the utility of SPIO for clinical applications [122].

Quantitative imaging of tumor angiogenesis and protein-protein interactions that modulate angiogenesis will lead to more robust and effective monitoring of personalized molecular cancer therapy. Multidisciplinary approaches and cooperative efforts from many individuals, institutions, industries and organizations are needed to quickly translate multimodality tumor angiogenesis imaging into multiple facets of cancer management [123]. Targeted delivery is a highly desirable strategy to improve the diagnostic imaging and therapeutic outcome due to enhanced efficacy and reduced toxicity. Hong *et al.* have used the anticancer drug doxorubicin (DOX), magnetic resonance imaging (MRI) as a contrast agent, superparamagnetic ion oxide Fe_3O_4 , in the core of micelles self-assembled from amphiphilic block copolymer of poly(ethylene glycol) (PEG), and poly(epsilon-caprolactone) (PCL) with a targeting ligand (folate) attached to the distal ends of PEG (Folate-PEG-PCL). The *in vitro* tumor cell targeting efficacy of these folate functionalized and DOX/SPIO-loaded micelles (Folate-SPIO-DOX-Micelles) was evaluated upon observing cellular uptake of micelles by human hepatic carcinoma cells (Bel 7402 cells) which overexpresses surface receptors for folic acid. The potential of Folate-SPIO-DOX-Micelle as a novel MRI-visible nanomedicine platform suggested that the multifunctional polymeric micelles, folate-SPIO-DOX-Micelles, have better targeting tropism to the hepatic carcinoma cells *in vitro* than their nontargeting counterparts, and that the cell targeting events of micelles can be monitored using a clinical MRI scanner [124].

7.8 Nanoemulsions

Micelles are formed in solution as aggregates in which the component molecules are generally arranged in a spheroidal structure with hydrophobic cores shielded from the water by a mantle of hydrophilic groups. These dynamic systems, which are usually below 50 nm in diameter, are used for the systemic delivery of water-insoluble drugs. Drugs or contrast agents may be trapped physically within the hydrophobic cores or can be linked covalently to component molecules of the micelle. Nanoemulsions consist of very stable nanodroplets of oil dispersed in an aqueous phase, typically below 300 nm in size. The presence of nanoemulsions in solution before spray-drying had a significant impact on microparticle size, distribution, and morphology known as Trojan

microparticles, which encapsulate, protect, and release the lipid nanodroplets [125]. The unique properties of nanomaterials related to structural stability and quantum-scale reactive properties open up a world of possibilities that could be exploited to design and to target drug delivery or create truly microscale biological sensors for veterinary applications. Low-energy nanoemulsions find application in the delivery of controlled amounts of drugs into the beverage of breeding animals (such as poultry, cattle, pigs) or can be used for the controlled release of injectable poorly water-soluble drugs [126].

Use of lipid nanoemulsions as carriers of drugs for therapeutic or diagnostic purposes was tested whether modifications of core particle constitution could affect the characteristics and biologic properties of lipid nanoemulsions. The nanoemulsions are stable for a long period, with good size, oxidation status, and cell survival during its uptake in all tested cell lines [127]. Kou *et al.* have shown the effect of nanoemulsions of antioxidant synergy formulation (ASF) containing gamma, alpha, and delta tocopherol, and suggested enhanced anti-inflammatory properties and bioavailability with gamma tocopherol as compared to their normal suspensions [128].

Drug delivery systems have the ability to allow superior drug absorption, controlled drug release and reduced side-effects, enhancing their effectiveness using nanoenabled systems such as micelles, nanoemulsions, nanogels, liposomes, nanofibers, dendrimer [129]. The pharmaceutical nanocarriers, such as liposomes, micelles, nanoemulsions, polymeric nanoparticles and many others are showing a variety of useful properties, such as longevity in the blood allowing for their accumulation in pathological areas with compromised vasculature; specific targeting to certain disease sites due to various targeting ligands attached to the surface of the nanocarriers; enhanced intracellular penetration with the help of surface-attached cell-penetrating molecules; contrast properties due to the carrier loading with various contrast materials allowing for direct carrier visualization *in vivo*; stimuli-sensitivity allowing for drug release from the carriers under certain physiological conditions, etc. The engineering of multifunctional pharmaceutical nanocarriers combining several useful properties in one particle can significantly enhance the efficacy of many therapeutic and diagnostic protocols [130].

7.9 Nanofibers

Nanofibers may be designed to present high densities of bioactive molecules such as those which promote cell adhesion and growth. For example, amphiphiles that present the pentapeptide epitope IKVAV, an amino acid sequence of laminin that promotes neurite adhesion, can self-assemble in aqueous media, or when injected directly into a tissue, to form fibers with a diameter of 5–10 nm. Indeed, these scaffolds were shown to induce rapid differentiation of cells to neurons, while discouraging the development of astrocytes. This presumably suggest that synthetic materials may have the ability to modulate selective gene expression. Another interesting approach was the design of a synthetic

collagen substitute, based on a material composed of a long hydrophobic alkyl group on one end and a hydrophilic peptide on the other that self-assembles into nanocylindrical structures. These nanocylinders guide the formation of hydroxyapatite crystallites with orientations and sizes similar to those in natural bone [131-132]. Nanofibrous scaffolds provide a superior environment for promoting cell functions due to their nanometer pore sizes, e.g., biodegradable and cytocompatible poly(DL-lactide-co-glycolide) (PLGA) nanofibers [133].

A self-assembling peptide, P4 (Ac-NH-LDLKLELKLDLKLELK-CONH₂), has 16 amino acids ~5 nm in size, with an alternating polar and nonpolar pattern. The ability of the peptide to stabilize the hydrophobic anticancer agent ellipticine was tested using two cancer cell lines including SMMC7721 and EC9706 [134]. RADA16-I peptide hydrogel, is a type of nanofiber scaffold extensively applied to regenerative medicine and tissue repair in order to develop novel nanomedicine systems. Using phase contrast microscopy, the appearance of the representative ovarian cancer cells encapsulated in RADA16-I peptide hydrogel suggested that precultured ovarian cancer cells had a twofold to fivefold higher anticancer drug resistance than the conventional two-dimensional petri-dish culture. So the 3D cell model on peptide nanofiber scaffold is an optimal type of cell pattern for anticancer drug screening and tumor biology [135]. The nanofiber-cell-hydrogel composite enables replication of the cellular and matrix architecture found in many natural tissues, offering a novel protocol for electrospun nanofibers in regenerative medicine and bioengineering [136]. Designed scaffolds comprised of natural and synthetic materials are now widely used in the reconstruction of damaged tissues. Utilization of absorbable and nonabsorbable synthetic and natural polymers with unique characteristics can be an appropriate solution to repair damaged nerve tissues. Polymeric nanofibrous scaffolds with properties similar to neural structures can be more effective in the reconstruction process. Better cell adhesion and migration, more guiding of axons, and structural features, such as porosity, provide a clearer role for nanofibers in the restoration of neural tissues [137].

Surface adhesion between nanofibers and nanoparticles has attracted attention for potential biomedical applications. Adhesion forces were measured using a polystyrene (PS) nanoparticle attached to an atomic force microscopy (AFM) tip/probe [138]. Gelain *et al.* have reported the designer self-assembling peptide scaffolds facilitate with a slow and sustained release of active cytokines that are extremely relevant to many areas of regenerative medicine. They have suggested that they not only provide evidence for long-term molecular release from self-assembling peptide scaffolds, but also inspiration for a plethora of slow molecular release strategies for clinical applications [139].

Kunduru *et al.* have demonstrated that scaling down the surface texturing from the micro- to the nanoscale enhances the amplitude of the measured detected signal strength. They suggested that the nanoscale fiber morphology provides size-matched spaces for trapping and immobilizing the protein biomolecule, resulting in improved detection signal strength. Nanotechnology-based biosensors for vulnerable coronary vascular plaque rupture were

designed and fabricated using micro- and nanotextured polystyrene polymer structures [140]. Nanofibers, which mimic collagen fibrils in the extracellular matrix (ECM), can be created from a host of natural and synthetic compounds and have multiple properties that may be beneficial to burn wound care. These properties include a large surface-area-to-volume ratio, high porosity, improved cell adherence, proliferation and migration, and controlled *in vivo* degradation rates. The large surface area of nanofiber mats allows for increased interaction with compounds and provides a mechanism for sustained release of antibiotics, analgesics, or growth factors into burn wounds; high porosity allows diffusion of nutrients and waste. Improved cell function on these scaffolds will promote healing [141].

Neurodegenerative disorders including Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis and stroke are rapidly increasing as the population ages. The diagnosis and treatment of devastating human diseases are possible with nanomedicine using liposomes, nanoparticles, polymeric micelles, block ionomer complexes, nanogels, and dendrimers for delivery of drugs, genes, and imaging agents. Nanomaterials like fullerenes, have antioxidant properties to eliminate reactive oxygen species in the brain to mitigate oxidative stress [142]. Nanomaterials and structures, such as nanoparticles, nanofibers, nanosurfaces, nanocoatings, nanoscaffolds and nanocomposites, are considered for various applications in orthopedics and traumatology [143]. Continuous nanostructures embedded with proteins may synergistically present topographical and biochemical signals to cells for tissue engineering applications. The co-axial electrospinning of aligned poly(epsilon-caprolactone) nanofibers encapsulated with bovine serum albumin and platelet-derived growth factor-bb for controlled release and bioactivity retention, is shown to be a versatile technique in achieving the delivery of biochemical signals in a controlled manner for regenerative medicine applications [144]. Poly(alpha-hydroxy acids), especially lactic acids and glycolic acid and their copolymers with epsilon-caprolactone, are the most widely known and used among all biodegradable polymers. Electrospun matrices made of lactic acids, glycolic acid and epsilon-caprolactone homo- and copolymers have been attracting increasing attention for fabrication of novel materials for medical use [145].

Biocompatible titanate nanofiber scaffolds are prepared on the surface of titanium foil/mesh via a one-step hydrothermal reaction. The nanofibers can self-organize into macroporous scaffolds potentially useful for developing new bioscaffolds, photocatalysts, sensors, and drug delivery vehicles [146]. Amoxicillin nanobelts, nanofibers, nanoparticles, and microparticles can be obtained by changing the pH in solutions of amoxicillin sodium and controllable growth of amoxicillin can be realized [147]. Electrospinning is a simple and versatile technique that can produce a macroporous scaffold comprising randomly oriented or aligned nanofibers. Endowed with both topographical and biochemical signals such electrospun nanofibrous scaffolds may provide an optimal microenvironment for the seeded cells for biomedical applications such as drug delivery and tissue engineering [148].

7.10 Nanoshells

The gold-coated silica nanosphere type of nanoshells, embedded in a drug-containing tumor-targeted hydrogel polymer, and then injected into the body, accumulate near tumor cells. As treated with an infrared laser, the nanoshells selectively absorb a specific infrared frequency, melting the polymer and releasing the drug payload at a specific site. Nanoshells might prove useful in treating diabetes. A patient would use a ballpoint-pen-sized infrared laser to heat the skin site where nanoshell polymer had been injected, releasing a pulse of insulin. Unlike injections, which are given several times a day, the nanoshell-polymer system could remain in the body for months. The stimuli responsive polymeric capsules and nanoshells are formed via layer-by-layer (LbL) approach for the multifunctionality and responsiveness to stimuli such as physical (light, electric, magnetic, ultrasound, mechanical, and temperature), chemical (pH, ionic strength, solvent, and electrochemical) and biological (enzymes and receptors).

Cancer is a leading cause of death. Conventional methods of treating cancer involve surgical removal of easily accessible tumors, radiation therapy, and chemotherapy, with no full treatment. Nanoshells are optically tunable core/shell nanoparticles that can be fabricated to strongly absorb in the near-infrared (NIR) region where light transmits deeply into tissue. When injected systemically, these particles have been shown to accumulate in the tumor due to their enhanced permeability and retention (EPR) effect, and induce photothermal ablation of the tumor when irradiated with an NIR laser [149]. Current modalities of diagnosis and treatment of cancer are based on nanoparticles using contrast agents, fluorescent materials, molecular research tools and drugs with targeting antibodies. Paramagnetic nanoparticles, quantum dots, nanoshells and nanosomes are a few of the nanoparticles used as a cancer diagnostic. The modalities of therapy, such as heat induced ablation of cancer cells by nanoshells and gene therapy, are also developed [150]. Nanomedicine has the potential to increase the specific treatment of cancer cells while leaving healthy cells intact through the use of novel nanoparticles to seek and treat cancer in the human body. There are various nanocarriers such as nanoshells, nanocrystals, nanopolymers, quantum dots, and dendrimers, and their role in early cancer detection and treatment have been developed [151].

Liposome-supported plasmon resonant gold nanoshells are degradable into components of a size compatible with renal clearance, potentially enabling their use as multifunctional agents in applications in nanomedicine, including imaging, diagnostics, therapy, and drug delivery. The laser illumination at the wavelength matching the plasmon resonance band of a gold-coated liposome leads to the rapid release of encapsulated substances, which can include therapeutic and diagnostic agents. The development of spectrally-coded release can find applications in controlled delivery of multiple agents to support complex diagnostic tests and therapeutic interventions [152].

Diagaradjane *et al.* have reported noninvasive modulation of *in vivo* tumor radiation response using gold nanoshells. Mild-temperature hyperthermia

generated by near-infrared illumination of gold nanoshell-laden tumors, noninvasively quantified by magnetic resonance temperature imaging, causes an early increase in tumor perfusion that reduces the hypoxic fraction of tumors [153].

Inorganic nanoparticles, such as carbon nanotubes, quantum dots and gold nanoshells, have been adopted for biomedical use, due to their unique optical and physical properties. Also inorganic porous nanomaterials are fundamentally advantageous for developing multifunctional nanomaterials, due to their distinctive inner and outer surfaces [154].

Much research and development is progressing in the areas of cancer diagnostics, devices, biosensors, and microfluidics, using nanoshells, carbon nanotubes, dendrimers, quantum dots, superparamagnetic nanoparticles, and liposomes [155]. Xie *et al.* have demonstrated a new nondestructive optical assay to estimate submicron solid particle concentrations in whole blood using Triton X-100 in blood samples containing gold (Au) nanoshells to act as a quantitative scattering standard and blood lysing agent [156].

Nanotechnology-based tools and techniques are rapidly emerging in the fields of medical imaging and targeted drug delivery. Employing constructs such as dendrimers, liposomes, nanoshells, nanotubes, emulsions and quantum dots, these advances lead toward the concept of personalized medicine and the potential for very early, even presymptomatic, diagnoses coupled with highly-effective targeted therapy [157]. One of the ultimate goals of nanomedicine is to create medically useful nanodevices that can function inside the body. It is envisioned that nanodevices will be hybrids of biologic molecules and synthetic polymers that can enter cells and the organelles to interact directly with DNA and proteins. Among the newly developed nanomedicine and nanodevices, such as quantum dots, nanowires, nanotubes, nanocantilevers, and nanopores, nanoshells and nanoparticles are the most promising applications for various cancer treatments [158].

7.11 Quantum Dots

QDs are nanoscale crystalline structures made from a variety of different compounds, that can transform the color of light. Quantum dots absorb white light and then re-emit it a couple of nanoseconds later at a specific wavelength. By varying the size and composition of quantum dots, the emission wavelength can be tuned from blue to near infrared. Cell membranes are impermeable to most molecules that are not actively imported by living cells, including all macromolecules and even small molecules whose physiochemical properties prevent passive membrane diffusion. However, cell-penetrating peptides (CPPs), short peptides believed to enter cells by penetrating cell membranes, have attracted great interest in the hope of enhancing gene therapy, vaccine development and drug delivery using cargo molecule quantum dots [159]. He *et al.* have presented the investigation of bioconjugating ability of near-infrared (NIR) CdSeTe/ZnS quantum dots (QDs) (710nm) and visible CdSe QDs

(595nm) in immunofluorescent staining for cancer biomarkers in gastric cancer tissues probed with the homemade Hadamard transform (HT) spectral imaging microscope and a commercial multispectral imaging system.

NIR QDs hold more promise for real-time imaging of tumor tissues due to its higher sensitivity and contrast [160]. Pompa *et al.* have proposed a systematic and reproducible evaluation of nanoparticles (NPs) toxicology in living systems, based on a physical assessment and quantification of the toxic effects of NPs by the experimental determination of the key parameter affecting the toxicity outcome (i.e., the number of NPs) and of the NPs toxicity factor. This approach may pave the way to a systematic classification of nanomaterials, leading to important developments in risk assessment and regulatory approval, as well as in a wide range of nanomedicine applications [161]. Ferritin is the protein to store iron that the cell does not require immediately for metabolic processes, thereby protecting against the toxic effects of free Fe^{2+} . Ferritin is itself a catalytic bionanoparticle. Its internal cavity can be used as a nanoreactor to produce non-native metallic nanoparticles. Moreover, its external protein shell can be chemically modified, allowing ferritin to be used as a precursor for a library of metallic nanoparticles, some of which may have potential applications in biomedicine, especially as multimodal imaging probes [162]. The efficiency with which NIR pulses can deliver nanoparticles and any adverse effects on living cells needs thorough testing. Toward this end, the efficacy and toxicity of delivering quantum dots (QDs) into cells of *Xenopus laevis* embryos by NIR laser injection was determined. Higher intensities of NIR pulses caused permanent damage to the targeted cells, and thus NIR pulses may also prove useful for ablation of specific cells within tissues [163].

Liposome structure with quantum dots (QD) for the construction of multifunctional nanoparticles are reporting the construction of drug-loaded liposome-QD hybrid vesicles (L-QD) by incorporating TOPO-capped, doxorubicin (Dox). The drug-loaded L-QD-Dox hybrid vesicles presented here constitute a promising multifunctional delivery vector capable of transporting combinations of therapeutic and diagnostic modalities [164]. Inorganic nanoparticles offer exceptional advantages to the field of multimodal imaging owing to their unique characteristics, such as nanometer dimensions, tunable imaging properties, and multifunctionality. Nanoparticles mainly based on iron oxide, quantum dots, gold, and silica have been applied to various imaging modalities to characterize and image specific biologic processes on a molecular level. A combination of nanoparticles and other materials such as biomolecules, polymers, and radiometals continue to increase functionality for *in vivo* multimodal imaging and therapeutic agents [165].

Quantum dots (QDs), as novel bioimaging and drug delivery agents, are generally introduced into the vascular system by injection, and are thus directly exposed to vascular endothelial cells (ECs). It was suggested that CdTe QDs could not only impair mitochondria but also exert endothelial toxicity through activation of mitochondrial death pathway and induction of endothelial apoptosis [166]. Quantum dots (QDs) have powerful inorganic fluorescent

probes for long-term, multiplexed imaging and detection. QDs have been used in the research of the drug delivery system from the following aspects: surface modification strategies of QDs for drug delivery, QDs as drug nanocarriers, QD-labeled drug nanocarriers, QD-based fluorescence resonance energy transfer (FRET) technique for drug release study as well as the development of multifunctional nanomedicines [167].

Nanooncology, is the molecular diagnosis of cancer by the use of gold nanoparticles and quantum dots, one such example being the sensitive detection of multiple protein biomarkers by nanobiosensors. Nanoparticles enable targeted drug delivery and nanoparticulate anticancer drugs cross the biological barriers and achieve therapeutic concentrations in tumor and check the surrounding normal tissues from toxic effects. Monoclonal antibody nanoparticle complexes are under investigation for diagnosis as well as targeted delivery of cancer therapy. Nanoparticle-based optical imaging of tumors as well as contrast agents to enhance detection of tumors by magnetic resonance imaging can be combined with delivery of therapeutic agents for cancer [168].

The cytotoxicity and genotoxicity of CdTe QDs were examined in human umbilical vein endothelial cells (HUVECs) and suggested that CdTe QDs have cytotoxic and genotoxic effects on HUVECs, and that ROS generation may be involved in QD-induced DNA damage [169]. Quantum dots (QDs) have shown noninvasive imaging and monitoring of cancer cells *in vivo* peptide-linked near-conjugated fluorescent QDs to label human buccal squamous cell carcinoma cell line (BcaCD885). It was found that they did not affect the growth, proliferation, apoptosis, and tumorigenicity ability, and support the application of near-infrared fluorescent QDs in noninvasive imaging and monitoring of cancer cells *in vivo* [170].

A lipid-coated quantum dot system doubles the fluorescence of PEG-grafted quantum dots at half the dose by using a tumor-targeted near-infrared imaging agent composed of cancer-specific monoclonal antinucleosome antibody 2C5 coupled to quantum dot (QD)-containing polymeric micelles, prepared from a polyethylene glycol/phosphatidylethanolamine (PEG-PE) conjugate. It represents a development that may potentially serve to enhance early detection for metastases [171].

Nanoparticles, with their superior physical properties, have become the materials of choice. Owing to inherent magnetic, optical or acoustic attributes, these nanoparticles can be detected by corresponding imaging modalities in living subjects at a high spatial and temporal resolution. These features allow implanted cells to be separated from host cells; and have advantages over traditional histological methods, as they permit noninvasive, real-time tracking *in vivo* [172].

Chakravarthy *et al.* have reported the ability of nanoconjugates of CdSe/CdS/ZnS quantum dots (QDs) and doxorubicin (Dox) to target alveolar macrophages (aMØs), cells that play a critical role in the pathogenesis of inflammatory lung injuries. They have demonstrated the nanoparticle platforms which can provide targeted macrophage-selective therapy for the treatment of pulmonary disease [173].

Theranostic nanomedicine is emerging as a promising therapeutic paradigm. It takes advantage of the high capacity of nanoplatforms to ferry cargo and loads onto them both imaging and therapeutic functions. Iron oxide nanoparticles, quantum dots, carbon nanotubes, gold nanoparticles and silica nanoparticles, have been previously well investigated in the imaging setting and are candidate nanoplatforms for building up nanoparticle-based theranostics [174]. Nanoparticles (NPs) are small in size with large surface areas, having unique properties and applications distinct from those of bulk systems. When exposed to biological fluid, NPs may become coated with proteins and other biomolecules due to their dynamic nature. The protein fibrillation utilizing the NPs as nucleation centers may be possible. Protein fibrillation is reported in many fatal neurodegenerative diseases. The protein-NP interaction brings about many key issues and concerns with respect to the potential risks to human health and the environment. The effects of NPs and semiconductor quantum dots (QDs) in the process of protein fibril formation using human serum albumin (HAS) suggested that an increased rate of fibrillation occurs. This opens up an understanding and possibility of controlling biological self-assembly processes for use in nanobiotechnology and nanomedicine [175].

Nanomedicine is an interdisciplinary field still in its infancy, where an accurate scientific assessment of potential risks and benefits is needed. There is increasing interest in improving our understanding of the interactions between nanomaterials and living systems, with regard to both the underlying chemistry and the physics of effects on the nanoscale. Imaging and therapeutic components, including metallic radioisotopes, semiconductor quantum dots, and magnetic materials, may be used to construct nanocarriers (by encapsulation or conjugation) by rapid and simple (covalent and supramolecular) chemistry. The biomedical functions of the resulting materials are as yet largely unexplored. Encapsulation in nanocarriers could achieve delivery of the reagents (imaging and therapeutic drugs) to the sites of action in the body, while minimizing systemic toxicity and enzymatic degradation. These functional systems have the potential to become a general solution in drug delivery [176].

Glioblastoma is the most malignant brain tumor and it is important to search for new diagnostic and therapeutic approaches for its treatment. Application of superparamagnetic nanoparticles of iron oxide, as well as monoclonal antibodies of immunophenotypic significance, conjoined to quantum dots for the ultrastructural assessment of glioblastoma cells. The process of tumor cell labeling using nanoparticles can successfully contribute to the identification of tumorigenic cells and consequently for better understanding of glioblastoma genesis and recurrence. In addition, this method may help further studies in tumor imaging, diagnosis, and prognostic markers detection [177].

NH₂ functionalized CdSe/ZnS quantum dot (QD)-doped SiO₂ nanoparticles (NPs) with both imaging and gene carrier show that QD-doped SiO₂ NPs are internalized by primary cortical neural cells without inducing cell death *in vitro* and *in vivo*. The ability to bind, transport and release DNA into the cell allows GFP-plasmid transfection of NIH-3T3 and human neuroblastoma

SH-SY5Y cell lines. QD-doped SiO_2 NPs properties are valuable tools for future nanomedicine application [178]. Subnanometer photoluminescent gold quantum dots (GQDs) are functionalized with a peptide moiety that contains both nuclear export signal (NES) and nuclear localization signal (NLS) sequences. By taking advantage of its small size and great photostability, the functionalized GQDs are used to mimic the actions of nucleus shuttle proteins, especially of those activated during cell apoptotic death, to work as protease-mediated cytoplasm-nucleus shuttles for dynamic monitoring of apoptosis. The resulting construct demonstrates activation of the nuclear pore complex (NPC) of cells for bidirectional transport between nucleus and cytoplasm. A caspase-3 recognition sequence (DEVD), placed within the NLS/NES peptide, serves as a proteolytic site for activated caspase-3. Upon the induction of apoptosis, the activated caspase-3 cleaves the functional peptide on GQDs resulting in changes of subcellular distribution of GQDs. Such changes can be quantified as a function of time by the ratios of GQDs photoluminescence in nucleus to that in cytoplasm. As such, the NES-linker-DEVD-linker-NLS peptide enables the GQDs to function as molecular probes for the real-time monitoring of cellular apoptosis [179].

Quantum dots (QDs), also known as semiconducting nanoparticles, are promising zero-dimensional advanced materials because of their nanoscale size and because they can be engineered to suit particular applications such as nonlinear optical devices (NLO), electro-optical devices, and computing applications. QDs can be joined to polymers in order to produce nanocomposites which can be considered a scientific revolution of the 21st century. One of the fastest moving and most exciting interfaces of nanotechnology is the use of QDs in medicine, cell and molecular biology. Recent advances in nanomaterials have produced a new class of markers and probes by conjugating semiconductor QDs with biomolecules that have affinities for binding with selected biological structures. The nanoscale of QDs ensures that they do not scatter light at visible or longer wavelengths, which is important in order to minimize optical losses in practical applications. Moreover, at this scale, quantum confinement and surface effects become very important and therefore manipulation of the dot diameter or modification of its surface allows the properties of the dot to be controlled. Quantum confinement affects the absorption and emission of photons from the dot. Thus, the absorption edge of a material can be tuned by control of the particle size [180]. Nanoprecipitation was utilized to synthesize biodegradable and surfactant-free nanoparticles loaded with quantum dots. This protocol also yielded nanoparticles coloaded with both quantum dots and hydrophobic drug (Coenzyme Q10) molecules. Importantly, even though surfactants were not utilized during the nanoprecipitation procedure, these loaded nanoparticles did not aggregate. Dialysis efficiently removed unencapsulated quantum dots from nanoparticle suspensions without altering the physical properties of the quantum-dot-loaded nanoparticles. The resultant purified, quantum-dot-loaded nanoparticles were biocompatible in differentiated PC12 cell cultures, which facilitated their use as nanoparticles in microscopy. In fact, confocal imaging studies showed that

purified, quantum-dot-loaded nanoparticles were associated with PC12 cells after one day *in vitro*. These novel and multifunctional coloaded nanoparticles may prove advantageous in future simultaneous drug delivery and imaging applications [181]. Al-Jamal *et al.* have reported the construction of lipid-quantum dot (L-QD) bilayer vesicles by incorporation of the smallest (2nm core size) commercially available CdSe/ZnS QD within zwitterionic dioleoylphosphatidylcholine and cationic 1,2-dioleoyl-3-trimethylammonium-propane lipid bilayers, self-assembling into small unilamellar vesicles. They suggested that this constituted a novel delivery system that offers the potential for transport of combinatory therapeutic and diagnostic modalities to cancer cells *in vitro* and *in vivo* [182].

Zeta potential (ZP) is a powerful tool to probe the electrostatic surface potential of biological materials. It remains an untapped method for studying the interaction of nanoparticles with cells to aid in the development of future therapeutic strategies. ZP measurements on viral nanoparticles is useful to elucidating the characteristics of the surface charge and the potential interaction modes with cell surfaces [183]. Nanoparticles (NPs) and nanosized objects are available for translation into clinical practice. Computer tomography and MRI with iron-based magnetic NPs are promising methods for radiological detection of cancers. Semiconductor fluorescent NPs (quantum dots) are being developed for simultaneous detection and localization of multiple breast cancer biomarkers, enabling the personalization of therapeutic regimens for each patient. Additionally, inorganic NPs can be conjugated with tumor-specific ligands and used for tumor-selective delivery of chemotherapeutic or hormonal agents.

NPs bearing tumor-targeted antibodies and oligonucleotides for anticancer gene therapy are a novel and rapidly developing therapeutic approach in oncology [184]. Ligand exchanged gold quantum dots (GQDs) conjugated with cell-penetrating peptides are a new class of photoluminescent probes for nuclear targeting and intracellular imaging [185]. Molecular imaging, first developed to localize antigens in light microscopy, now encompasses all imaging modalities including those used in clinical care: optical imaging, nuclear medical imaging, ultrasound imaging, CT, MRI, and photoacoustic imaging. Molecular imaging always requires accumulation of contrast agent in the target site, often achieved most efficiently by steering nanoparticles containing contrast agent into the target. This entails accessing target molecules hidden behind tissue barriers, necessitating the use of targeting groups. For imaging modalities with low sensitivity, nanoparticles bearing multiple contrast groups provide signal amplification. The same nanoparticles can in principle deliver both contrast medium and drug, allowing monitoring of biodistribution and therapeutic activity simultaneously (theranostics). Nanoparticles with multiple bioadhesive sites for target recognition and binding will be larger than 20 nm diameter. They share functionalities with many subcellular organelles (ribosomes, proteasomes, ion channels, and transport vesicles) and are of similar sizes. The materials used to synthesize nanoparticles include natural proteins and polymers, artificial polymers, dendrimers, fullerenes and other carbon-based

structures, lipid-water micelles, viral capsids, metals, metal oxides, and ceramics. Signal generators incorporated into nanoparticles include iron oxide, gadolinium, fluorine, iodine, bismuth, radionuclides, quantum dots, and metal nanoclusters. Newly emerging diagnostic imaging applications include sentinel node localization and stem cell tracking [186].

Nanomedicine has major potential applications in diabetes. These include solving needs such as noninvasive glucose monitoring using implanted nanosensors, with key techniques being fluorescence resonance energy transfer (FRET) and fluorescence life time sensing, as well as new nanoencapsulation technologies for sensors such as layer-by-layer (LBL) films. The latter might also achieve better insulin delivery in diabetes by both improved islet encapsulation and oral insulin formulations. An “artificial nanopancreas” could be an alternative closed-loop insulin delivery system. Other applications of nanomedicine include targeted molecular imaging *in vivo* (e.g., tissue complications) using quantum dots (QDs) or gold nanoparticles, and single-molecule detection for the study of molecular diversity in diabetes pathology [187]. Functionalized-quantum-dot-liposome (f-QD-L) hybrid nanoparticles are engineered by encapsulating poly(ethylene glycol)-coated QD in the internal aqueous phase of different lipid bilayer vesicles. Moreover, f-QD-L offer many opportunities for the development of combinatory therapeutic and imaging (theranostic) modalities by incorporating both drug molecules and QD within the different compartments of a single vesicle [188]. Magnetic nanoparticles/quantum dots are used for measuring, understanding, and manipulating stem cells for labeling, *in vivo* tracking, intracellular delivery of genes/oligonucleotides, protein/peptides and engineered nanometer-scale scaffolds for stem cell differentiation and transplantation [189].

Nanopharmaceuticals have seen a number of advancements with a focus on the engineering of novel applications. Nanotechnology also offers the ability to detect diseases at much earlier stages, such as finding hidden or overt metastatic colonies often seen in patients diagnosed with breast, lung, colon, prostate, and ovarian cancer. Diagnostic applications could build upon conventional procedures using nanoparticles, such as colloidal gold, iron oxide crystals, and quantum dots. Additionally, diseases may be managed by multifunctional agents encompassing both imaging and therapeutic capabilities, thus allowing simultaneous monitoring and treatment [190].

Quantum dots (QDs) are semiconductor nanocrystals that emit fluorescence on excitation with a light source. They have excellent optical properties, including high brightness, resistance to photobleaching and tunable wavelength. The surface modification of QDs enable their potential application in cancer imaging. QDs with near-infrared emission could be applied to sentinel lymph-node mapping to aid biopsy and surgery. Conjugation of QDs with biomolecules, including peptides and antibodies, could be used to target tumors *in vivo*. QDs for cancer diagnosis and treatment from a clinical standpoint are very crucial [191]. The enhanced diagnostic and therapeutic medicine against cancer and other diseases are possible due to nanomedicine. The special properties exhibited by the nanoscale size of quantum dots, metal colloids, superparamagnetic

iron oxide, and carbon-based nanostructures give rise to their nanotechnologic behavior (i.e., theranostic) making them both diagnostic and therapeutic by design [192].

Biomedical imaging depends on the development of probes that combine low toxicity with high sensitivity, resolution, and stability. Fluorescent core-shell silica nanoparticles with narrow size distributions and enhanced photostability (C dots), provide appealing *in vivo* applications in cancer biology. C dots are nontoxic at biologically relevant concentrations and are used in a broad range of imaging applications within the emerging field of nanomedicine including: intravital visualization of capillaries and macrophages, sentinel lymph node mapping, peptide-mediated multicolor cell labeling for real-time imaging of tumor metastasis, and tracking of injected bone marrow cells in mice [193]. Man-made nanostructured materials such as fullerenes, nanoparticles, nanopowders, nanotubes, nanowires, nanorods, nanofibers, quantum dots, dendrimers, nanoclusters, nanocrystals, and nanocomposites are widely produced in large quantities. This is due to their great potential in applications including skincare, consumer products, healthcare, electronics, photonics, biotechnology, engineering products, pharmaceuticals, drug delivery, and agriculture. However, human exposure is caused by these nanomaterials entering the body through the lungs or other organs via food, drink, and medicine and this can affect different organs and tissues such as the brain, liver, kidney, heart, colon, spleen, bone, blood, etc., and may cause toxicity and various diseases in humans and animals. Nanomaterials interactions with biological systems depend upon their properties, such as size, concentration, solubility, chemical and biological properties, and stability [194]. Nanotechnologies enable diagnosis at the single-cell and molecule levels as biochips and nanobiosensors, which are promising for potential clinical applications in the areas of biomarker discovery, cancer diagnosis, and detection of infectious microorganisms [195]. Inorganic nanoparticles, such as carbon nanotubes, quantum dots and gold nanoshells, have been adopted for biomedical use, due to their unique optical and physical properties.

Compared to conventional materials, inorganic nanomaterials have several advantages such as simple preparative processes and precise control over their shape, composition and size. In addition, inorganic porous nanomaterials are fundamentally advantageous for developing multifunctional nanomaterials due to their distinctive inner and outer surfaces. In this review, we describe recent developments of hollow and porous inorganic nanomaterials in nanomedicine, especially for imaging/diagnosis and photothermal therapy [196]. Bioconjugated nanoparticles and quantum dots are among the most exciting nanomaterials with promising application potential in the nanomedicine field. These applications include biosensing, bioimaging, bioassay, targeted drug delivery and new therapeutic agents or method development. Although most of these applications are based on the optical properties of nanoparticle materials such as surface plasmon resonance, surface enhanced Raman scattering and strong photoluminescence, other aspects of nanoparticles such as the catalytic effect and amplification effect associated with the nanoscale dimension have also been explored [197].

7.12 Nanoimaging

Imaging techniques, including nanoimaging, molecular imaging, and medical imaging provide ways of understanding structure and function in biology at different scales, and are widely used in nanomedicine, regenerative medicine, and nuclear medicine. Structural misfolding and aggregation of a particular protein are main causes of numerous neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's diseases. They are confirmed by X-ray crystallography, nuclear magnetic resonance, electron microscopy, and atomic force microscopy.

Also, protein misfolding and assembly of protein molecules into aggregates of various morphologies link a number of important human diseases, including numerous neurodegenerative diseases, cataracts, arthritis, medullary carcinoma of the thyroid, and late-onset diabetes mellitus. Nanoimaging provides a method to monitor the aggregation process, visualize protein aggregates and analyze their properties, and provides fundamental knowledge of key factors that lead to protein misfolding and self-assembly in various protein-misfolding pathologies [198–200]. Most ocular diseases are treated by topical drug application in the form of solutions, suspensions and ointment. However, these conventional dosage forms suffer from the problem of poor ocular bioavailability. Therefore, the applications of various nanoparticulate systems like microemulsions, nanosuspensions, nanoparticles, liposomes, niosomes, dendrimers and cyclodextrins in the field of nanodiagnostics, nanoimaging and nanomedicine can be utilized to explore the frontiers of ocular drug delivery and therapy [201].

7.13 Inorganic Nanoparticles

Polyhedral oligomeric silsesquioxane (POSS) with a distinctive nanocage structure consisting of an inner inorganic framework of silicon and oxygen atoms, and an outer shell of organic functional groups is one of the most promising nanomaterials for medical applications. The application of POSS nanocomposites in combination with other nanostructures has also been investigated, including silver nanoparticles and quantum dot nanocrystals. The highly versatile POSS derivatives have created new horizons for the field of biomaterials research and beyond [202]. Amorphous silica (SiO_2) is a mesoscopic, with tunable pore dimensions in the molecular size range and a high pore volume and surface area, with a selective surface functionality that provides shelter for incorporated agents (drugs, proteins, imaging agents, photosensitizers) leaving the outer particle surface free for further modification. The unique features make these materials particularly amenable to modular design, whereby functional moieties and features may be interchanged or combined to produce multifunctional nanodelivery systems combining targeting, diagnostic, and therapeutic actions in cancer therapy and diagnostics [203].

Inorganic nanoparticles, mainly zinc oxide (ZnO) and titanium dioxide (TiO_2), are used for sunscreen applications due to their intrinsic physical properties.

These properties cause attenuation of the ultraviolet light by absorption and scattering in one way, and in another way by the formation of free radicals (i.e., phototoxicity) [204]. The development of nanoparticles-based drugs seems to be effective in providing low side effects and targeted action on cancer cells. The nano drugs are targeted selectively and specifically only towards tumors resulting in better treatment. The important molecules used for preparation of nano drugs are cisplatin, carboplatin, bleomycin, 5-fluorouracil, doxorubicin, dactinomycin, 6-mercaptopurine, paclitaxel, topotecan, vinblastin, etoposide, etc. The most commonly used materials for preparing nanoparticles carriers are dendrimers, polymer, liposome, micelles, inorganic and organic nanoparticles, etc. [205]. Various forms of nanocarriers, such as nanoparticles (polymeric, inorganic, and solid lipid), liposomes, polymeric micelles, dendrimers, cyclodextrins, and cell-based nanoformulations have been studied for delivery of drugs intended for HIV prevention or therapy. Nanocarriers provide a means to overcome cellular and anatomical barriers to drug delivery. Their application in the area of HIV prevention and therapy may lead to the development of more effective drug products for combating this pandemic disease [206]. The unique physical properties of nanoscale materials can be utilized to produce novel and effective sensors for cancer diagnosis, agents for tumor imaging, and therapeutics for cancer treatment. Functionalizing inorganic nanoparticles with biocompatible polymers and natural or rationally designed biomolecules offers a route towards engineering responsive and multifunctional composite systems. Nanocomposite materials based on functionalized metal and semiconductor nanoparticles promise to transform the way cancer is diagnosed and treated [207]. Das *et al.* have developed a gold nanoparticle and silica nanoparticle mediated drug delivery system containing NO donors, which could be used for potential therapeutic application in chronic liver disease [208].

Inorganic nanomaterials (INMs) and nanoparticles (NPs) are important in our lives because of their use as drugs, imaging agents, and antiseptics. Among the most promising INMs being developed are metal, silica, dendrimers, organic-inorganic hybrids, and bioinorganic hybrids. Gold NPs are important in imaging as drug carriers and for thermotherapy of biological targets. Gold NPs, nanoshells, nanorods, and nanowires have the extensive potential to be an integral part of our imaging toolbox and are useful in the fight against cancer. Metal NP contrast agents enhance magnetic resonance imaging and ultrasound results in biomedical applications of *in vivo* imaging. Hollow and porous INMs have been exploited for drug and gene delivery, diagnostic imaging, and photothermal therapy. Silver NPs show improved antimicrobial activity. Silica NPs have been used in drug delivery and gene therapy. Biomolecular inorganic nanohybrids and nanostructured biomaterials have been exploited for targeted imaging and therapy, drug and gene delivery, and regenerative medicine. Dendrimers find use as drug or gene carriers, contrast agents, and sensors for different metal ions [209]. Inorganic nanomedicine refers to the use of inorganic or hybrid nanomaterials and nanosized objects to achieve innovative medical breakthroughs for drug and gene discovery and delivery, discovery of biomarkers, and molecular diagnostics.

Potential uses for fluorescent quantum dots include cell labeling, biosensing, *in vivo* imaging, bimodal magnetic-luminescent imaging, and diagnostics. Biocompatible quantum dot conjugates have been used successfully for sentinel lymph node mapping, tumor targeting, tumor angiogenesis imaging, and metastatic cell tracking. Magnetic nanowires applications include biosensing and construction of nucleic acids sensors. Magnetic cell therapy is used for the repair of blood vessels. Magnetic nanoparticles (MNPs) are important for magnetic resonance imaging, drug delivery, cell labeling, and tracking. Superparamagnetic iron oxide nanoparticles are used for hyperthermic treatment of tumors. Multifunctional MNPs applications include drug and gene delivery, medical imaging, and targeted drug delivery. MNPs could have a vital role in developing techniques to simultaneously diagnose, monitor, and treat a wide range of common diseases and injuries [210]. Inorganic nanoparticles, such as carbon nanotubes, quantum dots and gold nanoshells, have potential for biomedical applications due to their unique optical and physical properties. In addition, inorganic porous nanomaterials are fundamentally advantageous for developing multifunctional nanomaterials, due to their distinctive inner and outer surfaces in nanomedicine, especially for imaging/diagnosis and photothermal therapy [211]. Inorganic hollow nanoparticles and nanotubes are of great interest in nanomedicine because of the generic transporting ability of porous material and a wide range of functionality that arises from their unique optical, electrical, and physical properties especially for drug/gene delivery [212]. Target-specific drug/gene delivery and early diagnosis in cancer treatment are one of the priority research areas. The diagnosis and treatment of cancer or tumor at the cellular level will now be greatly enhanced using organic and inorganic nanoparticles that interface with biological systems in the fields of biology and medicine. Nanoparticles have the potential as novel intravascular or cellular probes for both diagnostic (imaging) and therapeutic (drug/gene delivery), which is expected to generate innovations and play a critical role in medicine [213].

7.14 Conclusion

Nanomedicine is a large industry that uses nanoparticles that act as biological mimetics (functionalized carbon nanotubes), nanomachines (DNA parts and DNA scaffolds), nanofibers and polymeric self-assembly and nanofibers of peptides, molecular switches, nanoporous membranes, and nanoscale micro-fabrication-based devices, sensors, laboratory diagnostics, etc. The nanoparticle applications in medicine are geared towards drug delivery, catalytic and photo-stability, multiplexing capabilities, and as nano-probes to the tumor that make them either release the drug load or heating them in order to destroy the surrounding tissue. Research into the rational delivery and targeting of pharmaceutical, therapeutic, and diagnostic agents via intravenous and interstitial routes of administration with nanosized particles is the main achievement of

nanomedicine. This chapter critically evaluates key aspects of nanoparticulate design and engineering, as well as recent breakthroughs and advances in cellular and intracellular targeting. It also discusses the advantage of its nanometer-scale size range, biological behavior, and safety profile.

Acknowledgement

R.P.S., A.T., J.W.C. and A.C.P. contributed equally to this work which was supported by Nano/Bioscience & Technology Program (M10536090001-05N3609-00110) of the Ministry of Education, Science and Technology (MEST), and Nano-Mission (SR/NM/NS-87/2008), Department of Science and Technology, Government of India.

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Gold Nanoparticle-based Electrochemical Biosensors for Medical Applications

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Abstract

Recent developments concerning the attractive properties of nanoparticles have increased interest about their applicability in biosensing areas resulting in great progress in the development of nanomaterial-based biosensors. The use of metal nanoparticle superstructures for the organization of electrochemical sensing devices is an extremely promising prospect. In electroanalytical applications, metal nanoparticles provide some important functions including the roughening of the conductive sensing interface and some catalytic properties that result in the amplification of the electrochemical signal. In addition, the conductivity properties of nanoparticles at nanoscale dimensions allow the electrical contact of redox-centers in proteins with electrode surfaces.

Gold nanoparticle (AuNP) is a kind of metal nanoparticle that can help proteins retain their biological activity upon adsorption. Because of its large surface area and good electronic properties, AuNP has been extensively used in the construction of electrochemical sensors and biosensors. AuNP can be defined as good biocompatible material since modification of electrode surfaces with this metal nanoparticle provides a suitable microenvironment similar to that of redox protein with more freedom in orientation. Moreover, AuNP is claimed to reduce the insulating effect of the protein shell by providing direct electron transfer through the conducting tunnels of gold nanocrystals, causing the penetration of nanomeric edges of gold particles. In this way, the distance between the electrode and biomolecular redox sites for electron transfer is decreased.

On the other hand, the activities of the immobilized antibodies can be largely influenced by the surface properties of the transducer in clinical immunoassays. It was demonstrated that use of gold colloids favored a particle-enhanced immobilization of antibodies due to the unique physical and chemical features of nanoparticles. The aim of this chapter is to give detailed information about AuNP-based electrochemical biosensors and their usage in medical application [1–10].

Keywords: Gold nanoparticles, electrochemical biosensors, medical applications

8.1 Introduction

It is very important to monitor health status, disease onset, and progression and treatment outcome in health care research. On the other hand, early disease diagnosis is crucial for patient survival, therefore sensitive and specific methods are required for that. In recent years, the demand has grown in the field of medical diagnostics for simple and disposable devices that are practical, economical, robust and suitable for mass production. Biosensor technologies that are combined with nanotechnology, chemistry and medical science have potential to fulfill these demands [11].

Moreover, some clinical analyses are carried out in hospitals or the patient's home rather than in a clinical chemistry laboratory. Considering this situation, biosensors become more advantageous over methods like spectroscopy and chromatography [12].

8.2 Electrochemical Biosensors

A biosensor can be generally defined as a device that consists of a biological recognition system and a transducer for a selective bioanalysis, which means signal processing to deduce and quantify a particular analyte [13, 14]. If this transducer is an electrode and produces an electrochemical signal such as potential and current change, this system is defined as an electrochemical biosensor. At first electrode-based biosensors measured the signal that is produced with an enzyme attached onto the electrode surface. However especially for clinical analysis, microorganisms, biological tissues, antigens or antibodies have also been used as molecular recognition elements [14].

8.2.1 Gold Nanoparticles

Gold has been a very important object as an ancient medicine and ornament [15–17]. In the old days, medieval artists used "red gold" which was known to be "finely-divided" gold, dispersed in plain glass to make stained glass [15–18]. The differences in color are because of nanoscale particles of gold that has dimensions of ~100 nm or less [19]. As a kind of metallic nanoparticle, AuNP has been extensively used in electrochemical biosensors due to its important properties. It is claimed that AuNP acts as a conducting tunnel, penetrates the insulating shell of protein and provides direct electron transfer [3]. AuNP also provides a suitable microenvironment similar to that of redox protein with more freedom in orientation. In many oxido reductase enzyme-based biosensors the oxidation of hydrogen peroxide (H_2O_2) is followed. It has been shown that AuNPs exhibit good catalytic activity towards this analyte. This result has been attributed to Au-nps high surface area-to-volume ratio and its interface-dominated properties [20].

8.2.1.1 Preparation of AuNPs

Preparation procedure is very important in terms of nanoparticle behaviors. It has been demonstrated that catalytic effects of some nanoparticles have been shown to be closely connected to the support on which it is deposited [21]. Also, stabilizing agents which are controlled by the preparation conditions have directly affected the properties of these nanostructured particles. On the other hand, the size and the uniformity of the nanoparticles are affected by the type and the amount of reducing agent employed in synthesis [1].

AuNPs can be prepared electrochemically or via chemical reduction methods, which often leads to colloidal gold. Colloidal gold consists of octahedral units of gold homogeneously dispersed in a liquid phase [22–26]. The chemical preparation method usually involves simple reduction of metal salts by reducing agents in a controlled way. Some of the most well-known and frequently used methods to synthesize spherical gold nanoparticles include the reduction of gold chloride by citrate to produce 15 nm gold particles in boiling water. For smaller (~2 nm) gold nanoparticles, borohydride reduction was applied after an aqueous solution of gold ions is transferred to an organic phase that includes a phase transfer agent. The microemulsion method wherein gold salts are reduced in the aqueous core of inverse micelles, and the seeding method which utilizes gold seed particles (prepared by one of the other methods) to grow more gold in the presence of a weak reducing agent, are the other two methods used for this purpose [19]. For the examination of obtained structure, transmission electron microscopy (TEM) [24] and X-ray photoelectron spectroscopy [25] have been used (Figure 8.1).

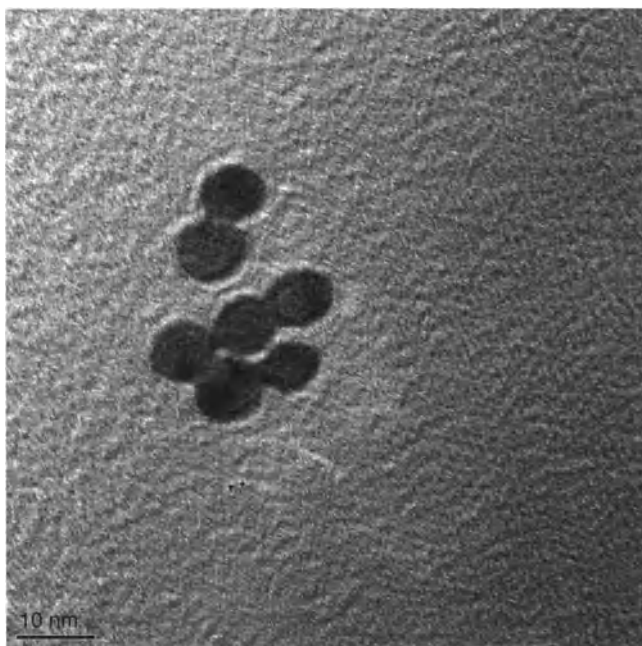


Figure 8.1 TEM image of AuNP at 200 kV.

8.2.1.2 *Au-np Modified Electrodes*

Solid electrodes like glassy carbon (GCE), crystalline gold and highly oriented pyrolytic graphite electrodes [25, 27] and composite electrodes including carbon paste electrode (CPE) and glassy carbon paste electrode (GCPE) [3, 22] have been modified with colloidal gold. Modification of CPE and GCPE [3, 22] with AuNP was achieved directly by mixing of colloidal gold into the paste at the production stage. For preventing the leakage of resulting electrode's structure, in some works, dialysis membrane has been used [3]. Besides this, proper additives like albumin [22] has been utilized for obtaining better dispersion of gold particles into composite structure. In some examples, instead of putting AuNP into composite structure, AuNP was immobilized onto electrode surface via electrodeposition [28, 29], cysteamine self-assembly monolayers (SAM)s [30], and by means of physical adsorption [31].

8.2.1.3 *AuNP-based Electrochemical Biosensors for Medical Applications*

Because of the important properties that were mentioned in the above sections, AuNPs have been widely used in electrochemical biosensors [32]. A literature survey has been done and summarized in Table 8.1.

On the other hand, the usage of this electrode as an electrochemical biosensor transducer for medical applications has increased recently. For clinical applications usually immunosensors were constructed. For example, in one study a flow-injection electrochemical immunoassay system based on a disposable immunosensor for the determination of interleukin-6 (IL-6) was proposed. IL-6 is a pleiotropic cytokine that has a critical role in the inflammatory response. It has been implicated in the pathogenesis of a number of inflammatory conditions, such as psoriasis, rheumatoid arthritis, cardiovascular disease, and inflammatory bowel disease [79–81]. The immunosensor was prepared by entrapping horseradish peroxidase (HRP)-labeled IL-6 antibody into AuNP-modified composite membrane at a screen-printed graphite electrode. The formed antigen–antibody complex partly inhibited the active center of HRP, and decreased the amount of immobilized HRP that participated in H_2O_2 reduction. After the optimization of experimental parameters, the linear range was investigated as 5–100 ng L^{-1} with a detection limit of 1.0 ng L^{-1} for IL-6 [82].

In another immunosensor study, an immunosensing assay was developed for the detection of α -fetoprotein (AFP) in human serum. AFP is an oncofetal glycoprotein with a molecular weight of approximately 70,000 Da, and it is an important tumor marker. The average concentration of AFP is approximately 25 ng ml^{-1} in healthy human serum but rises greatly in patients with liver cancer [83–86]. Thus, the detection of serum AFP level plays an important role in the diagnosis and management of original liver carcinoma. The immunosensor was prepared by immobilizing AFP antigen onto GCE modified by AuNP and carbon nanotubes (CNT) doped chitosan (AuNP/CNT/Ch) film. AuNP/CNT hybrids were produced by one-step synthesis based on the direct redox reaction. During the examination of the film, it was found that AuNP/CNT nano-hybrid acted as an electron promoter and accelerated the electron transfer.

Table 8.1 Gold nanoparticle-based electrochemical biosensors

Electrode	Analyte	Linear Range (M)	LOD (M)	Reference
Au-np-CS / Gold	DNA	$1.0 \times 10^{-9} - 5.0 \times 10^{-7}$	5.0×10^{-4}	33
Au-np-CS / Gold	DNA	$1.0 \times 10^{-11} - 8.0 \times 10^{-10}$	5×10^{-12}	34
Au -np CS / HRP / ITO	H ₂ O ₂	$1.0 \times 10^{-5} - 5 \times 10^{-4}$	5.0×10^{-6}	35
Au -np associated with a cysteamine monolayer HRP / gold	H ₂ O ₂	$1.4 \times 10^{-6} - 2.8 \times 10^{-3}$	5.8×10^{-6}	36
Covalent linking Au-np SH monolayer HRP / ITO	H ₂ O ₂	$5 \times 10^{-6} - 1.5 \times 10^{-3}$	1.0×10^{-6}	37
Au -np / HRP / CPE	H ₂ O ₂	$4.8 \times 10^{-7} - 5 \times 10^{-5}$	2.1×10^{-6}	38
Covalent incorp. of CNTs and Au-np onto poly(thionine)film HRP / GCE	H ₂ O ₂	$5.0 \times 10^{-6} - 7.0 \times 10^{-3}$	3.0×10^{-6}	39
Au -np / HRP / GCE	H ₂ O ₂	$2.6 \times 10^{-6} - 8.8 \times 10^{-3}$	6.4×10^{-7}	40
Au -np SAM / HRP / gold	H ₂ O ₂	$8.0 \times 10^{-6} - 15 \times 10^{-3}$	2.4×10^{-6}	41
Au -np HRP / SPE	H ₂ O ₂	$1.0 \times 10^{-5} - 11.3 \times 10^{-3}$	6.5×10^{-7}	42
Au -np SAM / HRP / gold	H ₂ O ₂	$5.0 \times 10^{-6} - 1.0 \times 10^{-2}$	2×10^{-6}	43
Au -np / HRP / platinum	H ₂ O ₂	$5.0 \times 10^{-5} - 30.6 \times 10^{-3}$	2×10^{-5}	44
Au -np cyst monolayer / HRP / gold	H ₂ O ₂	$0.39 \times 10^{-6} - 3.3 \times 10^{-4}$	1.5×10^{-7}	45
Au -np cyst monolayer / HRP / gold	H ₂ O ₂	$1.5 \times 10^{-7} - 8.6 \times 10^{-3}$	7.0×10^{-8}	46
Au -np / HRP / ITO	H ₂ O ₂	$2.0 \times 10^{-5} - 8.0 \times 10^{-3}$	8.0×10^{-6}	47

Table 8.1 (Continued)

Electrode	Analyte	Linear Range (M)	LOD (M)	Reference
l-Cysteine-gold particle nanocomposite immobilized HRP/CPE	H ₂ O ₂	1.60×10^{-5} – 1.10×10^{-3}	5.50×10^{-6}	48
multilayer films of sulfonate-capped Au-np / thionine GOx/HRP / Gold	Glucose	6.0×10^{-6} – 1.1×10^{-3}	3.5×10^{-5}	49
Au-np SAM / ADH/ gold	Ethanol	1.0×10^{-5} – 1.3×10^{-2}	8.0×10^{-6}	50
Au-np SAM / LDH/ gold ADH/ gold	Lactate	-	0.1×10^{-6}	51
GOx on SAM Au-np / gold	Glucose	-	23×10^{-6}	52
Au-np adsorben on 3-mercaptopropyltrimethoxy-silane –SAM / Gold	DNA	1.0×10^{-8} – 1.0×10^{-6}	5.0×10^{-3}	53
Au-np-SAM / CPE	DNA	-	-	54
Au-npSAM / GOx / GCE /	Glucose	1×10^{-5} – 10^{-2}	7.0×10^{-6}	9
CS-Au-np-GOx / platinum	Glucose	5×10^{-4} – 32×10^{-3}	-	10
GOx/CGE Tyr / GCE	Phenol, glucose	1.0×10^{-6} – 2.0×10^{-5}	3×10^{-8} (Ph) 2×10^{-6} (Glu)	55
SNS-NH ₂ / Au np/GOx on graphite electrode	Glucose	2.0×10^{-6} – 5.0×10^{-3}	-	56
Au-np -GOx / Gold	Glucose	2.0×10^{-5} – 5.7×10^{-3}	8.2×10^{-6}	57
GOx/Au-np / CPE	Glucose	4×10^{-5} – 2.8×10^{-4}	1.0×10^{-5}	58
Au-np GOx / CNT-Teflon	Glucose	5.0×10^{-5} – 1.0×10^{-3}	17×10^{-6}	59
GOx/ Au-np and PANI / GCE	Glucose	1.0×10^{-6} – 8.0×10^{-4}	5.0×10^{-7}	60

Table 8.1 (Continued)

Electrode	Analyte	Linear Range (M)	LOD (M)	Reference
GOx Au-np / Gold	Glucose	5×10^{-6} - 2.4×10^{-3}	2.7×10^{-6}	61
Au-np -GOx / gold	Glucose	1.0×10^{-5} - 1.3×10^{-2}	-	62
Au-np -GOx / ITO	Glucose	Up to 1.5×10^{-3}	17×10^{-6}	63
Multilayer Au-NP / MWNT / GOx membrane / gold	Glucose	Up to 9.9×10^{-3}	128×10^{-6}	64
Multilayer film of (PDDA / GNPs)n / PDDA GOx / gold	Glucose	-	-	65
electrodeposited chitosan-Au-np hybrid film GOx / GCE	Glucose	1.0×10^{-6} - 1.6×10^{-3}	6.9×10^{-7}	66
electrodeposited Au-np / Gold	DNA	1.0×10^{-9} - 1.0×10^{-11}	5×10^{-9}	67
electrodeposited Au-np / Gold	DNA	-	-	68
Au-np hybridized / GCE	DNA	1×10^{-10} - 5×10^{-9}	5×10^{-11}	69
electrodeposited Au-np / GCE	DNA	5×10^{-7} - 8×10^{-5}	5×10^{-9}	70
electrodeposition Au-np on PDC / GCE	DNA	1.0×10^{-10} - 1.0×10^{-5}	2.4×10^{-11}	71
Au-np / GCE	DNA	1.52×10^{-10} - 4.05×10^{-8}	10^{-10}	72
Au-np hybridized / SPE	DNA	-	1.5×10^{-9}	73
oligo-nucleotides conjugated Au-np / SPE	DNA	-	2.17×10^{-12}	74

Table 8.1 (Continued)

Electrode	Analyte	Linear Range (M)	LOD (M)	Reference
Au-np / ITO	DNA	4×10^{-7} – 1×10^{-6}	2.0×10^{-7}	75
Au-np label / ITO	DNA	5×10^{-12} – 10×10^{-9}	2.0×10^{-6}	76
electrodeposited Au-np XOD / CPE	Hypoxanthine	5.0×10^{-7} – 10×10^{-6}	2.2×10^{-7}	29
XOD/GCPE–Au-np	Xanthine	5.0×10^{-7} – 1.0×10^{-5}	-	3
Lectrodeposited Au-np / AChE / Gold	Carbofuran	1.0×10^{-8} – 1.35×10^{-7}	33×10^{-9}	77
Au-np composite coated / AChE / / GCE	Methhyl	-	-	78
Au-np / Tyr / CPE	Phenol,	4×10^{-6} – 48×10^{-6}	6.1×10^{-9}	4

AChE: Acetylcholinesterase, **AuE:** Gold disk electrode, **Au-np:** Gold nanoparticle, **AuColl:** Colloidal gold, **G-AuNP:** Graphene-gold nanocomposite, **CPE:** Carbon paste electrode, **CS:** Chitosan, **Cyst:** Cysteamine, **CNT:** Carbon nanotubes, **GCE:** Glassy carbon electrode, **GCPE:** Glassy carbon paste electrode, **Glu:** Glutaraldehyde, **GOx:** Glucose oxidase, **GNP:** Gold nanoparticles, **GE:** Gold electrode, **Glu:** Glucose, **HRP:** Horseradish peroxidase enzyme, **ITO:** Indium tin oxide, **MWNT:** Multiwalled carbon nanotube, **MPA:** 3-mercaptopropionic acid, **PAMAM:** Polyamidoamine, **PANI:** Polyaniline nanofibers, **PDDA:** Poly(diallyldimethylammonium chloride), **PDC:** 2,6-Pyridinedicarboxylic acid, **PEM:** Polyelectrolyte multilayer, **Ph:** Phenol, **PyOx:** Pyranose oxidase, **RSH:** 3-mercaptopropionic acid, **SAM:** Self-assembly monolayer, **SCE:** Saturated calomel electrode, **SPE:** Screen-printed electrode, **SNS-NH2:** 4-(2,5-di(thiophen-2-yl)-1H-pyrrolyl)benzenamine, **XOD:** Xanthine oxidase, **Tyr:** Tyrosinase.

Sample AFP, immobilized AFP, and alkaline phosphatase (ALP)-labeled antibody were incubated together for the determination based on a competitive immunoassay format. The amperometric response of 1-naphthyl phosphate was followed via the bound ALP label on the modified GCE, after the immunoassay reaction. Under the optimal experimental conditions, linear range of developed immunosensor was found in the range of 1 to 55 ng ml⁻¹ with a detection limit of 0.6 ng ml⁻¹ [87].

Another AFP biosensor is composed of functionalized AuNPs (SV-AuNP) which were synthesized by covalently capping the surface of AuNP with 1,1'-bis-(2-mercapto)-4,4'-bipyridinium dibromide, a kind of sulfhydryl viologen (SV). First SV-AuNP was coated onto a GCE surface, then anti-AFP was adsorbed by this prepared electrode and AFP detection was conducted. As a result, a linear range between 1.25 and 200 ng/mL with a correlation coefficient of 0.9983 was obtained. Moreover, the detection limit at a signal to noise ratio of 3 was calculated as 0.23 ng/mL under optimal conditions [88].

The other AFP immunosensor was prepared by attaching AuNP-enclosed titania nanoparticle (AuTi)-labeled secondary antibody onto a gold-silver-graphene hybrid nanosheet (AuAgGP)-functionalized GCE. It was stated that the presence of AuAgGP nanosheets enhanced the immobilized amount of biomolecules and also improved the electrochemical properties of the immunosensor. Under optimal conditions, the sensitivity and dynamic range of the immunosensor were evaluated by using the labeled HRP on the AuTi as trace and H₂O₂ as enzyme substrate. As a result, a wide dynamic range of 0.001–200 ngmL⁻¹ with a low detection limit of 0.5 pgmL⁻¹ was obtained. Developed methodology was evaluated for 8 positive serum specimens obtained from hepatocarcinoma patients and 19 negative sera. The validation of the developed system was conducted with the commercially available Roche 2010 Electrochemiluminescent Automatic Analyzer. As it was reported, no significant differences at the 95% confidence level were encountered between the two methods [89].

A microfluidic immunosensor was developed for the detection of IgG antibodies specific to *Echinococcus granulosus* in human serum samples, which represents an alternative tool that can be used for the immunodiagnosis of hydatidosis in an automated way. Hydatidosis is a common disease of humans and animals, resulting from infection with the larval stage or metacestode of *Echinococcus granulosus*. The developed device consists of a plexiglas system with a central channel and a gold electrode. For immobilization of the *E. granulosus* antigen, the gold electrode was modified with AuNP. The immunoassay reaction was based on reaction of immobilized antigen with IgG anti-*E. granulosus* antibodies in samples where quantification was made by HRP enzyme-labeled secondary antibodies specific to human IgG using catechol (Q) as enzymatic mediator. HRP in the presence of H₂O₂, catalyzes the oxidation of Q to o-benzoquinone. Following this, the electrochemical reduction back to Q was detected on the gold electrode at -0.15 V. The obtained current was proportional to the activity of the enzyme and to the concentration of antibodies of interest. The detection limit for electrochemical detection was 0.091 ng ml⁻¹, and the within- and between-assay coefficients of variation were below 6.7% [90].

The other HRP AuNP included immunosensor was constructed by using AuNP/CNT hybrids platform with HRP-functionalized Au-np label for the sensitive detection of human IgG. It was claimed that the AuNP/CNT nano-hybrids covered on the GCE provides an effective antibody immobilization matrix and allowed the immobilized biomolecules to retain high stability and bioactivity. Besides a linear response range between 0.125 and 80 ng/mL, a detection limit of 40 pg/mL was obtained with this system [91].

Another immunosensor was constructed by modifying gold electrode with a passivating organic layer 4-thiophenol, where AuNPs were attached on it. Then a biotin derivative as a model epitope was immobilized onto nanoparticles in order to detect anti-biotin IgG. The measurement of the developed system was based on analyzation of $\text{Fe}(\text{CN})_6^{4-/3-}$ redox couple as a probe by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The impedance change, due to the specific immuno-interaction at the immunosensor surface was utilized to detect anti-biotin IgG. As a result, linear range of 5–500 ng mL^{-1} , with a detection limit of 5 ng mL^{-1} was obtained [92].

Human chorionic gonadotrophin (hCG) is an important diagnostic marker of pregnancy and many other diseases. It is a secretion of the placenta during pregnancy and gestational trophoblastic diseases, and increases as a consequence of abnormal placental invasion and placental immaturity. A novel amperometric immunosensor for determination of hCG was constructed by immobilizing hCG antibody on the Pt–Au alloy nanotube array. In this study the “usual” AuNP has not been used. Instead Pt–Au alloy nanotube array was produced by electrodepositing these nanometals on a nanopore polycarbonate membrane at -0.35 V. The vertically aligned Pt–Au alloy nanotube was accepted as a nanoelectrode. The measurement principle was based on following the electrocatalytic reduction of H_2O_2 by Pt–Au alloys after the hCG antigen was bounded on the surface of electrode through immunoreactions. Under the optimal condition, the current response of the immunosensor is in linear relationship with concentration of hCG ranging from 25 to 400 mLU/mL with a detection limit of 12 mLU/mL. The method was applied for determination of hCG in serum samples and satisfactory results were obtained [93].

GCE electrode was modified by nanoporous gold film and label-free electrochemical immunosensor for ultrasensitive detection of cancer biomarker prostate specific antigen (PSA) was obtained. Then the adsorption of anti-PSA antibody on the electrode surface was made and amperometric measurements of $\text{K}_3\text{Fe}(\text{CN})_6$ was conducted. After the optimization of experimental conditions, the analytical characteristics were done. The linear range was found to be between 0.05–26 ng/mL PSA concentration with a limit of detection of 3 pg/mL. This system was also applied for the PSA detection in human serum samples and very promising results were obtained [94].

A reagentless amperometric immunosensor was constructed based on antibody-embedded AuNP and SiO_2 /Thionine nanocomposition self-assembled layers. For electrode modification, L-cysteine was first modified on a bare gold electrode by potential cycling to prepare a uniform orientation SAM with functional groups of $-\text{NH}_2$. Then two-double Au-np layers were constructed by

introducing SiO_2 /Thionine nanocomposition as linker. Lastly, carcinoembryonic antibody, as immunoreagent model, was immobilized onto the Au-np, SiO_2 /Thionine nanocomposition and AuNP self-assembled sandwiched layers. EIS and CV were used for the characterization and electrochemical properties of the composite film. After the optimization of working conditions, a linear range between 1.00 and 100.00 ng/mL with a detection limit of 0.34 ng/mL for carcinoembryonic antigen were calculated [95].

Besides immunosensors, AuNP included electrochemical DNA sensors were also used in medical applications. For example AuNP-based biosensors have been constructed in recent years to detect *Mycobacterium tuberculosis* (MTB) for simple and rapid diagnosis in realtime samples. Currently, about 54 million people are infected with MTB. Each year approximately 8 million new cases occur in which nearly 2.4 million people die. Due to the vulnerability and rapid spread of MTB, highly sensitive detection methods are required for clinical diagnostics. MTB infections of diagnostic investigations are at risk due to the difficulty in detecting the tiny population of *Mycobacteria* or the immunological markers associated with the infections they cause. On the other hand, AuNP-conjugated nucleotide probes using colorimetric assays showed a rapid and sensitive detection of MTB with a limit about 0.75 lg of total DNA using PCR [96], and similarly unamplified DNA of pathogenic MTB detection was reported with limit determined as 18.75 ng/10 μL [97]. AuNP-based immunoassay executed to detect MTB in a clinical sample shows enhanced sensitivity compared with traditional analytical methods and visually detected up to 50 ng of genomic DNA [98]. As a result an electrochemical DNA assay was developed for *Mycobacterium* sp. genomic DNA detection in a clinical specimen using a signal amplifier as dual-labeled AuNPs. The DNA electrochemical biosensors were fabricated using a sandwich detection strategy involving two kinds of DNA probes specific to *Mycobacterium* sp. genomic DNA. The probes of enzyme ALP and the detector probe both conjugated on the AuNPs and subsequently hybridized with target DNA immobilized in a SAM/ITO electrode. The characterization of the developed system was made by CV, EIS, and differential pulse voltammetry using the electroactive species para-nitrophenol generated by ALP through hydrolysis of para-nitrophenol phosphate. A detection limit of 1.25 ng/ml genomic DNA was determined under optimized conditions. The dual-labeled AuNP-facilitated electrochemical sensor was also evaluated by clinical sputum samples, showing a higher sensitivity and specificity and the outcome was in agreement with the PCR analysis [99].

The other system was developed for the electrochemical detection of the mutated *BRAF* gene associated with papillary thyroid carcinomas (PTC). PTC is the most common carcinoma of the thyroid gland and the most frequent type of endocrine cancers. Over the past six decades, the incidence of PTC has increased in many countries [100–102]. PTC is sporadic in around 95% of cases and familial in the remaining 5%. PTC patients have a very good prognosis provided that the carcinoma is detected and treated at an early stage. Given prompt intervention, the mortality rate is typically less than 10%, even 10 years after surgery [103]. For this purpose, a biotinylated 30-nucleotides

probe DNA was immobilized in a streptavidin-modified 96-well microtiter plate and the free active sites of the streptavidin were blocked using biotinylated BSA. The biotinylated target DNA was then added and allowed to hybridize with the immobilized probe DNA for 30 min. Subsequently, streptavidin-labeled AuNPs were added, and a nanoparticle enlargement process was performed using gold ion solution and formaldehyde reductant. AuNPs were then dissolved in bromide and DNA hybridization detection process was performed using a square wave stripping voltammetry technique. The detection limit was estimated to be 0.35 aM, based on a signal-to-noise ratio of 3:1. This value was compared with regular protocol where no gold amplification method was applied. As a result, it was found that approximately three orders of magnitude lower results were obtained with gold amplification method included assay. Finally, the proposed approach was applied in differentiating between the mutant and wildtype *BRAF* sequences that are present in genuine 224-nucleotides DNA and successful results were obtained [104].

Besides DNA biosensors, AuNP-modified aptasensors were also used for the same purpose. A practical EIS aptasensor based on an antithrombin aptamer as a molecular recognition element was prepared [105]. Thrombin is the last enzyme protease involved in the coagulation cascade, and it converts fibrinogen to insoluble fibrin that forms the fibrin gel both in physiological conditions and in a pathological thrombus [106]. Thrombin also has hormone-like properties, and is involved in thrombosis and platelet activation. Therefore, thrombin plays a central role in a number of cardiovascular diseases [107] and it is thought to regulate many processes in inflammation and tissue repair at the blood vessel wall [105]. For the construction of aptasensor, AuNPs were self-assembled on the surface of a gold electrode by using 1,6-Hexanedithiol as a medium. To quantify the amount of thrombin, changes in the interfacial electron transfer resistance (R_{et}) of the aptasensor were monitored using the redox couple of an $[\text{Fe}(\text{CN})_6]^{3-/4-}$ -probe. The plot of $(R_{et} - R_{et0}) / R_{et0}$ against the logarithm of thrombin concentration is linear with over the range from 0.1 nM to 30 nM with a detection limit of 0.013 nM. The results were compared with that of bare electrode with no AuNPs on it. It was observed that the total amount of aptamer probes immobilized on the gold nanoparticle surface is six-fold higher than that on the bare electrode. The interference studies were also conducted and as a result it was found that prepared aptasensor also showed good selectivity for thrombin without being affected by the presence of other proteins [105].

8.3 Conclusion

It is important to have precise, practical, economical and robust devices for a disease diagnosis. Biosensors can cover these requirements. Introducing AuNP in these sensors increases sensitivity and provides better environment for biological component immobilization. Because of these advantages, development of various configurations of these systems are still in progress.

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Impedimetric DNA Sensing Employing Nanomaterials

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Abstract

This chapter describes variants of DNA sensors (genosensors) that employ electrochemical impedance signal for detecting a target DNA. In this way, the clinical diagnostic-related sought gene or gene variant, can be very simply detected, with an electrically addressable device, and potentially, without the use of any label. Existing variants for measuring and the different formats for the assay are presented. To improve the performance of these devices, current nanobiotechnology utilizes nanocomponents, either at the transducer level or integrated in the procedure itself, to improve the detection or to amplify the signal. Carbon nanotubes and nanowires, graphene or gold nanoparticles can be used to produce or to modify transducers, fostering their electrical characteristics, or helping in the immobilization of the recognition element. Metal nanoparticles or even quantum dots may be used to improve signal to noise ratio. The chapter ends by summarizing existing applications related to clinical diagnostic, and discussing the latest trends.

Keywords: DNA biosensor, genosensor, carbon nanotube, gold nanoparticles, quantum dots, electrochemical impedance spectroscopy

9.1 Introduction

The detection of DNA sequences has gained important attention in recent years, as their quick identification is important in a number of different fields. The determination of nucleic acid sequences from humans, animals, bacteria and viruses is a departure point to solve different kinds of problems, such as the investigation about infection or food and water contamination caused by microorganisms, the detection of fraud in appreciated food variants, the identification of genetic disorders and genetic diseases, the studies on tissue matching and breeding origin, and the application to forensic issues. In medical application, DNA analysis may be of great help for a better diagnosis, or

prevention and treatment of many human diseases. Apart from this, in relation to the health field, pharmacological studies on modification of expression of certain genes by certain active principles are one of the promising alternatives to minimize animal experimentation. In diagnostic medicine two important applications can be derived: (1) identifying infectious microorganisms, such as viruses or bacteria responsible for disease, or (2) forecasting or confirming a genetically inherited disease, and thus initiating prognosis treatment, even prior to onset of the disease. With these assumptions, DNA biosensor technologies are rapidly developing as an alternative to the classical gene assays, due to certain advantages they present such as low cost, rapid analysis, simple equipment, possibility of miniaturization and analysis *in situ* [1, 2]. The biosensor technologies are those promising a simpler integration of high technology diagnostic tools for a quicker response in the point of care.

Electrochemical impedance spectroscopy (EIS) is a powerful technique to characterize the electrical properties of a system, being sensitive to surface phenomena and changes of bulk properties. Due to its effectiveness to directly probe the interfacial properties (capacitance, electron transfer resistance) of modified electrodes, EIS is becoming an attractive electrochemical tool for numerous applications such as immuno and genosensing, enzyme activity determination, studies of corrosion and characterization of surface phenomena [3]. Thanks to these properties, this electrochemical technique is rapidly being adopted for monitoring affinity interaction of biomolecules. In more recent years, EIS has been widely used for the detection of DNA hybridization occurring at the surface of a sensing electrode.

On the other hand, the use of nanoscale materials for electrochemical biosensing has seen an explosive growth during the last decade [4]. The huge interest in nanomaterials is due to the ability to tailor their size and structure to the biosensing function and to the exceptional properties they present compared to the bulk material. The exceptional electrochemical properties derived from nanobiomaterials offer excellent prospects for transducing biorecognition events, or exhibiting novel operation modes. The use of nanobiomaterials in impedimetric genosensing is focused either on the development of new platforms or on the conjugation of DNA sequences with the former in order to enhance the impedimetric signal, thus improving overall sensitivity. This chapter describes the recent advances in genosensors employing EIS as detection technique and the improvements obtained by the use of different nanobiomaterials in their construction or operation, such as carbon nanotubes or nanofibers, graphene, gold and other metal nanoparticles and quantum dots. The main goal is to discuss issues and performance of nanomaterial-based DNA sensors that may be used in medical application as tools for disease diagnostic and control.

9.1.1 DNA Biosensors (Genosensors)

A biosensor can be defined as an analytical device which combines a biorecognition element that provides selectivity with a transducer conferring sensitivity and converting the biorecognition event into a measurable electronic signal [5].

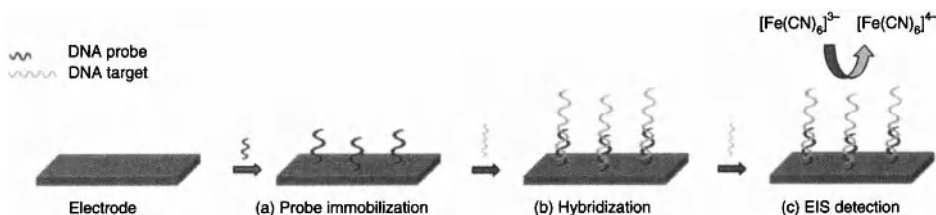


Figure 9.1 Scheme for a DNA biosensing experiment, employing EIS detection.

Genosensors are biosensors in which the biorecognition element consists of a ssDNA sequence (DNA probe) [6].

The detection of DNA sequences by using DNA biosensors is based on the hybridization phenomenon, which is the union of two complementary single-stranded DNA (ssDNA) sequences into one double-stranded DNA (dsDNA). A DNA probe, complementary to the target gene sequence is used as the biorecognition element for the biosensing platform. The formation of a double-stranded hybrid between the probe and the target occurs with high efficiency and extremely high specificity in the presence of a mixture of many different, noncomplementary nucleic acids. The general scheme for the analysis of DNA using an impedimetric biosensor, depicted in Figure 9.1 consists of three main steps: a) the ssDNA probe immobilization onto the electrode surface; b) the hybridization with a complementary single strand sequence (called DNA target); c) the detection.

The most common electrode surfaces used as biosensing platforms for DNA detection are based on carbon materials such as glassy carbon [7, 8], carbon paste [9, 10], graphite [11, 12], graphite composites [13], carbon nanotubes [14, 15] and graphene [16, 17]; and metals such as gold [18, 19], mercury [20, 21], silver [22] and platinum [23]. On these different platforms DNA probes have been immobilized following a variety of protocols like physical adsorption [24, 25], affinity interactions [26, 27], chemical binding [28–30] or entrapment into a polymeric matrix [31, 32]. While adsorption is the simplest method because it does not require additional reagents or specific DNA modifications, DNA probes immobilized by covalent bond onto the electrode surface can benefit by higher structural flexibility and chemical stability.

The hybridization step is then achieved if incubating the probe-modified electrode surface in a solution containing the DNA target, which can be either synthetic or a sequence coming from a real sample after the required pretreatment (extraction, purification and amplification-labeling by polymerase chain reaction [33]).

Consequently the biosensing event is converted by the transducer into a measurable signal. Depending on the nature of this signal, the biosensor can be classified into three main groups: optical, piezoelectric or electrochemical.

Optical genosensors generally require the use of DNA sequences which are modified with different labels such as organic dyes, enzymes, gold nanoparticles, and quantum dots for the detection [34]. One variant is the use of Surface

Plasmon Resonance (SPR) transduction [35], which in this case, does not require labeling. Piezoelectric genosensors correlate the hybridization event to the change in the oscillation frequency of the transducer, allowing a label-free detection [36].

Electrochemical genosensors exploit electrochemical transduction for the detection of specific DNA sequences. The following paragraph describes in detail the different electrochemical techniques and protocols employed in the detection of DNA sequences.

9.1.2 Electrochemical Genosensors

Electrochemical genosensors have been the most widely used class of DNA biosensors, due to their simplicity, small dimensions, low cost, high sensitivity, fast response and compatibility with microfabrication technology [37–39]. A variety of schemes have been employed so far for the electrochemical detection of DNA sequences.

A very well known approach for the detection of DNA hybridization is represented by the use of different electrochemical-active labels during the biosensing event. These labels can be used as a tag the DNA sequences are modified with (such as enzymes, electroactive molecules or metal nanoparticles [39]) or as dsDNA intercalators (such as cationic metal complexes [10], anticancer drugs [7], or organic dyes [40]) which can form complexes with specific fragments of DNA sequences. The magnitude of their electrochemical signal, e.g., current, as given by these labels is then correlated to DNA hybridization yield.

A different approach is represented by label-free protocols, in which the direct electrochemical oxidation/reduction peaks of DNA nitrogenous bases (namely, guanine) are measured. In this scheme, the difference in the signal intensity before and after DNA hybridization reveals the presence of the target sequence [8, 41–44]. Essentially, label-free approaches are SPR or piezoelectric genosensors. Another electrochemical technique, very rapidly developing and employed in recent years for biosensing events is electrochemical impedance spectroscopy (EIS). This technique also allows the label-free detection of DNA hybridization. The following describes in detail EIS fundamentals and applications for genosensing.

9.2 Electrochemical Impedance Spectroscopy for Genosensing

The history of impedance spectroscopy began in 1886, when the mathematician and physicist Oliver Heaviside introduced impedance into electrical engineering [45]. His work was soon extended to include vector diagrams, representations in the complex plane and use of equivalent circuits to represent the impedimetric response [46].

Impedance spectroscopy (IS) is a general term that involves the small-signal measurement of the linear electrical response of the material of interest and the consequent analysis of the response to yield useful information about the physicochemical properties of the system. Electrochemical impedance spectroscopy (EIS) is one main category of IS which has been widely used in many fields of electrochemistry, e.g., electrode kinetics, double-layer studies, batteries, corrosion, solid-state electrochemistry, and bioelectrochemistry [47].

EIS can be defined as a characterization technique which provides electric information in the frequency domain [48, 49]. With this technique, a process occurring in an electrochemical cell can be modeled using a combination of electrical circuits that give the same current response provided by the electrochemical system. By the use of equivalent circuits [50] the experimental spectra can be fitted with the theoretical curve corresponding to the selected circuit model, thus obtaining the values of the electrical parameter (i.e., resistance, capacitance, etc.) which are directly correlated to specific electrochemical phenomena occurring in the system under study. Due to its high ability for the characterization of electrode-electrolyte interfaces [51] and its high sensitivity for probing the interfacial properties of a modified electrode surface, EIS have been widely applied for biosensing purpose during the last years [52–54].

In order to gain more insight into EIS a brief description of the basics of impedance spectroscopy is given in the following paragraph.

9.2.1 Theoretical Background

Electrochemical impedance is generally measured by applying a small-amplitude, frequency dependant AC signal, to the electrochemical cell and measuring the obtained response. Assuming that an AC potential E_t is applied to the system under study:

$$E_t = E_0 \cdot \sin(\omega \cdot t) \quad (9.1)$$

(E_t : time dependant potential; E_0 : signal amplitude, $\omega = 2\pi f$: radial frequency). The current response provided by the electrochemical cell will be:

$$I_t = I_0 \cdot \sin(\omega \cdot t + \phi) \quad (9.2)$$

(I_t : time dependant potential; I_0 : signal amplitude, $\omega = 2\pi f$: radial frequency; ϕ : phase angle between E_t and I_t).

The magnitude of the obtained current as well as the presence of a phase shift are dependent on the impedance of the system (see Figure 9.2). In analogy to the Ohm's law for an ideal resistor, the impedance through the cell can be expressed in the following equation:

$$Z = \frac{E_t}{I_t} = \frac{E_0 \cdot \sin(\omega \cdot t)}{I_0 \cdot \sin(\omega \cdot t + \phi)} = Z_0 \cdot \frac{\sin(\omega \cdot t)}{\sin(\omega \cdot t + \phi)} \quad (9.3)$$

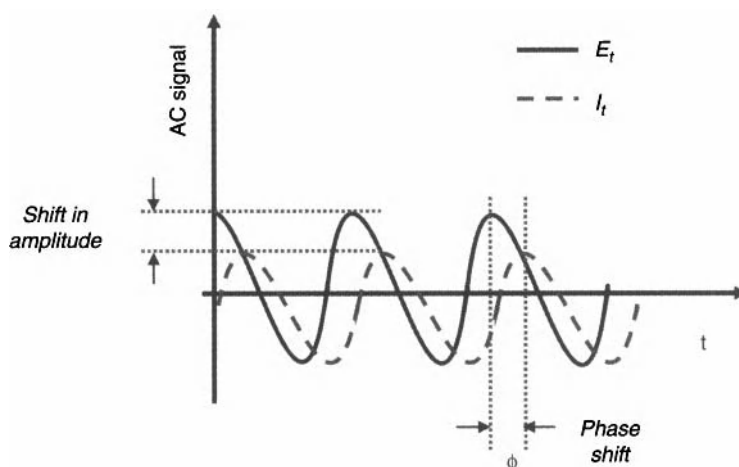


Figure 9.2 AC excitation signal applied and sinusoidal current response in the system under study.

The total impedance of the system Z is therefore defined by a magnitude Z_0 and a phase shift ϕ .

By using Euler's relationship the impedance can be written as complex function:

$$Z = Z_r + jZ_i \quad (9.4)$$

(Z_i , imaginary component of Z ; Z_r , real component of Z ; $j = \sqrt{-1}$ imaginary unit).

If the real part Z_r is plotted on the x-axis and the imaginary part Z_i on the y-axis of a chart, the well-known "Nyquist plot" is obtained. In this plot, the impedance function is represented as a vector with magnitude Z_0 , and a direction given by the phase angle ϕ . Another popular presentation of impedance results is given by the Bode plot, in which both the absolute value of the impedance and the phase shift are plotted in the y-axis versus the frequency which is represented in the x-axis. Albeit having the disadvantage of not explicitly showing the frequency information, the Nyquist plot continues to be the most used representation for impedance data, especially in electrochemical studies.

Figure 9.3 shows a typical example of Nyquist plot of a generic electrochemical reaction taking place at an electrode. The corner insert schematizes the electrical equivalent circuit representing its behavior, called Randles' equivalent circuit [55]. Each point plotted in the spectrum corresponds to a different recorded frequency. Low frequency data are on the right side of the plot and high frequencies are on the left. The theoretical electrical circuit (Randles' equivalent circuit) comprises a resistance R_1 in series with the R_2C element which also includes a Warburg impedance term (W). This circuit has been extensively employed in electrochemistry for the fitting of data coming from different experiments. As stated before, each element of the circuit can

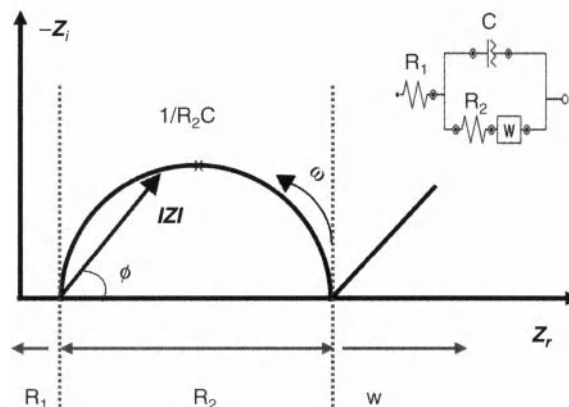


Figure 9.3 Nyquist plot in the complex plane and Randles equivalent circuit (top right) used for the fitting.

be associated to a specific electrochemical phenomenon occurring in the cell under study. The parameter R_1 is generally associated to the resistance of the electrolytic solution, R_2 corresponds in most cases to the resistance (R_{ct}) to the charge transfer between the electrolytic solution and the electrode surface and C is associated to the capacitance of the double layer (due to the interface between the electrode and the electrolytic solution).

Since the electrochemical system frequently does not conform to the assumptions made in the models, especially those associated with electrode uniformity, a constant phase element (CPE) is often introduced in the electrical circuit to replace the capacitor (C) and to compensate for the behavior of a nonhomogeneous electrode surface [47].

The impedance of a CPE is given in the following equation:

$$Z_{CPE} = (j \cdot \omega)^{-\alpha} / C \quad (9.5)$$

(ω : radial frequency; C : capacitance; α : empirical coefficient corresponding to 1 for ideal capacitors; $j = \sqrt{-1}$ imaginary unit).

In the right part of the Nyquist plot, corresponding to the low frequencies, a diagonal line with a slope of 45° can be observed. This is called "Warburg impedance" and is associated to the diffusion of the species from the solution to the electrode. The equation for the Warburg impedance is the following:

$$Z_w = \sigma \cdot (\omega)^{-1/2} \cdot (1 - j) \quad (9.6)$$

(ω : radial frequency; σ : Warburg coefficient -constant for a defined system; $j = \sqrt{-1}$ imaginary unit)

As a general rule, EIS is commonly measured using small excitation signals in order to obtain a pseudo-linear cell's response. Thus, the current response to a sinusoidal potential will be a sinusoid at the same frequency but shifted in phase. In this way it is possible to perform the impedance analysis using linear

assumptions, which provides a much easier interpretation of the system under study [48].

As mentioned above, impedance spectroscopy is a versatile technique which has so far been widely used in different studies, such as corrosion, semi-conducting electrodes, surface coatings, batteries and fuel cells, electrochemical kinetics and mechanism, biomedical and biological systems, electronic and ionic conducting polymers, data processing or solid-state systems [56].

Due to its ability of directly probing the interfacial properties of a modified electrode, the technique is rapidly developing as a tool for studying biorecognition events at the electrode surface or as the basis for signal transduction [54, 57]. In particular, EIS is becoming an attractive electrochemical tool for numerous applications either based in the the immune system [53, 58] or in genosensing field [52, 59], especially in the last decade.

9.2.2 Impedimetric Genosensors

The main advantages of employing EIS for genosensing purpose are its label-free and real-time detection capabilities. The former is possible because of the high sensitivity of EIS in the detection of any modification occurring at the electrode surface, performed by monitoring the electrode-solution interface characteristics. In this way the analytical signal comes from the interactions occurring during the biorecognition event and each step of the biosensing event (as shown in Figure 9.2), can be monitored without the use of any additional labeling to the DNA sequences under study, thus allowing the so called *label-free* detection.

In impedimetric applications to DNA detection, two *modus operandi* are generally followed: *Faradaic* and *non-Faradaic* impedance measurements. The first one involves the use of a redox marker in the bulk solution and it is mainly focused in monitoring the charge transfer resistance due to the electrochemical process [53, 60]. In non-Faradaic impedance spectroscopy no additional reagent is required for the measurements and the parameter of interest in the study is generally the capacitance of the double layer formed between the solution and the electrode surface [61, 62]. Both approaches have been applied for studies on the formation of DNA probe recognition layer on the electrode surface [63, 64], the detection of the hybridization event [65, 66] and the presence of a single nucleotide polymorphism (SNP) in a DNA sequence [67, 68].

In Faradaic impedance spectroscopy, which is so far the most used approach in impedimetric genosensors, the analytical signal comes from the variation of the charge transfer resistance (R_{ct}) between the solution and the electrode surface. The charge transfer process is due to the presence of a redox marker, e.g. ferrocyanide/ferricyanide, in the electrolytic solution. Two main factors can influence the variation of this parameter: the steric hindrance and the presence of a certain charge density on the electrode surface. Any modification of the electrode surface generates a change in the R_{ct} that can be associated to the specific step of the biosensing. In the case of genosensors, negatively charged redox species are usually employed. In fact, since nucleic acid/DNA

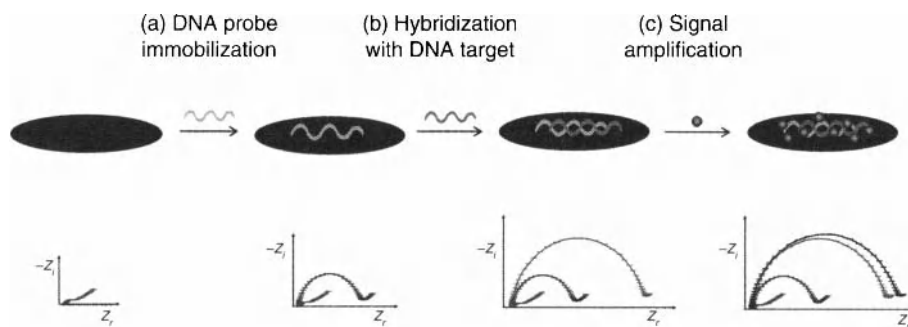


Figure 9.4 Generation of the impedance signal when an impedimetric genosensor detects its target.

complexes (both single-stranded and double-stranded DNA) are oligoanionic polymers, their immobilization on surfaces generates a repulsion of the redox marker, thus inhibiting the redox reaction and enhancing the charge transfer resistance value (see Figure 9.4).

During the last few years, several signal amplification strategies have been developed with the purpose of improving the sensitivity of the technique, that is, to obtain lower detection limits. These are based on either increasing the steric hindrance on the electrode surface or enhancing its charge density. The former can be achieved by the enzymatic precipitation of an insoluble compound on the sensing interface, which may generate a significant impedance change after the hybridization step [69, 70]. On the other hand, tagged negatively charged species (i.e., liposomes) [71] or positively charged metal ions [68, 72] which interact in different ways with dsDNA can be successfully used to generate a significant change in the charge density on the electrode surface, with the effect of enhancing the impedimetric signal.

With the advent of nanotechnologies, the use of nanomaterials for electrochemical biosensing has seen explosive growth. A wide choice of nanoscale materials with different shape, size and composition have been used during the recent years for designing novel sensing systems and improving the performance of bioanalytical assays [73–75].

Nanomaterials have been adopted in impedimetric-based biosensing applications with the main goal of amplifying electrical signals generated by bio-recognition events occurring at the electrode surface. Different nanomaterials have been reported to label DNA oligonucleotides in order to achieve significant signal amplification [76–78]. In impedimetric genosensing, nanomaterials have been utilized also with a second purpose: to study and develop new sensing platforms with the aim of improving the analytical response [14, 79, 80]. The nanomaterials that were mostly used in impedimetric genosensors have been nanostructured carbon, nanostructured gold and different kinds of quantum dots. Specific paragraphs are dedicated below to the description of the use of these materials for DNA detection using electrochemical impedance spectroscopy.

9.3 Nanostructured Carbon Used in Impedimetric Genosensors

9.3.1 Carbon Nanotubes and Nanostructured Diamond

In the past few years carbon nanotubes (CNTs) have attracted increasing interest of many researchers due to their remarkable tensile strength, high resistance, flexibility and other unique structural, mechanical, electrical and physicochemical features [81, 82]. Not only these excellent properties, but also their high specific surface area and the possibility to directly functionalize them with different biomolecules [14] make CNTs extremely attractive for electrochemical sensing [83, 84].

Carbon nanotubes have been recently used as transducers for enhanced electrical detection of DNA hybridization [75, 85]. In impedimetric genosensors, numerous platforms employing different kinds of CNTs either alone or combined in a composite with different polymer or nanoparticles were recently fabricated, characterized, and employed for the detection of DNA sequences [4].

Bonanni *et al.* employed commercial screen-printed carbon electrodes modified with carboxyl functionalized multiwalled carbon nanotubes (MWCNTs-COOH) for the covalent immobilization of amino-modified DNA probes by EDC-NHS chemistry. The platform was then employed for the impedimetric detection of oligonucleotide sequences specific for either a transgenic insect resistant Bt maize [80] or correlated to the H1N1 influenza A virus gene [86]. Caliskan *et al.* [87] employed carboxylic acid functionalized single-walled carbon nanotubes modified graphite sensors to compare voltammetry and impedance spectroscopy studies for the monitoring of direct DNA hybridization related to a specific sequence of Hepatitis B virus. The authors found an enhanced electrochemical signal when using the carbon nanotube platform compared with that provided by bare graphite. A similar platform was used by Weber and coworkers for the impedimetric detection of *Salmonella enterica* serovar Typhimurium related sequences [88].

A carboxylic group-functionalized multiwalled carbon nanotube (MWNTs-COOH) modified platform was also used by Xu *et al.* [89] to perform a doping of nucleic acid probes within electropolymerized polypyrrole (PPy) film deposited on the electrode surface. After the hybridization reaction a decreased impedance value was obtained, which was attributed to the lower electronic transfer resistance of dsDNA than ssDNA. The developed sensing interface revealed an increased conductivity and active surface area. In a different work, the same group used a similar platform to monitor the impedance changes provoked by the metallation of DNA double helix after hybridization [90]. Several metals such as Zn^{2+} , Co^{2+} , and Ni^{2+} were used for the formation of metal-DNA complex (M-DNA). Results showed that Zn^{2+} -DNA complex had the best ability to transport electrons in M-DNA double-stranded chains when compared with the others M-DNAs.

A different composite was used by Jiang and coworkers [91] for the impedimetric detection of transgenic plants gene fragment. DNA probes were

immobilized on a polylysine/single-walled carbon nanotubes modified electrode through electrostatic adsorption on polylysine films. The obtained platform presented an enhanced conductivity, with an estimated detection limit in the pM range.

A nanocomposite membrane, including nanosized shuttle-shaped cerium oxide (CeO_2) and single-walled carbon nanotubes (SWCNTs) was developed by Zhang *et al.* [92] for the electrochemical sensing of the immobilization and hybridization of DNA. The synergistic effect of nanocomposite could dramatically enhance the sensitivity of DNA hybridization recognition. The charge transfer resistance of the electrode surface increased after the immobilization of ssDNA probes and rose further after the hybridization with the complementary target, thus achieving a detection limit in the pM range by working in a label-free protocol.

In a different work, a polyaniline nanofiber modified carbon paste electrode was used as a platform to bind nanogold-carbon nanotubes composite nanoparticles [93]. On this electrode surface the immobilization and hybridization of DNA sequences was investigated by differential pulse voltammetry, cyclic voltammetry, and electrochemical impedance spectroscopy. The loading of the DNA probes on the developed platform was greatly enhanced and the sensitivity for the DNA target detection was markedly improved. The sequence-specific DNA of phosphinothricin acetyltransferase (PAT) gene and the polymerase chain reaction (PCR) amplification product of nopaline synthase (NOS) gene from transgenically modified beans were determined with this label-free impedimetric method.

In recent years diamond has become an attractive alternative candidate for its use as a transducer material in bioelectronics, due to its compatibility with processes applied in microelectronics, its biocompatibility and its ability to be biofunctionalized [94]. In impedimetric genosensors, different forms of nanostructured diamond platform have been employed for DNA sequence immobilization and detection. Yang and coworkers [95] used H-terminated nanocrystalline diamond (NCD) to covalently immobilize ssDNA sequences. They showed that measurements at open-circuit potential displayed a significant decrease in real-time impedance measurements at frequencies of $>10^4$ Hz, when the NCD electrode was exposed to complementary ssDNA target. With this platform they could easily discriminate 4-mismatch DNA sequences. A similar platform was used by Vermeeren *et al.* to investigate the possibility of SNP detection. ssDNA probes were covalently attached to carboxylic groups present on NCD surface. The frequency-dependent change in impedance was analyzed in real-time during hybridization with complementary, 1-mismatch and noncomplementary ssDNA target. SNP discrimination was detectable in a frequency region around 1,000 Hz [96]. Nebel *et al.* [97] employed vertically aligned diamond nano-wires to geometrically control the immobilization of DNA probes. Ultra-hard vertically aligned diamond nano-wires were electrochemically modified to bond phenyl linker molecules to their tips which provide mesospacing for DNA molecules on the transducer. Electro- and biochemical sensor properties were investigated using cyclic and differential

pulse voltammetry as well as impedance spectroscopy. The developed genosensor provided high sensitivities and superior DNA bonding stability over 30 hybridization/denaturation cycles.

9.3.2 Graphene-based Platforms

Graphene is one-atom-thick layer of graphite with a honeycomb lattice structure. Since its successful isolation in 2004 [98], numerous researches have been conducted to explore and understand its superior electrical [99], electrochemical [100], optical [101] and mechanical [102] properties. Even though the earliest samples were produced by micromechanical cleavage, recent efforts have been focused on the production of large scale amounts of graphene. To this aim, three main approaches have been used so far: direct growth of graphene on metallic surfaces by chemical vapor deposition method (CVD) [103]; direct exfoliation of graphite by using different solvents [104]; reduction of the graphene oxide obtained by oxidation and further exfoliation of graphite, thus obtaining the so called chemically modified graphenes (CMGs) [105]. While with the first two protocols a higher percentage of monolayers of pristine graphene are present in the product, the third protocol is highly recommended for the production of bulk amounts. In this case, the presence of a few layers structure in the final product is dominant.

Graphene-based nanomaterials exhibit advantages over other electrode materials in terms of high 2D electrical conductivity, very fast heterogeneous electron transfer, and high surface area [100, 106, 107]. For these reasons a large number of research reports have emerged recently, suggesting the importance of utilizing graphene for electrochemical sensing and biosensing [16, 108].

In the impedimetric detection of DNA sequences, linear or hairpin shaped DNA probes have been immobilized on the graphene surface either by physical adsorption [17, 109, 110] or by chemical binding [111, 112]. In a typical example of physical adsorption, Bonanni and Pumera [17] used hairpin DNA sequences as DNA probes to be immobilized onto graphene platforms consisting of different numbers of same-sized graphene layers. The π -stacking interactions between the nucleobases and the hexagonal cells of graphene make the platform a stable substrate for genosensing. When the hybridization with a complementary target takes place, a partial release of the hpDNA probes from the graphene surface occurs, as also confirmed by different authors [113]. This is due to the formation of stable hydrogen bonds among the nucleobases and their shielding inside the phosphate backbone after hybridization. The above mentioned release generates a significant decrease in the charge transfer resistance value. This decrease is less significant when hybridization is performed with a sequence containing one mismatch and almost negligible in the case of a noncomplementary sequence (see Figure 9.5).

With this label-free protocol the presence of a single nucleotide polymorphism correlated to the development of Alzheimer's disease was detectable at the pM concentration level. The same authors used a similar scheme to perform the impedimetric detection of DNA hybridization on different chemically

modified graphene platforms containing different defect densities and amounts of oxygen-containing groups, namely graphite oxide (GPO), graphene oxide (GO), thermally reduced graphene oxide (TR-GO) and electrochemically reduced graphene oxide (ER-GO) [109]. These CMG materials were obtained from the oxidation of graphite to graphite oxide and subsequent exfoliation to graphene oxide. The latter was electrochemically reduced to obtain ER-GO. The thermal treatment of graphite oxide led to TR-GO (see Figure 9.6). The authors compared the four CMGs as platforms for impedimetric genosensing,

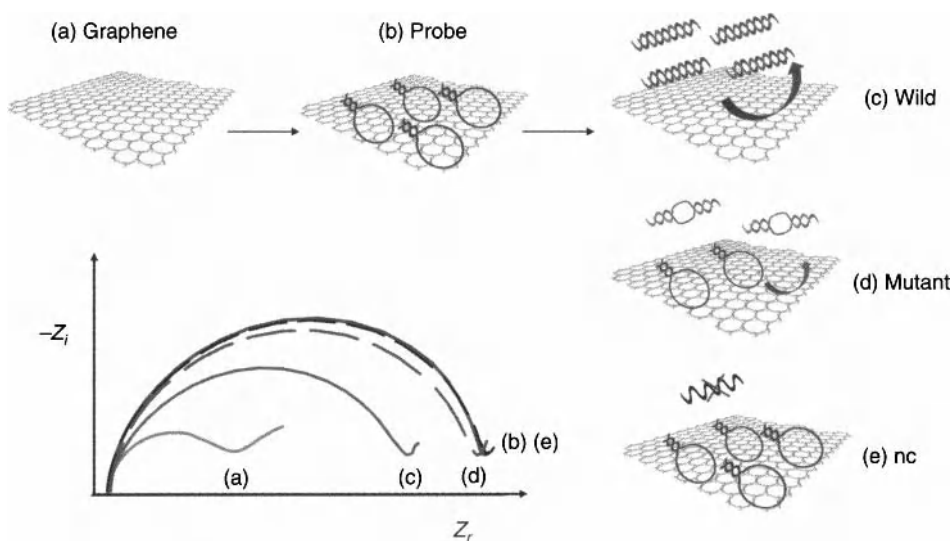


Figure 9.5 DNA biosensing on graphene platform and relative variation of charge transfer resistance (modified from [17]).

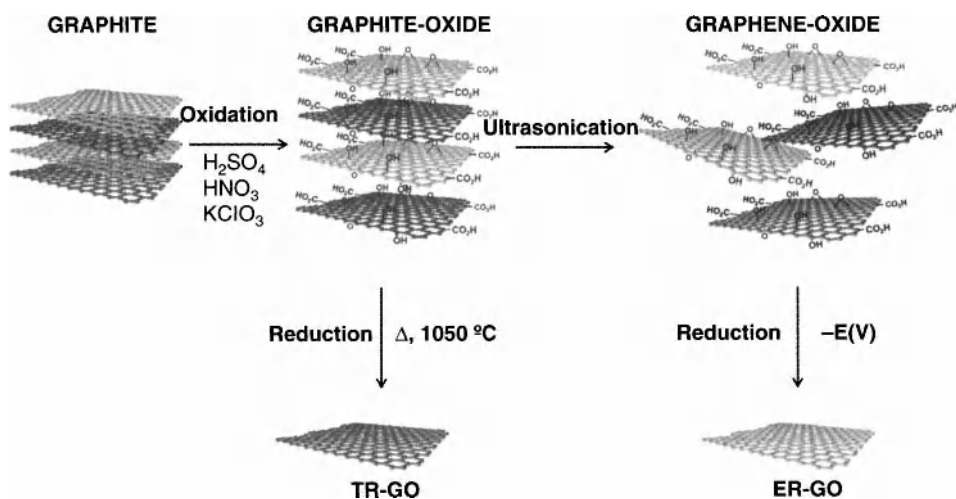


Figure 9.6. Synthesis of chemically modified graphene materials starting from the oxidation of graphite (modified from [109]).

finding that graphene oxide provided the best ability to discriminate a complementary sequence from one carrying a SNP.

The same platforms were used by Bonanni *et al.* [111] to investigate the utility of graphenes prepared by different methods and carrying on their surface various oxygen containing groups, for the covalent functionalization with single-stranded DNA. The functionalization was performed via carbodiimide chemistry and a covalent bond was formed between the amino linker of ssDNA probes and the carboxylic group present on the graphene surface. The platforms were then employed for the impedimetric detection of DNA hybridization and polymorphism. The best sensitivity and reproducibility were obtained when using electrochemically reduced graphene oxide. On this platform, a higher amount of DNA probes was immobilized, as also confirmed by the chronocoulometry results. These results were consistent with the surface characterization previously performed by XPS in which a higher content of carboxylic group was found in ER-GO.

Dubuisson *et al.* [112] employed anodized epitaxial graphene (EG) as platform for label-free DNA detection by electrochemical impedance spectroscopy. In two different protocols, DNA probes were immobilized either by physical adsorption or by covalent grafting and the results of biosensing were compared. It was found that covalently-bound DNA on anodized EG affords a larger dynamic range and a more sensitive response than the π - π stacked DNA probe.

In a different immobilization procedure, Hu [114] *et al.* decorated graphene oxide sheets with perylene tetracarboxylic acid (PTCA) in order to introduce more carboxylic sites for the covalent immobilization of DNA sequences. The same authors used a similar platform for the reduction of HAuCl_4 to gold nanoparticles [115]. DNA probes were then immobilized on the gold nanoparticle decorated graphene via electrostatic interaction and adsorption effect. The platform was used for the impedimetric detection of the conserved sequence of the pol gene of human immunodeficiency virus type-1.

In order to improve the amount of DNA probes that could be immobilized on the electrode surface Wang *et al.* [110] employed an aminopropyltriethoxysilane (APTES) modified glassy carbon electrode to adsorb graphene oxide sheets that were successively electrochemically reduced to obtain APTES-rGO platform. The latter was successfully used for the label-free impedimetric detection of methicillin-resistant *Staphylococcus aureus* DNA.

9.4 Nanostructured Gold Used in Impedimetric Genosensors

The use of nanostructured gold for the development of electrochemical biosensors with enhanced analytical performance has seen exponential growth in recent years [116, 117, 76]. This is due to the well known advantage of the gold material which can be easily functionalized with biomolecules through

thiol chemistry, together with the improvement arising from the novel chemical and physical properties of the nanostructures and the possibility to tailor its size.

In impedimetric genosensors, nanostructured gold has been routinely used either for the development of improved sensing platforms or for the labeling of DNA sequences in order to enhance the analytical response [78, 118].

9.4.1 Gold Nanoelectrodes

For the application on DNA detection by impedance spectroscopy, nanostructured gold has been used either as the transducer material or to be included in modified electrodes. Both schemes were adopted with the aim of either improving the electron transfer or enhancing the amount of biomolecule immobilized on the electrode surface.

Gold interdigitated nanoelectrodes were used by Bonanni and coworkers [118] by measuring the single-frequency capacitance change for the detection of the breast cancer related BRCA1 gene mutation. The nanometric size of the transducer allowed an improved sensitivity if compared with other similar systems, which enabled a label-free detection. Meyyappan *et al.* employed a comparable system to introduce a multiplexing capability for multiple analyte detection [119].

A self-assembled two-dimensional bilayer comprised of silane and gold nanoparticles on a gold substrate was employed by Fu *et al.* [120] for the label-free impedimetric detection of DNA hybridization. The improved sensitivity obtained with this novel platform was mainly due to enhanced immobilization of DNA probes, thanks to the increased surface area of the modified electrode, with nanoparticles both inside the network and on the surface.

Yang *et al.* [121] developed a new platform by depositing a gold nanoparticle (NG) layer on a poly-2,6-pyridinedicarboxylic acid film (PDC) on a glassy carbon electrode (GCE). Then the ssDNA probes were immobilized on the NG/PDC/GCE by the interaction of NG with DNA. An increase in the electron transfer resistance in $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution was recorder either after the immobilization of the DNA probes or after the hybridization with cDNA sequence related to PAT gene in transgenic plants. The NG modified on the PDC dramatically enhanced the immobilization amount of the DNA probe and greatly improved the sensitivity of the label-free detection, allowing a detection limit of $2.4 \times 10^{-11} \text{ mol L}^{-1}$.

Nascimento and co-workers [77] prepared and characterized a novel gold nanoparticles-polyaniline hybrid composite (AuNpPANI) with SH-terminal groups that, due to its ability of immobilizing dengue serotype-specific primers 1,2 and 3 (ST1, ST2 and ST3), could be used for the development of a genosensors able to detect dengue serotype at picomolar concentration. Both electrochemical impedance spectroscopy and cyclic voltammetry were used for the detection of probe immobilization and hybridization with the target sequences. A similar platform was employed by Feng *et al.* [122] combining a

gold nanoparticle/polyaniline nanotube membrane on a glassy carbon electrode for the impedimetric sensing of the immobilization and hybridization of nonlabeled DNA, thus obtaining a much wider dynamic detection range and a lower detection limit for the DNA analysis.

A polyaniline nanofibers (PANI(nano))/carbon paste electrode (CPE) was designed by Zhou *et al.* [123] via clapping PANI(nano) in the carbon paste. Subsequently, a composite comprising nanogold (Au -nano) and carbon nanotubes (CNT) was bound to the PANI(nano)/CPE surface. The loading of the DNA probes on this novel platform was strongly enhanced as well as the sensitivity for the detection of PCR amplified sequences of transgenically modified beans, achieving a limit of detection of $5.6 \times 10^{-13} \text{ mol L}^{-1}$.

9.4.2 Gold Nanoparticles Used as Labels

In electrochemical genosensors, gold nanoparticles (AuNPs) have been widely employed as labels for DNA sequences, due to their size which can be comparable to that of DNA double helix diameter and the fact that they can provide a sensitive electrochemical signal [76].

In impedimetric genosensors, AuNPs were mainly used to achieve an amplification of the analytical signal. In fact, the presence of AuNPs on the electrode surface strongly influence the charge transfer process, thus increasing the variation of the charge transfer resistance, both for electrostatic repulsion and for sterical hindrance issues. To this aim, Bonanni *et al.* [78] used streptavidin-coated gold nanoparticles (strept-AuNPs) to label biotinylated DNA sequences and amplify the impedimetric signal generated in a biosensor for the detection of DNA hybridization. With this system a 90% amplification of the impedimetric signal was obtained. In a similar scheme, the same authors [124] carried out the detection of double tagged DNA coming from polymerase chain reaction (PCR) amplification of *Salmonella spp.* in real samples. The amplification of impedimetric signal was performed by conjugating the duplex with anti-dioxigenin antibody from mouse. This was followed by a secondary labeling with AuNPs-labeled anti-mouse IgG. In an alternative protocol, an amplification scheme using protein G was also proposed. With the AuNPs labeling, a detection limit in the fM range was achieved.

The detection of a synthetic sequence correlated to cystic fibrosis was also performed by Bonanni *et al.* [30] using MWCNTs platform and strept-AuNPs amplification in a sandwich scheme. Different protocols for the impedimetric detection of DNA hybridization were compared in this work, finally achieving a LOD of 100pM.

Moreno-Hagelsieb and coworkers [125] labeled the DNA target with AuNPs in order to amplify the capacitance recorded between interdigitated aluminium electrodes imprinted over an oxidized silicon wafer. In addition, a further signal amplification was attained by a silver enhancement treatment on the AuNPs.

9.5 Quantum Dots for Impedimetric Genosensing

Quantum dots (QDs) are nanometric scale semiconductor crystals (mainly sulphides, selenides, or tellurides of heavy metals Cd, In, Zn or Tl) with unique properties originated in the quantum confinement effect, that are advantageous for the development of novel bioassays, chemical sensors and biosensors [126]. Their exceptional physical and chemical properties, together with their capability to easily bind to biomolecules have attracted significant interest in the biondiagnostic field [127]. QDs are successfully used to label biomolecules in order to either obtain an analytical signal, as for colorimetry [128] or electrochemical detection [129, 130], or to enhance the response in impedimetric genosensing [131].

Travas-Sejdic [132] and coworkers employed cadmium sulphide (CdS) nanoparticles to amplify the electrochemical signal after the detection of specific oligonucleotide sequences. The sensor platform was based on electropolymerization of a conducting polymer (polypyrrole) in the presence of the probe oligonucleotide. The hybridization was then performed with a CdS-labeled complementary target. A significant improvement in sensor sensitivity was observed comparing this system with the label-free detection.

CdTe nanoparticles were used by Kjallman [133] *et al.* for the modification of a hairpin DNA probe. The stem-loop-shaped probes were then self-assembled on the gold electrode through Au-SH bonding. Impedance spectra were recorded at the modified gold electrode surface before and after hybridization with the target DNA. The sensor showed reliable and sensitive detection of 4.7 fM of target sequences.

Xu *et al.* [131] immobilized DNA probes onto a self-assembled mercaptoacetic acid monolayer modified gold electrode by the formation of a covalent bond. Hybridization was then performed with ssDNA-CdS nanoconjugate target. An increased R_{ct} value was observed only when complementary DNA sequence was used in comparison with a three-base mismatched or non-completely matched sequences. The sensitivity of the assay using CdS nanoparticle labels was improved by two orders of magnitude when compared with nonlabeled DNA sequences.

9.6 Impedimetric Genosensors for Point-of-Care Diagnosis

DNA analysis for point-of-care medical diagnosis is focused on two main issues: the identification of a specific DNA sequence belonging to some microorganisms or virus which are the vectors for the development of a certain disease, and the identification of nucleotide polymorphisms on specific genes, the presence of which is related to the development of a specific disease. A single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome differs between members of the same

species. SNPs can occur in every 100–300 base pairs and have been correlated to the development of different inherited diseases and genetic disorders.

In the application for the detection of a real sample, synthetic sequences are immobilized on the electrode surface, which are complementary to the gene of interest. The kind of nitrogenous bases and their order in the specific target gene can be found in the several “gene banks” or public databases available on the web. After that, for the detection of the target gene, the real sample (e.g., microorganism or virus culture, cell culture, blood cells or other human fluids) needs to be pretreated in order to extract the nucleic acid (DNA or RNA), purify it, and finally amplify it by polymerase chain reaction (PCR).

For the first purpose, impedimetric genosensors employing nanomaterials have been applied for the identification of DNA sequences specific for human immunodeficiency virus (HIV) [125, 134, 135] or belonging to H1N1 influenza A virus [86]. Also, impedimetric genosensors have been used to detect the Hepatitis B virus [87]. In the detection of polymorphisms on a specific gene, impedance spectroscopy was successfully employed for the identification of DNA sequences correlated to the development of Alzheimer’s disease [17, 109, 111], kidney syndrome [68], metabolic syndrome [136], and cystic fibrosis [30, 137]. Also, in connection with cancer, impedimetric genosensors constructed using vertically aligned diamond nanowires and the superior electrochemical sensing properties of diamond as transducer material were employed to detect the CK20 marker [97]. In another work, specific DNA probe immobilized onto interdigitated gold nanoelectrodes was successfully employed to detect the breast cancer gene BRCA1 through the associated change in capacitance [118].

9.7 Conclusions (Past, Present and Future Perspectives)

This chapter has presented current technology typically employed with genosensors which make use of electrochemical impedance spectroscopy (EIS) as the detection technique. Its operational principles and the essential protocols employed for impedimetric genosensing have been introduced. Although impedance is commonly used to investigate a variety of electrochemical systems, only recently has it been applied in the field of biosensors. Given its ability to monitor the charge transfer resistance and the double-layer capacitance, it is possible to derive applications for different types of sensing schemes, with numerous recognition agents, by direct signal acquisition, or with the use of simple and inexpensive redox markers. One main advantage of impedimetric genosensing is that it can provide potentially label-free assays, as hybridization with the DNA probe immobilized on a surface can be directly monitored. In general, impedimetric genosensors are extremely simple in operation, and capable of achieving low detection limits even when used without any amplification step. If combined with additional signal amplification strategies, their absolute detection limits may be comparable to other genosensing strategies.

The contribution of nanostructured materials in the development of genosensors is an active research area, and the use of nanoparticles, nanotubes,

graphene or other nanostructured materials have been pointed out as part of the significant research with impedimetric nanosensors. Sensitivity of a biosensor depends on the dimensions and morphological shape of the nanobio-materials involved. Therefore, some morphological- (nanotube, nanowires) based biosensing transducers could function as effective mediators and facilitate the electron transfer between the active site of probe DNA and surface of the electrodes. Other nanocomponents can be used as aids in improving detection limits or improving detection capability. The topic of impedimetric genosensors using nanobiomaterials is common in the biosensing literature, where many formats and designs are proposed to improve performance of biosensors, especially if devised for medical applications. The resulting nanostructures could be substantially stronger and lighter than conventional equivalents currently used in hospitals or point of care facilities.

For a wider acceptance of these genosensors, further research should be mainly focused on the improvement of their reproducibility and stability. Scientists should also increase efforts to optimize the proposed electrode assemblies for use in real clinical samples, overcoming all problems associated with the complexity of clinical matrices. The future application in this field, together with the successful commercialization of a device may depend on improvements in several different areas, including minimization of the effects of nonspecific adsorption.

It is clear that electrochemical impedance sensors are particularly promising for portable, on-site applications, in combination with simplified discrete-frequency instruments. In addition, impedance technique is fully compatible with multiplexed detections in electrically addressable DNA chips, which is one of the clear demands in genosensing in future years. Some of the DNA sensor technology described in this chapter could be transferred from single analyte devices to electrochemical methods offering the possibility of simultaneous measurements of a panel of targets. The multiplex detection only needs electrochemical addressing of a number of electrochemical cells, which may be prepared, for example, on a silicon chip. But, the modern sensing strategies, when coupled with the rich information contained in impedance spectra, can deduct the presence of more than one gene from the signal generated by a single sensing platform. In the work of Bonanni *et al.* [138] two different genes were immobilized on a single electrode, hybridization experiments were carried out and impedance spectra were treated with a purposely trained artificial neural network, which was capable of deducting the presence of one gene target, the other, both or none, illustrating the potentialities that can be extracted, in this case, from intelligent sensor array devices.

To conclude, it must be commented that most of the principles shown are also extensible to specific detection of proteins, in this case taking advantage of the DNA-protein interaction exploited by aptamer sensors [139] where in analogy to the protocols described before, electrochemical impedance spectroscopy can also be employed as the key transduction principle. As above, the unique and attractive properties of nanostructured materials may present new opportunities for the design of highly sophisticated electroanalytical

nanobiosensing devices. Due to the high surface area, biocompatibility and charge-sensitive conductance of nanomaterials they can act as more efficient transducers in nanoscale biosensing and bioelectronic devices.

Acknowledgements

Financial support for this work was provided by the Spanish Ministry of Science and Innovation, MCINN (Madrid) through projects Consolider-Ingenio CSD2006-00012 and CTQ2010-17099, and by the ICREA Academia program.

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Bionanocomposite Matrices in Electrochemical Biosensors

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Abstract

Bionanomaterials are increasingly used for the construction of electrochemical enzyme biosensors. The performance of enzyme-based biosensors usually depends on the physicochemical properties of the electrode materials, as well as the process of the enzyme immobilization and the enzyme concentration on the electrode surface. Most importantly, the method of immobilization and electrode matrices are both considered promising factors concerning the determination of the operational and storage stability of the biosensors. In this respect, nanoscale materials offer excellent prospects for interfacing biological recognition events with electronic signal transduction for designing a new generation of bioelectronic devices exhibiting novel functions. In particular, bionanomaterials such as noble carbon nanotubes (CNTs) and inorganic nanoparticles with charged biopolymeric matrix (i.e., chitosan) have been actively investigated for their applications in electrochemical biosensors, which have become a new interdisciplinary frontier between biological science and material science. In this chapter, we will discuss some main advances, application prospects, vital issues, approaches, and challenges, with the aim of stimulating a broader interest in the development of SiO₂-chitosan/CNT bionanocomposites-based electrochemical biosensors.

Keywords: Bionanomaterials, bioelectronic devices, carbon nanotubes, chitosan

10.1 Introduction

During the last decade bionanomaterials have emerged as a new frontier for multidisciplinary science namely with roots in biology, chemistry, and engineering, and are presenting an array of both opportunities and challenges across all areas of biomedical sciences. In particular, there has been rapid

progress in the use of engineered nanomaterials with diameters from 10–100 nm. This has led to significant new advances in the detection of biological molecules and organisms, and in the bioprocess [1–3]. Because of the nature of their targets, biosensors need to be faster, smaller, more sensitive, and more specific than nearly all of their physicochemical counterparts or the traditional methods that they are designed to replace [4]. Rapid and accurate detection of biomolecules are essential parameters in medical diagnosis. Small size and greater sensitivity mean a less invasive sampling and detection of molecules such as neurotransmitters or hormones at biologically relevant levels [2, 4]. Greater specificity allows assays to be performed in complex fluids such as blood or urine without false negative or false positive results. Nanotechnology promises to improve biosensing on all of these fronts [1]. Nanofabricated biomaterials can bind directly to biomolecules and/or act as transducers to extremely small and sensitive detectors. The unique electrical, chemical, thermal and catalytic properties of nanomaterials offer excellent prospects in the development of electrochemical biosensors [1–4]. The high sensitivity of such devices, coupled with their compatibility with modern microfabrication technologies, portability, low cost (disposability), minimal power requirements, and independence of sample turbidity or optical pathway, make them excellent candidates for clinical diagnostics.

On the other hand, organic-inorganic bionanocomposites based on biopolymer and silica are an important class of advanced materials. The combined organic-inorganic characteristics of the composites represent an improvement in their physical properties. The silicate-filled polymer composites often exhibit remarkable improvement in mechanical, thermal, and physicochemical properties when compared with their pure one. Sol-gel synthesis of composite materials allows the production of materials by which it is possible to control particle size, shape and sometimes even final packing of the colloidal particles [5–8].

Sol-gel derived nanobiomaterials have recently been used for the fabrication of biosensors [9,10]. In general, biosensors provide easy operation, accuracy, sensitivity and selectivity for bioanalytes. Several nanomaterials including sol-gel films [11–14] and self-assembled monolayers [15] have been used for the fabrication of biosensors using layer-by-layer technique, [16] physical adsorption, [17] hydrogel or sol-gel entrapment, [18] crosslinking and covalent techniques [19].

It is known that sol-gel derived silica (SiO_2)-based bionanomaterials possess high mechanical strength, tunable porosity, chemical inertness, thermal stability and negligible swelling in aqueous as well as nonaqueous medium [20,21]. The sol-gel derived silica prepared using tetraethyl orthosilicate (TEOS) precursor has been found to be more reactive towards the condensation reaction and has high affinity towards enzymes as compared to other precursors such as tetramethyl orthosilicate (TMOS), etc. [22].

Silica and biopolymer composites are attractive for the preparation of high performance and multifunctional materials using the sol-gel method [23]. TEOS has been extensively used as silica precursor for the preparation of various biopolymer-silica hybrid composites [24, 25]. The biopolymer silica

composites prepared by the sol-gel that process in aqueous solutions, owing to their promising properties, are of special interest [26–30]. It was observed that polysaccharides can serve as a template for silica generated *in situ* by the sol-gel processes, thus manipulating its synthesis as well as properties and structure. The polysaccharides promoted silica polymerization through acceleration and catalysis of processes. Their effect is explicable by the formation of hydrogen bonds between hydroxyl groups of macromolecules and silanols generated by the hydrolysis of precursor.

In this context, chitosan (CHIT) is a cationic polysaccharide which has attracted much interest owing to its interesting properties such as biocompatibility, nontoxicity, low-cost, good film-forming ability, high mechanical strength and high hydrophilicity [31]. Moreover, presence of amino and hydroxyl groups in CHIT can facilitate covalent enzymes immobilization for biosensor application [32, 33]. However, even though sol-gel-derived silica-CHIT (SiO_2 -CHIT) biocomposite exhibits interesting biosensor characteristics, very small response current has to be observed. The incorporation of carbon nanotubes (CNTs) into SiO_2 -CHIT nanocomposite film is likely to result in improved sensing characteristics. CNTs enhance electro-catalytic activity due to presence of edge-plane-like sites located at both ends and in the defect region [34]. Besides this, these provide high surface-to-volume ratio and faster electron-transfer kinetics due to curvature of CNTs that produce changes in energy bands close to Fermi level [35]. With this in mind, CNTs have recently been utilized for fabrication of electrochemical biosensor [36–39]. The aim of this chapter is to focus on preparation of sol-gel derived SiO_2 -CHIT/carbon nanotubes (SiO_2 -CHIT/CNTs) bio-nanocomposite, characterization, enzyme immobilization techniques and biosensor applications. In this review, strategies involved in the preparation of cholesterol, urea and creatinine biosensors based on SiO_2 -CHIT/CNTs bionanocomposite and their properties such as their electrochemical, morphological and biosensing behavior are discussed in details.

10.2 Fabrication of SiO_2 -CHIT/CNTs Bionanocomposites

The use of nanomaterials such as CNTs to fabricate matrices for biosensors is one of the most exciting approaches because nanomaterials have a unique structure and high surface-to-volume ratio [40]. The surfaces of nanomaterials can also be tailored in the molecular scale in order to achieve various desirable properties [41]. The diverse properties of nanocomposite materials such as unique structure and good chemical stability enable them to provide a wide range of applications in sensor technology [42]. Further, nanocomposites do not suffer from the drawback of sensing complications and synthesis complexities, are efficient and have a long shelf life. In addition, the fundamental electronic characteristics of CNTs could also be used to facilitate the flow of uniform current within the nanocomposite biosensing electrodes. There are many reports on integration of CNTs with sol-gel derived SiO_2 -CHIT to fabricate biosensors to gain synergistic action using organic-inorganic bionanocomposites.

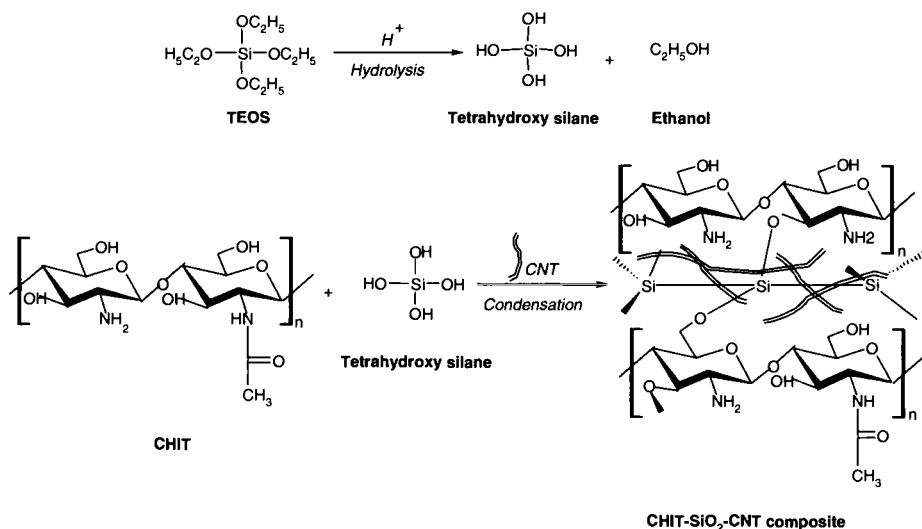


Figure 10.1 Reaction scheme for the preparation of SiO₂-CHIT/CNTs bionanocomposite.

The sol-gel SiO₂-CHIT is prepared by the mixing of alcoholic silica precursor, such as TEOS and CHIT solution, under magnetic stirring at room temperature. To this mixture, homogeneously dispersed CNTs in ethanol are added. The mixture initially comprising of two phases is made uniform by stirring vigorously until -SiO₂ is distributed evenly in the aqueous solution while the hydrolysis reaction occurs (see Figure 10.1). After a certain time period, the opaque and black sol is formed.

In a control process, tetraethoxysilane undergoes hydrolysis and forms tetrahydroxy silane (silanol) at acidic pH [43]. The resulted silanol then reacted with CHIT via a condensation reaction between the -OH groups and led to the formation of a CHIT-SiO₂ composite network, in which CNTs are homogeneously dispersed. Both CNTs and SiO₂ improves the mechanical properties of the CHIT-SiO₂-CNTs bionanocomposite. However, CNTs primarily enhance the electrical conductivity of the biocomposite.

10.3 Preparation of Bioelectrodes

The SiO₂-CHIT/CNTs sol thin film is fabricated by spreading it uniformly onto a substrate such as ITO glass plate using spin coating technique, and is subsequently dried at room temperature. SiO₂-CHIT/CNTs/substrate electrode is washed with deionized water followed by treatment with phosphate buffer saline of pH 7.0 in order to maintain pH over the electrode surface. SiO₂-CHIT/CNTs electrode is treated with aqueous glutaraldehyde as a cross-linker. The freshly prepared enzyme solution is uniformly spreaded onto glutaraldehyde treated SiO₂-CHIT/CNTs electrode and is kept in a humid chamber for 12 h at 4°C (see Figure 10.2).

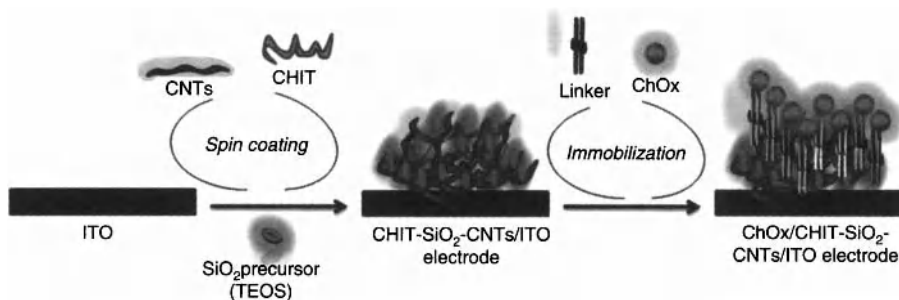


Figure 10.2 Schematic diagram of the fabrication of CHIT-SiO₂-CNTs/ITO and ChOx/CHIT-SiO₂-MWNT/ITO electrodes.

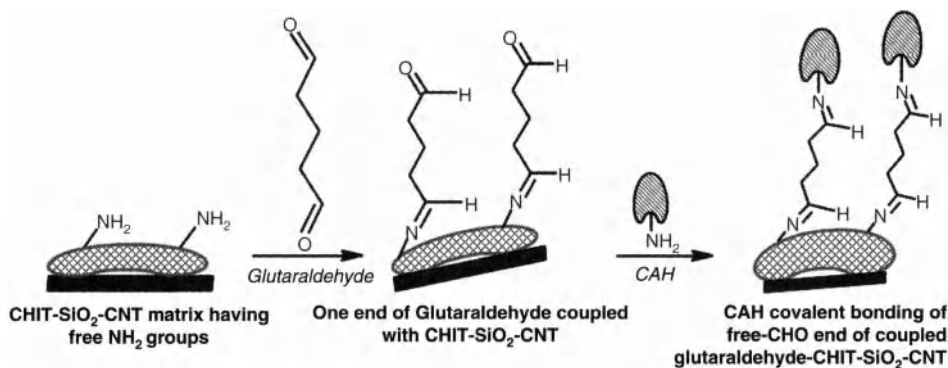


Figure 10.3 Covalent immobilization of the creatine amidinohydrolase (CAH) enzyme on SiO₂-CHIT/CNTs bionanocomposite matrices using glutaraldehyde as a linker.

The enzyme-SiO₂-CHIT/CNTs bioelectrode is immersed in phosphate buffer solution of pH 7.0 in order to wash out unbound enzyme from the electrode surface. A stable enzyme-substrate coupling is achieved with glutaraldehyde as a crosslinking agent [44]. At one end glutaraldehyde is attached to the -NH₂ side of the CHIT-SiO₂-CNTs/ITO electrode through a reaction between the -CHO end group of glutaraldehyde, while the other end of the glutaraldehyde is attached to enzyme such as creatine amidinohydrolase (CAH) through a reaction between the -CHO group of glutaraldehyde and the terminal -NH₂ group of enzyme, which resulted in an enzyme/CHIT-SiO₂-CNTs/ITO bioelectrode as shown in the Figure 10.3.

10.4 Characterizations

The controlled and enzyme immobilized SiO₂-CHIT/CNTs bionanocomposite can be characterized with Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and cyclic voltammetry (CV). In Figure 10.4, the infrared peaks of CHIT (curve A) in SiO₂-CHIT (curve B) become wider and sharper due to overlap of functional groups of CHIT and SiO₂, i.e., stretching vibration bands of Si-O-Si, Si-O-C and C-O bond. In addition, two new

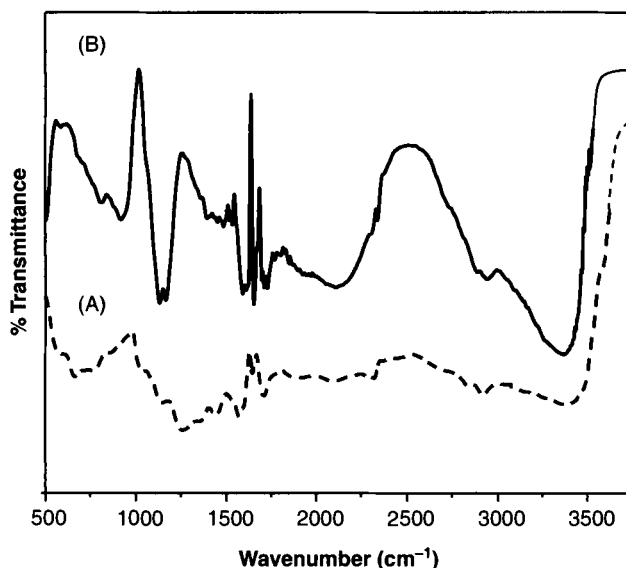


Figure 10.4 FTIR spectra of the (A) CHIT-SiO₂/CNTs nanocomposite and (B) CAH/CHIT-SiO₂/CNTs.

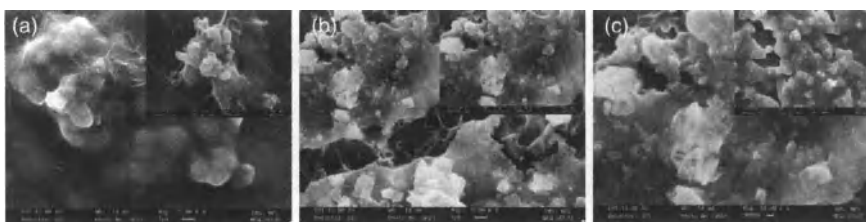


Figure 10.5 SEM images of the (A) CHIT-SiO₂/CNTs and (B-C) Enzyme/CHIT-SiO₂/CNTs.

peaks appear at 1300 and 785 cm⁻¹ pertaining to stretching vibration of C-Si and bending vibration of C-H corresponding to CH₃-Si group [45].

On incorporation of CNTs in CH-SiO₂ hybrid, the infrared band corresponding to SiO₂ becomes broader, and a new infrared band appears at 890 cm⁻¹ revealing presence of CNTs that affects vibration mode of CHIT and SiO₂ resulting in the formation of SiO₂-CHIT/CNTs bio- nanocomposite. After immobilization of enzyme, FTIR bands corresponding to -NH/-OH group in nanobiocomposite become broader suggesting interaction between amino and hydroxyl group of CHIT with. However, presence of 1672 and 1442 cm⁻¹ peaks (corresponding to amide bands) indicates immobilization of enzymes [46].

The surface morphology (Figure 10.5) of SiO₂-CHIT/CNTs (image A) reveals the monodispersed rope-like structure of CNTs surrounded with globular appearance of SiO₂ particles into CHIT matrix indicating that CNTs and SiO₂ are uniformly dispersed into the backbone of CHIT. We speculate that CNTs are wrapped with CHIT and SiO₂ via electrostatic interactions. The surface morphology of SiO₂-CHIT/CNTs nanobiocomposite further changes after the immobilization of enzyme revealing attachment of enzymes over the electrode surface (image B-C).

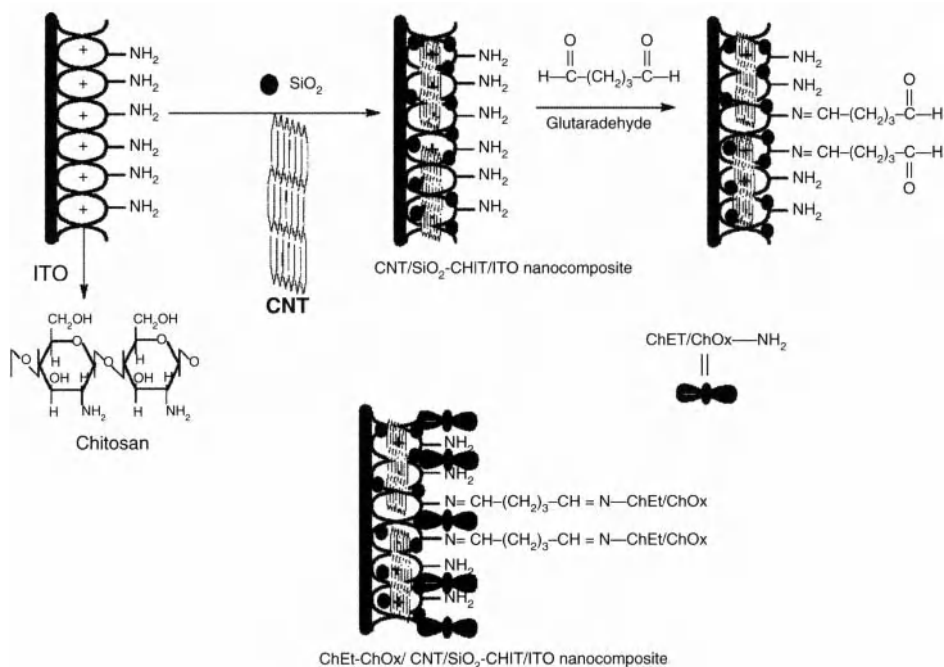


Figure 10.6 Proposed mechanism for preparation of ChEt-ChOx/CNTs/SiO₂-CHIT/ITO bioelectrode.

It may be noted that the appearance of CNTs is less predominant due to immobilization of enzymes onto bionanocomposite via electrostatic interactions and covalent binding. Multifunctional matrix presumably provides mesoporous surface resulting in enhanced enzyme loading at the enzyme loaded SiO₂-CHIT/CNTs bioelectrode. Figure 10.6 shows a probable mechanism of enzyme immobilization on to nanobiocomposite using glutaraldehyde as linker. It appears that available -NH₂ groups of CHIT get covalently attached with aldehyde group of glutaraldehyde at one end, and another aldehyde group gets linked with available -NH₂ groups of enzymes via covalent bonding and electrostatic interactions between bionanocomposite and enzymes.

10.5 Electrocatalytic Properties

Electrocatalytic properties of novel nanomaterials display a fundamental role in the organization of electrochemical biosensing devices. A variety of nanostructures have been investigated to determine their properties and were proposed for their possible applications in the development of electrochemical biosensing devices [1–4, 47]. These nanomaterials including metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles, polymeric nanomaterials, carbon nanotubes and even nanocomposite materials have been widely used in the construction of electrochemical biosensors because of their unique electrocatalytic properties. For example, metal-nanoparticles (e.g., platinum) catalyze the redox

process of some biomolecules with analytical interest, which can be monitored using electroanalytical techniques. Nanomaterials have high surface-to-volume ratio and electrocatalytic activity, as well as good biocompatibility and novel electron transport properties making them highly attractive materials for ultra-sensitive detection of biological macromolecules via bioelectronic devices [4]. Some nanoscale materials exhibited remarkable electron transport properties, which are strongly dependent on their nanocrystalline structure. Morphological-based nanomaterials show new capabilities that are generated by the combination of novel nanobuilding units and strategies for assembling them. These extraordinary electrocatalytic characteristics of the nanomaterials are exploited in the fabrication of efficient electrochemical biorecognition devices. The surface features of nanoscale materials such as shape, size, diameter, surface condition, crystal structure and its quality, chemical composition, crystallographic orientation along the axis, etc., are very important parameters, all of which influence the electron transport mechanism of the nanomaterials [3,4].

Electrochemical study measures the movement and separation of charge in matter, i.e., the study of the transfer of electrons. Most of the chemical reactions involve charge transfer, and matter may hold charge, either positive or negative. The charges could be discrete and measureable or partial and diffuse; charged matter can maintain separations leading to interesting effects. Figure 10.7 shows the CVs of the electrochemical cells using either CHIT-SiO₂-CNTs/ITO or CAH/CHIT-SiO₂-CNTs/ITO electrode at a constant 20 mVs⁻¹ scan rate in 50 mM phosphate buffer solution (pH 7.0, 0.9% NaCl) containing 5 mM Fe(CN)₆^{3-/4-}. The current of the electrochemical cell using the electrode CHIT-SiO₂-MWCNTs/ITO (2.5×10^{-3} A) was about five times of that using the CAH/CHIT-SiO₂-CNTs/ITO bioelectrode (0.5×10^{-3} A). Thus, immobilizing

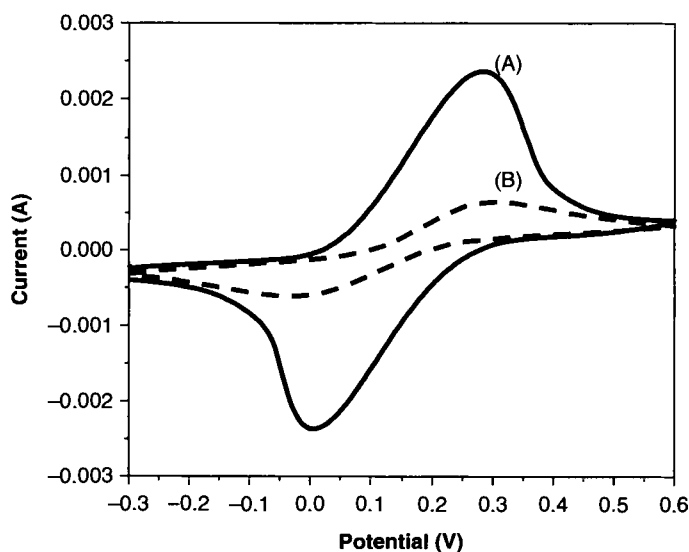


Figure 10.7 Cyclic voltammograms of the (A) CHIT-SiO₂-CNTs/ITO and (B) CAH/CHIT-SiO₂-CNTs/ITO electrodes in PBS (50 mM, pH 7.0, 0.9% NaCl, 5 mM Fe(CN)₆^{3-/4-}) at a 20 mVs⁻¹ scan rate.

CAH onto the bare electrode reduced the current. A decrease in current after the immobilization of CAH may be attributed to a slower redox behavior when compared with the bare CHIT-SiO₂-CNTs/ITO electrode. The covalent binding of CAH on the CHIT-SiO₂-CNTs/ITO electrode controls the moment of the supporting electrolytes [48]. Also, the nonconducting nature of the CAH molecules might have contributed to the decrease in current when using the CAH/CHIT-SiO₂-CNTs/ITO electrode. The study indicates a high affinity of CAH to the CHIT-SiO₂-CNTs nanocomposite matrix over the electrode surface, which may be attributed to: (1) the advantageous nanoporous surface of the CHIT-SiO₂-CNTs matrix for the enzyme immobilization that can favor conformational changes of the enzyme, and (2) the high surface-to-volume ratio, which can help to effectively immobilize CAH onto the CHIT-SiO₂-CNTs nanocomposite [49].

Particular morphology of nanomaterials including nanotubes, nanofibers, nanorods, nanowires, nanocombs, nanodisc, and nanorings have different electrical conductance and tune the surface behavior of the deposited thin film on the substrate [2–4]. Moreover, the electron transport properties of the nanomaterials can also be altered by introducing some doping materials into the matrix, which enhance the surface properties and electrical conductance of the nanomaterials. The small change in the surface properties of the nanomaterials can cause remarkable change in the transport behavior. It has been reported that the change in electrical conductivity of the bioelectrode is influenced by minor surface perturbations such as binding of biomacromolecular species on a long conduction channel. 1D semiconductor electronic nanomaterials, in particular, have active surfaces that can easily be modified for immobilization of numerous biomolecules [4]. Additionally, the sizes of biological macromolecules, such as proteins and nucleic acids, are comparable to nanoscale building blocks. Therefore, any interaction between such molecules should induce significant changes in the electrical properties of 1D nanostructures. Due to the infinitesimally size of these nanomaterials, it is possible to pack a large number of biomacromolecule-functionalized nanomaterials onto a remarkably small footprint array device. These nanometer-scale electronic transducers reduce the pathway for direct electron communication between redox biomolecule to the electrode for sensitive and speedy detection of analyte without any hindrance [2, 3].

This chapter illustrates the advantages nanoscale biocomposite materials for the designing of efficient electrochemical biosensing devices and also highlights the potential analytical applications in terms of nanostructured-based electrochemical biosensors and bioreactors. All these properties of the nanoscale composite materials strongly depend on the synthetic procedures used to grow them. Therefore, extensive efforts have been made to synthesize novel morphological-based nanosize biocomposite materials such as nanotubes, nanoglobules, nanoparticles, etc.. This is therefore these nanosize-biocomposite-materials-based electrochemical biosensing devices show higher performance such as sensitivity, selectivity, and real time detection limit compared to those fabricated from other forms of nanomaterials [4]. By controlling their size,

shape and structure, these novel nanomaterials can be tuned by altering the physical, chemical and biological routes.

The CV studies of CHIT/ITO, SiO_2 -CHIT/ITO, CHIT-CNTs/ITO, SiO_2 -CHIT/CNTs/ITO, ChEt-ChOx (cholesterol esterase-cholesterol oxidase)/ SiO_2 -CHIT/CNTs/ITO electrodes have been conducted to understand the synergy between the various components in phosphate buffer saline containing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at scan rate of 50 mV/s (Figure 10.8 a [a–c]). The CV of pure CHIT (curve a) shows well-defined reversible redox behavior attributed to highly positive charged species on matrix, indicating that electrons originate from the negatively charged medium. After addition of $-\text{SiO}_2$ - in CHIT, the magnitude of current decreases (curve b) due to formation of complex between SiO_2 and $-\text{OH}/-\text{NH}_2$ group of CHIT. In the case of CHIT and CNTs (curve c), the magnitude of current decreases and the potential is shifted towards the lower side at 0.213 V as compared to that of CHIT (0.301 V) (curve a). It appears that CHIT-CNTs biocomposite has an increased number of electrons compared to that of CHIT since ΔE_p is inversely proportional to the number of transferred electrons ($\Delta E_p \propto 1/n$) [50], while curve (d) exhibits the highest voltammetric response on SiO_2 -CHIT/CNTs/ITO electrode because SiO_2 /CNTs provides an electroactive surface area that enhances electron conduction pathway and promotes electron transfer between the enzymes and electrode. The redox potential of ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode (curve e) is much less as compared to the other electrode due to slow redox process during the biochemical reaction. The inset in Figure 10.8a shows oxidation (I_{pa}) and reduction (I_{pc}) peak current against $v^{1/2}$ (v is the scan rate) for SiO_2 -CHIT/CNTs/ITO and ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrodes. The diffusion control process yields linear response obtained by plotting peak current versus square root of the scan rate, and it depends on value of the diffusion coefficient. The values of slope of the peak oxidation current obtained using $d(I)/d(v^{1/2})$ (square root of the scan rate is proportional to $D^{1/2}$ for electrode and nanobiocomposite) have been found to be as 94.6 and 99.3 $\mu\text{A} (\text{mV/s})^{1/2}$, respectively. The high value of slope of the electrode compared to the other bioelectrode is probably due to the binding of ChEt and ChOx onto the electrode that possibly controls transport of the ions. Figure 10.8b exhibits CV of SiO_2 -CHIT/CNTs/ITO bioelectrode as a function of scan rate varying from 10–100 mV/s. The magnitudes of cathodic peak and anodic peak currents increase with increasing scan rate. The increased peak-to-peak separation reveals electron transfer between ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode and the medium. The variation of potential difference (ΔE_p) between cathodic (E_{pc}) and anodic (E_{pa}) peaks for SiO_2 -CHIT/CNTs/ITO nanobiocomposite matrix and ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode is shown in Figure 10.8c.

The decrease in ΔE_p value for the bioelectrode indicates faster kinetics of electron transfer on the surface [51]. The surface concentrations of CHIT/ITO, SiO_2 -CHIT/CNTs/ITO; ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode have been estimated from the plot of current verses potential (CV) using equation: $I_p = n^2 F^2 T A V / 4RT$ (Brown-Anson model) where n is the number of electrons transferred which is 1 in the case of CHIT/CNTs/ITO electrode, F is the Faraday

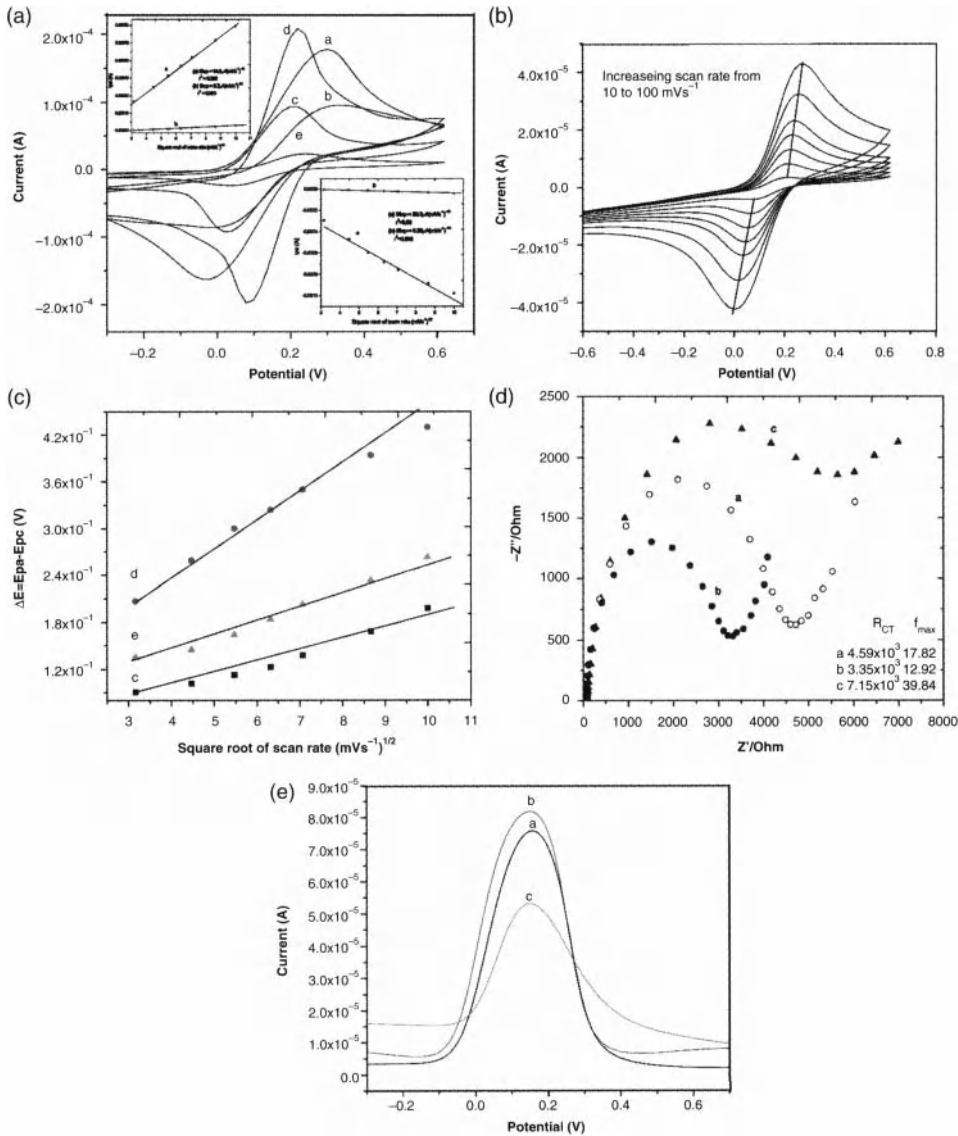


Figure 10.8 (A) Cyclic voltammogram of (a) CHIT/ITO electrode; (b) SiO_2 -CHIT/ITO electrode; (c) CHIT/CNTs/ITO electrode; (d) SiO_2 -CHIT/CNTs/ITO electrode; (e) ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode at 50 mV s^{-1} in PBS containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$. The inset shows oxidation and reduction peak current with square root of scan rate for SiO_2 -CHIT/CNTs/ITO electrode; (e) ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode. (B) CVs of ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode at as a function of scan rate (10-100 mV s^{-1}). (C) Square root of scan rate for (a) SiO_2 -CHIT/CNTs/ITO electrode; (b) ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode and difference between the cathodic and anodic peak shifts. (D) Nyquist plot of (a) CHIT/ITO electrode, (b) SiO_2 -CHIT/CNTs/ITO electrode, and (c) ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode. (E) DPV of (a) CHIT/ITO electrode, (b) SiO_2 -CHIT/CNTs/ITO electrode, and (c) ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode in PBS containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ at potential height as 0.4995 V, potential period 0.07 ms and interval period as 0.14 ms.

constant (96,584 C/mol), Γ is the surface concentration (mol/cm²) obtained for the SiO₂-CHIT/CNTs/ITO nanobiocomposite matrix, A is the surface area of the electrode (0.25 cm²), V is the scan rate (50 mV/s), R is the gas constant (8.314 J/mol K), and T is the absolute temperature (298 K). The values of surface concentrations for CHIT/ITO electrode and SiO₂-CHIT/CNTs/ITO nanobiocomposite matrix have been found as 11.8×10^{-8} and 13.0×10^{-8} mol/cm², respectively [52]. These results indicate that SiO₂-CHIT/CNTs/ITO matrix provides increased electroactive surface area for loading of enzymes (ChEt and ChOx).

The ability to mass produce biosensors in an efficient and cost-effective fashion has been a major achievement of this research. Hence, SiO₂-CHIT/CNTs bionanocomposite matrices can be potentially used for the production of enzyme electrodes, immunodiagnostic arrays or as a fluidic handling system in more elaborate high throughput analytical systems. The crucial role of diffusion membranes in both the kinetics and selectivity of amperometric biosensors must not be underestimated if devices are to be accurately reproduced.

Figure 10.8d demonstrates the Faradaic impedance spectra, presented as Nyquist plots obtained from real (Z') and imaginary ($-Z''$) in the frequency range 0.01–105 Hz for CHIT/ITO electrode, SiO₂-CHIT/CNTs/ITO nanobiocomposite and ChEt-ChOx/SiO₂-CHIT/CNTs/ITO in phosphate buffer saline (pH 7.0, 0.9% NaCl) containing 5 mM [Fe(CN)₆]^{3-/4-} that yields information about electrical properties at desired interfaces. The values of electron-transfer resistance (R_{CT}) derived from the diameter of semicircle of impedance spectra are obtained as $4.59 \times 10^3 \Omega$ for CHIT/ITO electrode (curve a), $3.35 \times 10^3 \Omega$ (curve b) for SiO₂-CHIT/CNTs/ITO electrode and $7.15 \times 10^3 \Omega$ for ChEt-ChOx/SiO₂-CHIT/CNTs bioelectrode, respectively. The semicircle of CHIT/ITO (curve a) exhibits charge transfer phenomena between electrode and medium. Compared to CHIT/ITO electrode, charge-transfer resistance (R_{CT}) value obtained for SiO₂-CHIT/CNTs/ITO electrode (curve b) decreases resulting in enhanced electron transfer or conductive pathway towards electrode. This suggests that presence of both SiO₂ and CNTs enhance ionic transport in CHIT and results in the formation of complex and improved charge transfer. It can be seen that R_{CT} increases after immobilization of ChEt and ChOx onto SiO₂-CHIT/CNTs/ITO nanobiocomposite (curve c) due to the hindrance provided by macromolecular configuration of ChEt and ChOx to electron transport between electrode and redox mediator indicating immobilization of ChEt and ChOx onto SiO₂-CHIT/CNTs/ITO surface. The value of R_{CT} is dependent on electrochemical reaction time constant, τ ($\tau = \frac{1}{2\pi} f_{\max} = R_p \cdot C_{dl}$, where $f_{\max}$ is the frequency at which maximum Z'' obtained, R_p is the polarization resistance and C_{dl} is the double layer capacitance). DPV studies have been carried out on SiO₂-CHIT/CNTs/ITO electrode (Figure 10.8e). DPV experiments have been conducted in phosphate buffer saline (50 mM, pH 7.0) containing 5 mM [Fe(CN)₆]^{3-/4-} in the range -0.1–0.6 V. The DPV measurements have been carried out at 0.49 V, potential period as 0.07 ms and interval period as 0.14 ms. The value of maximum response current obtained as 7.58×10^{-5} A for CHIT (curve a) increases to 8.19×10^{-5} A on incorporation of SiO₂ particles and CNTs into CHIT. This suggests that the conducting nature of CNTs results in increased ionic transport in CHIT enhancing electron transfer towards the electrode. The

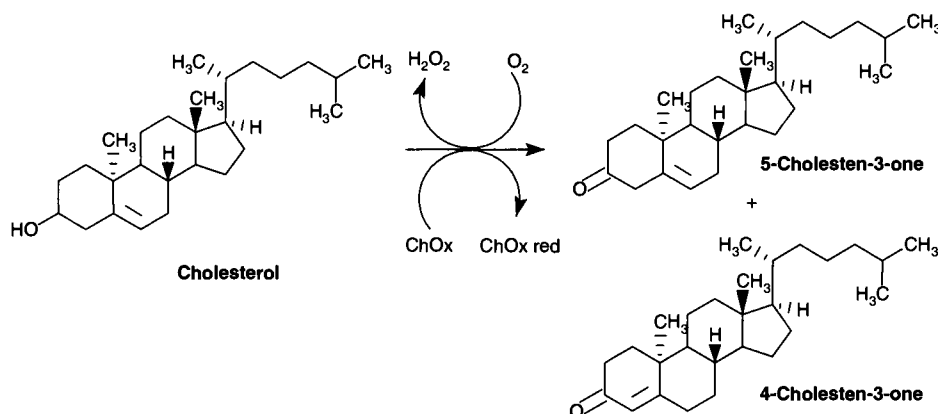


Figure 10.9 Liberation of electrically active H_2O_2 as a sensing element resulting from the reaction of cholesterol and ChOx.

magnitude of current decreases to 5.30×10^{-5} A for ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode indicating slow redox process at bionanocomposite due to insulating characteristics of ChEt and ChOx revealing immobilization of ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bionanocomposite. The electroactive molecule H_2O_2 is resulted from the enzymatic reaction between the cholesterol oxidase and the cholesterol (Figure 10.9).

In the biochemical reaction, the positive charge on the SiO_2 -CHIT/CNTs/ITO nanobiocomposite accepts electrons generated during reoxidation of ChEt and ChOx prior to the evolution of oxygen resulting in enhanced current response of the SiO_2 -CHIT/CNTs/ITO nanobiocomposite matrix. The enhancement in peak current suggests that this nanobiocomposite provides favorable microenvironment to enzyme wherein CNTs provide enhanced electron transfer to the electrode. Direct acceptance of electrons by the matrix is attributed to enhanced charge transport in the SiO_2 -CHIT/CNTs/ITO film due to electrons hopping via conductive CNTs that mediate electron transfer via bionanocomposite in presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as mediator.

The value of the enzyme-substrate kinetics parameter (Michaelis-Menten constant, K_m) estimated using the Lineweaver-Burke plot reveals affinity of enzyme for desired analyte. It is noted that K_m is dependent both on matrix and the method of immobilization of enzymes that often results in their conformational changes resulting in different values of K_m . Besides this; the value of K_m for the bound enzyme can be lower or higher than that of purified enzyme. In this study the value of K_m is obtained for the ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode as 3.4 mg/dL (0.052 mM) that is smaller than the reported value [52]. The lower K_m value indicates high affinity for cholesterol oleate attributed to the immobilization of ChOx and ChEt onto SiO_2 -CHIT/CNTs/ITO bionanocomposite for faster biochemical reaction. The value of sensitivity of ChEt-ChOx/ SiO_2 -CHIT/CNTs/ITO bioelectrode estimated from the slope of curve has been found to be 3.8 $\mu\text{A}/\text{mM}$. The values of standard deviation and correlation coefficient obtained from the linear regression analysis for the bioelectrode have been found to be 1.23 μA and 0.994, respectively. Table 10.1 compares the characteristics of

Table 10.1 Comparison of the characteristics of sol-gel CHIT-SiO₂-CNTs bionanocomposites-based biosensors.

CNTs Bio-nanocomposite electrode	Sensing element	Immobiliza- tion technique	Linearity with [enzyme]	Response time	Shelf-life	Sensitivity	Reference
ChOx/CHIT- SiO ₂ -CNTs/ ITO	Cholesterol esterase & Cholesterol oxidase	Covalent linkage	10-500 mg/dL	10s	2 months	3.8 μ A/ mM	[45]
ChOx/CHIT- SiO ₂ -CNTs/ ITO	Urease	Covalent linkage			8 months		[50]
ChOx/CHIT- SiO ₂ -CNTs/ ITO	Creatine amidi- nohydrolase	Covalent linkage	-	-	8 months	-	[54]
ChOx/CHIT- SiO ₂ -CNTs/ ITO	Cholesterol oxidase	Covalent linkage	50- 650 mg/dL	5 s	6 months	3.4 nA/ mgdl-1	[56]
Prussian blue- glass carbon/ chitosan/ silica/MWCNTs	Cholesterol oxidase	Covalent linkage	4 $\times$ 10 ⁻³ - 0.7 mM	13 s	<50 days	1.55 μ A/ mM	[57]

CNTs and sol-gel silica-based biosensors as reported in the literature. Hence, the CHIT-SiO₂-CNTs bionanocomposite matrices provided a longer shelf life, higher selectivity, and shorter response time to immobilized enzyme.

10.6 Photometric Response

The rate of enzyme reactions is usually measured with enzyme assays because enzymes are only not catalyse reactions without their consumption. The enzyme assays principally track the changes in the concentration of either substrates or products to measure the rate of reaction. There are many methods of measurement, among them spectrophotometric assays observe change in the absorbance of light between products and reactants. A photometric study was performed to calculate the apparent enzyme activity of the urease (Urs) enzyme. In the presence of urease, urea hydrolyzed into ammonia, which in turn reacts with the Nessler's reagent (K₂ Hg₂I₄) to form a colored product, NH₂Hg₂I₃ (Figure 10.10).

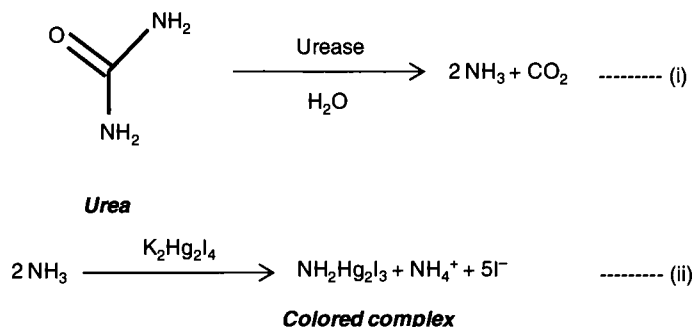
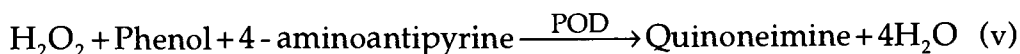
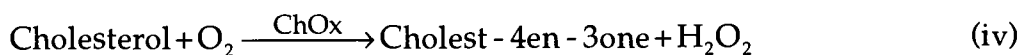


Figure 10.10 Production of photometrically active NH₂Hg₂I₃ as a sensing element (i.e., resulting from the reaction-ii of Nessler's reagent and librated NH₃; obtained from enzymatic hydrolysis of urea as Eq. i).

By means of taking the absorbance of NH₂Hg₂I₃ at 385 nm, apparent enzyme activity of the Urs enzyme can be determined [48]. The apparent enzyme activity ($^{enz}a_{app}$) was calculated using equation $^{enz}a_{app} = AV/ts$, where A is the difference in absorbance before and after incubation, V is the total volume of the solution, ϵ is the millimolar extinction coefficient, t is the reaction time and s is the surface area of the electrode. The apparent enzyme activity was calculated to be 89.02 mg/cm²; indicating 89.02 mg of Urs was actively immobilized per unit area of CHIT-SiO₂-CNTs matrix [52, 53].

The cholesterol oleate concentration can be measured by measuring the change in the intensity of color based on the following reaction:



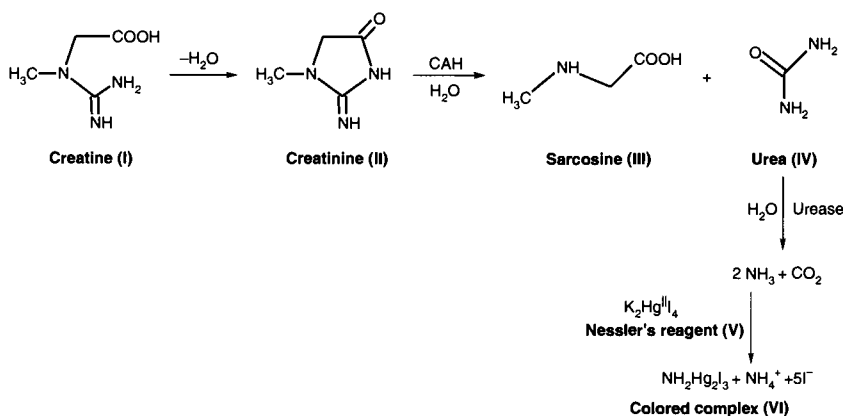


Figure 10.11 Production of photomerically active $\text{NH}_2\text{Hg}_2\text{I}_3$ as a sensing element resulting from the reaction of Nessler's reagent and liberated NH_3 (i.e., obtained from enzymatic hydrolysis of creatinine).

The value of apparent Michaelis-Menten constant (K_m) has been estimated using Lineweaver-Burke plot, i.e. graph between inverse of absorption and inverse of cholesterol oleate and has been found to be 0.14 mM revealing high affinity of ChEt-ChOx with cholesterol oleate in the presence of HRP. However, higher K_m value in comparison to that obtained using electrochemical response (i.e., 0.052 mM) may due to the presence of HRP [54].

A spectrophotometric assay was studied to calculate the apparent enzyme activity of the CAH enzyme. CAH catalyzes the hydrolysis of creatinine (II) to produce sarcosine (III) and urea (IV). In the presence of urease, urea further hydrolyzed into ammonia, which in turn reacts with the Nessler's reagent, $\text{K}_2\text{Hg}_2\text{I}_4$ to form a colored product, $\text{NH}_2\text{Hg}_2\text{I}_3$, Figure 10.11.

10.7 Conclusions

The fabrication of SiO_2 -CHIT/CNTs bionanocomposites can be obtained by the uniform dispersion of CNTs into -SiO_2 -CHIT matrix. The prepared SiO_2 -CHIT/CNTs bionanocomposites are widely applicable as matrices for various immobilization including CAH, Urs, ChEt and ChOx to fabricate electrochemical biosensors. It has been shown that obtained bionanocomposite electrode could potentially estimate bioanalytes from biological samples. Thus, utilization of SiO_2 -CHIT/CNTs bionanocomposite matrices is a novel way to immobilize enzymes for use in biosensors that show encouraging operational and storage stability.

Acknowledgements

The authors wish to thank the European Commission (PIIF-GA-2009-254955), LIST and IGEN Linköping University for their generous financial support.

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Biosilica – Nanocomposites - Nanobiomaterials for Biomedical Engineering and Sensing Applications

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Abstract

Biosilicification is a process by which silica-based nanostructures are formed (biosilica) through a biopolymer-based catalysis. Found in nature, biosilica is produced by many organisms and is renowned for its elaborate shapes down to nanoscale size. Biosilica is physically very strong, biocompatible, and has unique optical and chemical properties. Its surface is covered with chemically functional groups which are ideal for functionalization, protein adsorption and enzyme stabilization. Biomolecules entrapped within biosilica are highly stabilized from chemical denaturation, while protected from protease attacks. The ability to synthesize biosilica in the laboratory has set the grounds for the beginning of a new technological era which will provide us with novel devices and tools applicable to a variety of technologies. This chapter is devoted to the discussion of the engineering applications of biosilica in the biomedical and sensing areas.

Keywords: Biosilica, biosilicification, nanocomposites, biomedical engineering, biosensors, nanobiomaterials, silicic acid, silaffins, enzyme stabilization, denaturation, biocompatibility, artificial bone, implant

11.1 Introduction

Silicon is the eighth most abundant element in the universe, and the second in the earth's crust after oxygen, making up 27.7% of the crust by mass. It is usually found on earth in the form of silicon dioxide, or as it is usually called, silica. Silica is found in soil, in aqueous environments, and in plants and animals. Silicon-based materials are used in a wide range of applications. They are used as substrates in catalysis, polymers, coatings, in chemical analysis, microelectronic devices and in biotechnology [2]. Silicon is an essential element in biology, especially in marine-based species such as sea sponges and diatoms. The simplest form of silica, silicic acid (SiO_4H_4), is the basis upon

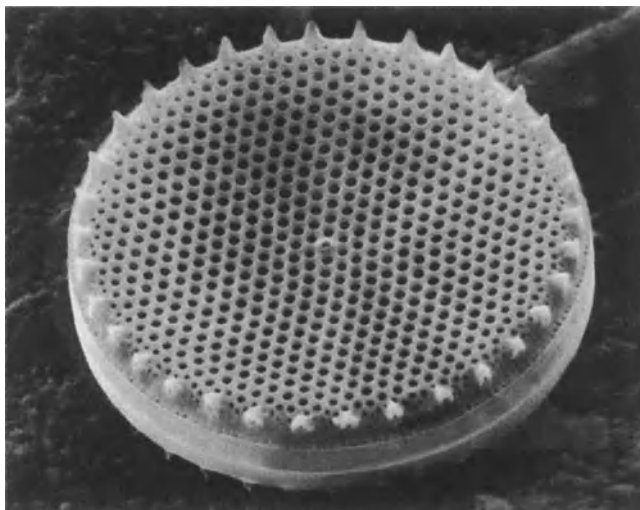


Figure 11.1 Centric diatom silica skeleton.

which the diatoms, a major group of algae, build their fascinating looking skeleton called frustule, Figure 11.1

It has been shown that both the physical structure of the silica skeleton, as well as the chemical functionalities of the walls of these structures, are very important in the life of the host organism. While the shape and strength of the structure provides a protecting armor against phytoplankton predators [3] it can also act as a proton buffer in order to improve the activity of various enzymes in the organism [4]. Moreover, it has been reported that due to their optical activity (they act as photonic crystals [5]) these silica structures can improve on the efficiency of photosynthesis in plants.

The synthesis of these elaborate nanostructures with an important biochemical role has recently become very intriguing to a variety of scientists. For them, understanding the mechanism(s) of biosilica formation was a priority. The elucidation of the synthetic pathways involved in the biosilicification process was of great importance in order for successful technological/industrial applications to follow.

While silica synthesis in the laboratory involves harsh and sometimes toxic conditions, long reaction times, and multiple steps, biosilicification is a synthetic pathway that requires very mild conditions. It usually occurs in nature under normal biological conditions producing exquisite hierarchical structures and multiple morphologies with precise nanoscale control [6].

From the early stages it was evident that occluded biopolymers and membranes of surrounding structures play a decisive role in biosilicification. Starting in 1999, it was evident that silicatein alpha could catalyze biosilicification based on the activity of the Ser/His and Cys/His proteases [7]. Since then, it has been discovered that several classes of biomolecules can mediate the formation of biosilica, including enzyme proteins [8–12], glycoproteins [13], polyamines [14] and polyamine-modified peptides [15–17]. The fact that the

protein filaments and their constituent subunits induce the polymerization of silica and silicone polymer network, both under chemical, and spatial control, has initiated a new era in silica applications. With this new class of materials easily accessible, biosilica-based nanomaterials have already found widespread applications in high-tech products.

Porous biosilica structures have found technological application as filters, adsorbents and thermal insulators. They offer large solid-gas interface and for this they have been used in gas sensors. In addition, their photoluminescent properties can be tailored by doping the biosilica during biosilicification. It has been shown that when some Si atoms are replaced by the dopant, the produced frustules exhibit both photoluminescent and electroluminescent properties [18, 19].

The unique characteristic of silica-based diatom cells is that their pore size is controlled from the nano to the micrometre range scale [20–22]. This is very important from the materials science point of view. During the last 10 years, there have been enormous strides into both better understanding of the mechanisms governing the formation of biostructures and the detailed characterization of these three-dimensional networks. We are now at the beginning of a very promising bioinspired-based nanotechnology era.

11.2 Silica Polymerization Process

There are many different chemical compounds based on silicon found in nature. The simplest one is that of the monomer orthosilicic acid, which is a weak acid with pK_a value of 9.8 [23]. This acid is found dissolved in all natural environments such as seawater, freshwater, soil water etc. Due to its low solubility, the concentration is usually very low, at a few parts per million (ppm). Many silicified organisms (diatoms, sponges and higher plants) are able to collect and utilize this monomer as the building block of their shell [24].

When the concentration of silicic acid exceeds 200 ppm, there is autopolycondensation which leads to the formation of elaborate structural motifs [25]. Although silicic acid has a high pK_a value (the conjugate base is mildly basic) when the polymerization initiates, the outer silanol groups become more acidic. This phenomenon provides a negative charge to the surface of the biosilica. The negative charge will then be able to interact with the environment, and any positively charged proteins or cell walls. The structures developed this way will end up with a large number of hydroxyl groups exposed to the environment. These are very active functional groups and will interact strongly with proteins, similarly to the activated carbon nanostructures. While the synthesis of silica is very well known and has been studied in depth [23], the formation of the elaborate silica structures found in nature, by organisms such as diatoms and sponges, was only recently uncovered [26, 27].

The formation of the siloxane bond (Si–O–Si) is the basis upon which the silica-based polymer is developed. The simplest precursor for the synthesis of the silica polymer is orthosilicic acid $[\text{Si}(\text{OH})_4]$.

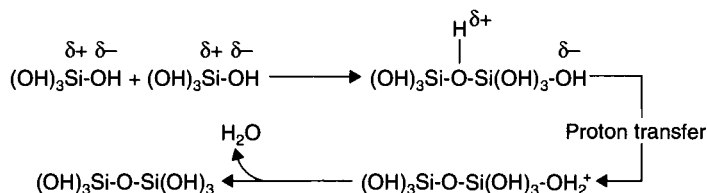


Figure 11.2 The polymerization process of the silicic acid.

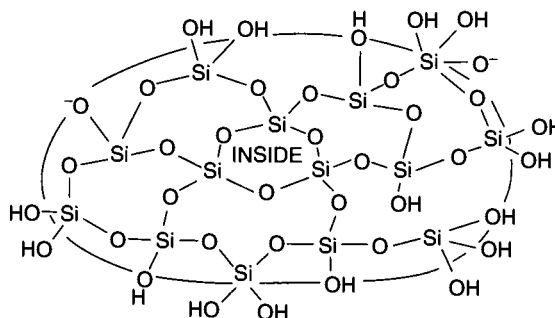


Figure 11.3 The silica structure with the negative hydroxy sites exposed.

This precursor can undergo self-polymerization condensation reactions forming the required siloxane bonds [28]. This reaction can initiate under neutral pH conditions, and when the concentration of the precursor, the silicic acid, is above the critical value of 1–2 mM [23, 29]. Depending on the initial pH value, the by-products of this condensation reaction can be just water without a change of the pH.

Alternatively, a hydroxyl ion will be liberated when the initial pH is in the basic region. It is interesting to note that the formation of silica oligomers results in an increase of the silanol group electrophilicity. This provides a further driving force and increases the rate of addition of the silicic acid monomers. Increasing the size of the polymer generates small particles, a process that is called the Ostwald ripening process [30]. This process leads to the growth of larger, insoluble particles [31] following a fractal growth process (Figure 11.3) [32]. This polymerization reaction is also aided by the addition of metal cations such as Mg^{2+} , Ca^{2+} and Sr^{+} , these positively charged ions interacting with the negatively charged silica particle surface, decreasing the effective negative charge [33]. The same behavior is observed when cationic polyelectrolytes are used [34], as we will see in the next section. Polyelectrolytes containing lysine, arginine, and allylamine are thus expected to promote the growth of the silica particles by adsorption of silicic acid monomers and oligomers onto the amino groups of these molecules.

The unique characteristic of the nanoparticles formed is that their surface is covered with free silanol groups [35]. It is these hydroxyl groups that are responsible for a strong adsorption of the negatively charged proteins onto the surface of the biosilica nanocomposites.

11.3 Biocatalytic Formation of Silica

Biomineralization is a precise *in vivo* mineralization process which leads to the formation of solid micro-nanostructures with fascinating physical characteristics. Biosilicification is the biomineralization that involves silica [27] and is defined as “the movement of silicic acid from environments in which its concentration does not exceed its solubility (<2 mmol/L) to intracellular or systemic compartments in which it is accumulated for subsequent deposition as amorphous hydrated silica” [36, 37].

The structured cell walls of diatoms (Figure 11.4) are spectacular examples of biomineralization and in particular biosilicification [38]. Diatoms are single-celled eukaryotic algae that are found in a variety of aqueous environments. Their cell walls are highly regular in shape and are made of silica. Their structure is highly hierarchical, which means that they possess pores that start from micrometers, and go down to a few nanometers. Such biominerals have exceptional physical characteristics, unique mechanical properties, and very valuable chemical functions. They are thus unique materials for the advancement of bionanotechnology.

The advancement in this area came about in 1969, when it was discovered that biosilicification is based on polycationic peptides (Figure 11.5) that exist on the diatom cell walls [39].

It was then suggested that the occurrence of these peptides in the protein of the diatom wall indicates a direct role in the silicification process [41]. Due to their affinity for silica, these proteins were named Silaffins (proteins with silica affinity). Silaffins had been implicated in the biosilicification process since 1999 [42] as they were found to promote silica condensation from a solution of monosilicic acid [42]. It has also been shown that silaffins can self-assemble into supramolecular structures which provide a template for the polycondensation of silicic acid [43].

It has been found that silicidin biomolecules such as phosphoproteins and silaffins induce silica nanoparticle formation [44–48]. As a result of biosilicification, elaborate and impressive silica structures are formed (Figure 11.6).

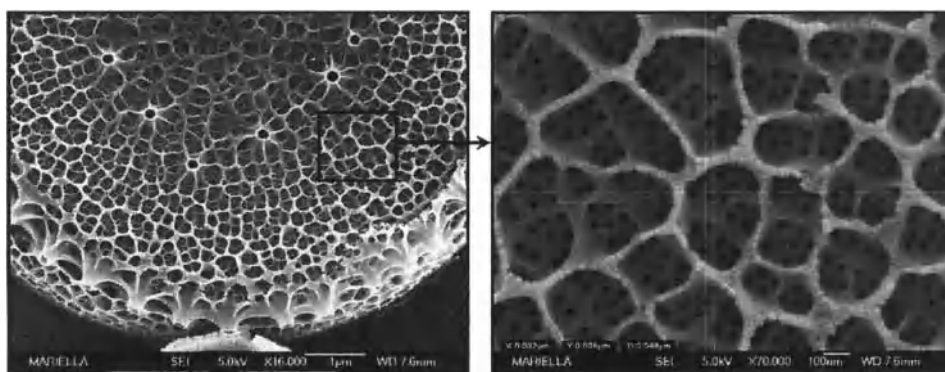


Figure 11.4 Microscope photographs of *Thalassiosira Rotula Meunier* [38].

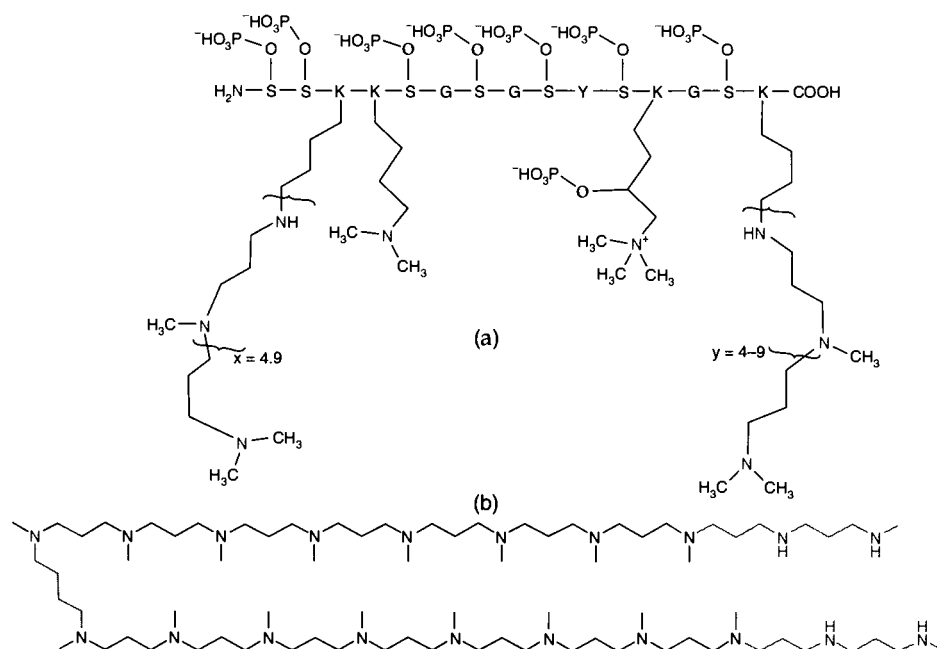


Figure 11.5 Molecular structure of (a) silaffins and (b) long chain polyamines (LCPA) [40].

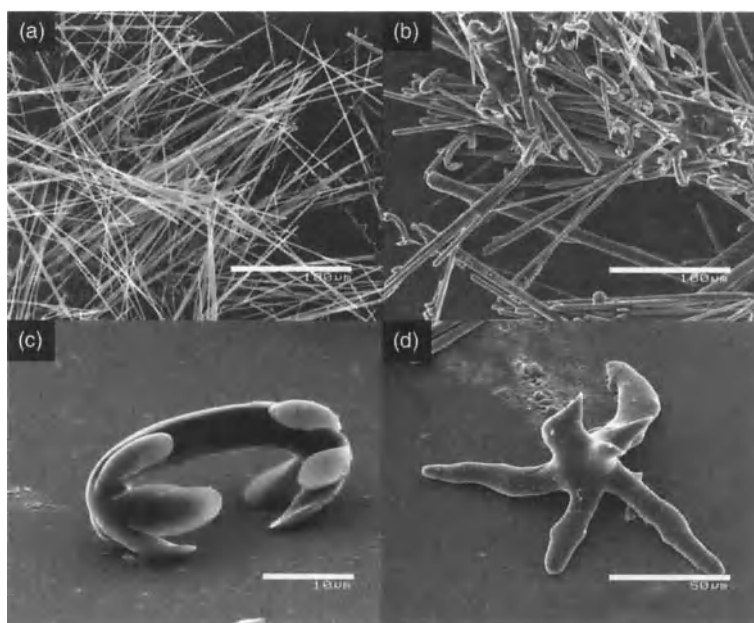


Figure 11.6 Examples of spicules formed under different concentrations of silicic acid [28].

In order to elucidate the formation of silica nanostructures by diatoms, a site-directed mutagenesis study of the non post-translationally modified silica precipitating R5 peptide (H2N-SSKSGSYSGSKGSKRRIL-COOH) found in silaffins was undertaken. Detailed studies on these proteins showed

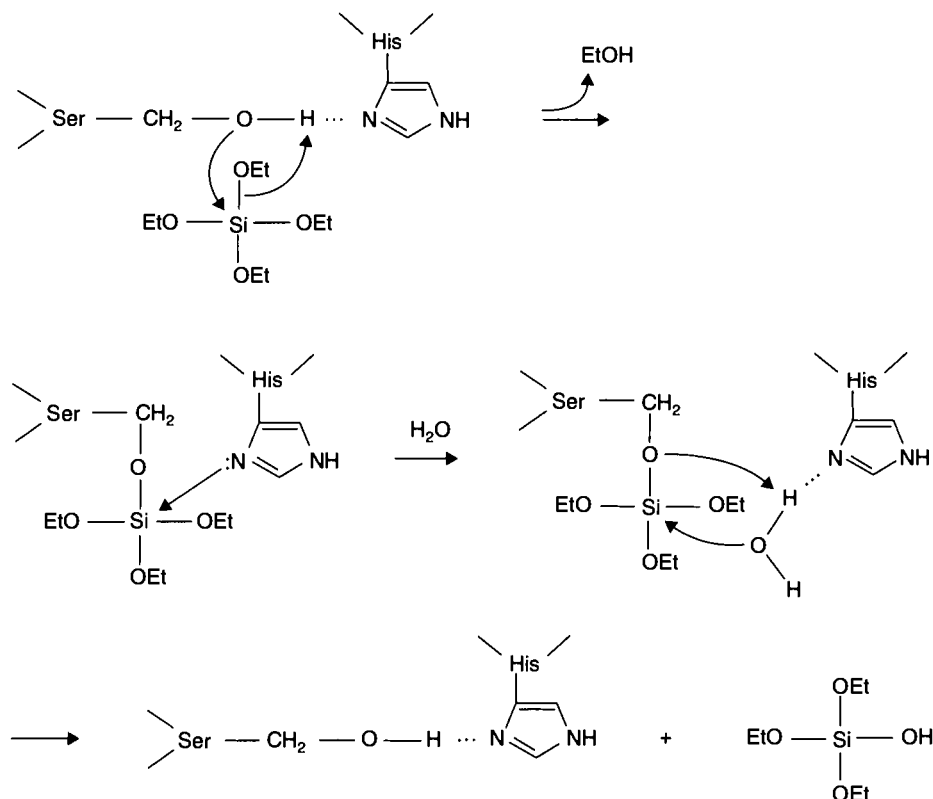


Figure 11.7 Mechanism of action of silicatein showing the key role of the amino acids present at the active site that are required for activity of the enzyme [25].

that they are a family of phosphoproteins which is composed of an acidic N-terminal domain (amino acids 20–107, theoretical $pI = 3.2$), and a strongly basic C-terminal part (amino acids 108–257, theoretical $pI = 11.7$) that is highly repetitive and has a very restricted amino acid composition with S (36%), K (20%), and G (15%) together constituting more than 70% of the amino acid residues [15].

The exact mechanism of the biosilicification catalyzed by these amino acids has been elucidated, as shown in Figure 11.7.

11.4 Biosilica Nanotechnology

Nanotechnology deals with the development of structures on a scale between 1–100 nanometers, and the exploitation of the physical phenomena that result from these structures. Although devices and processes based on nanotechnology can indeed provide humanity with novel tools, the establishment of such an industry depends almost entirely on the ability to manufacture nanoscale materials on a large scale. There are few such nanomanufacturing methods available today. To this, the biosilicification process can produce

nanostructured materials for a variety of nanotechnological applications. Biosilicification allows for the development of highly controlled nanopatterns that can be produced efficiently in large quantities and at low cost [49, 50]. Applications involving nanofabricated biosilica include selective separation [51], biosensors, microelectromechanical systems [52], and others [49]. Some of these will be addressed in the remaining part of this chapter.

11.5 Applications

11.5.1 Photonic Materials

Soon after the identification of polycationic peptides from the diatom *Cylindrotheca fusiformis*, simple silica nanospheres were synthesized *in vitro* [3, 4]. With the availability of such materials, the applications started to emerge. With the aid of the R5 peptide as a template, an organic/inorganic ordered array of silica nanospheres onto a polymer was created [53]. When a polymer hologram was exposed to a silicic acid, an ordered array of silica nanospheres was deposited onto the clear polymer substrate creating a system with a nearly 50-fold increase in diffraction efficiency. The ability to deposit biosilica combined with the ease of organic polymer processing gave rise to novel photonic materials [54].

11.5.2 Enzyme Stabilization

The term *stability* refers to the persistence of a proteins' molecular integrity or biological function in the face of adverse influences. During production, storage and experimental workup, enzymes undergo a variety of denaturation reactions. During denaturation, the enzymes, tertiary structure unfolds into a disordered polypeptide, the key residues no longer being aligned closely enough for continued participation in functional or structure stabilizing interactions [55]. The catalytic behavior of the protein is thus completely lost. A protein is also subject to chemical changes leading to an irreversible loss of activity or inactivation, particularly following unfolding [56].

There are many different methodologies that enable stabilization of proteins in general, and enzymes in particular. These methods involve protein engineering, chemical modification, use of additives or immobilization onto solid substrates. The first three methods are indirect, and usually involve substitutions of amino acids or chemical modifications of the side chains of the amino acids that can also be detrimental to the structure and function of the used protein.

More and more, the immobilization of the biological part onto solid substrates has become the preferred stabilization method. This method brings a huge advantage to the bioengineering applications involving proteins and enzymes. The choice of immobilization method used depends on the biomolecule to be immobilized, the chemical and physical nature of the immobilization

surface and, of course, on the specific application [57]. Whatever immobilization method is eventually chosen, careful consideration should be given in order to preserve the activity of the enzyme, not to reduce its specificity, to ensure that nonspecific binding does not occur and that the enzymes' active center can be easily reached by its substrate [57]. The immobilization of enzymes onto different substrates also allows for the reuse of the biocatalyst, leading to significant reduction in cost [58]. In addition to the obvious advantages, these immobilization methods also present a number of disadvantages. Either the proteins are not bound to the substrate strongly enough, or they are inactivated during work up. It has been lately shown that combining functional surfaces and well-structured nanocavities can provide a solution to the protein stabilization problem. The developments that nanotechnology brought through a large variety of nanomaterials, now provide the scientific world with materials that offer new perspectives and possibilities for the stabilization of enzymes.

In recent years, biosilica has proven to be a suitable alternative for enzyme stabilization. It is an inert material to many chemicals, it has a stable nanostructure, and a high specific surface area. In addition, the synthesis of biosilica under laboratory conditions allows for the development of materials with controllable pore size that can be tailored to the dimensions of a specific protein/enzyme and for a specific application [59]. Nanostructured materials such as biosilica, not only protect proteins from denaturation, but they also protect them from protease attacks which can destroy the enzymes very quickly [60].

In 2004, a method was presented for the first time for the encapsulation of enzymes and nanoparticles into silica matrices obtained by mimicking the biosilicification process that takes place in nature [61, 62]. A network of fused spherical silica particles with an average diameter of 500 nm is created, the enzyme butyrylcholinesterase [61], horseradish peroxidase or catalase [62]

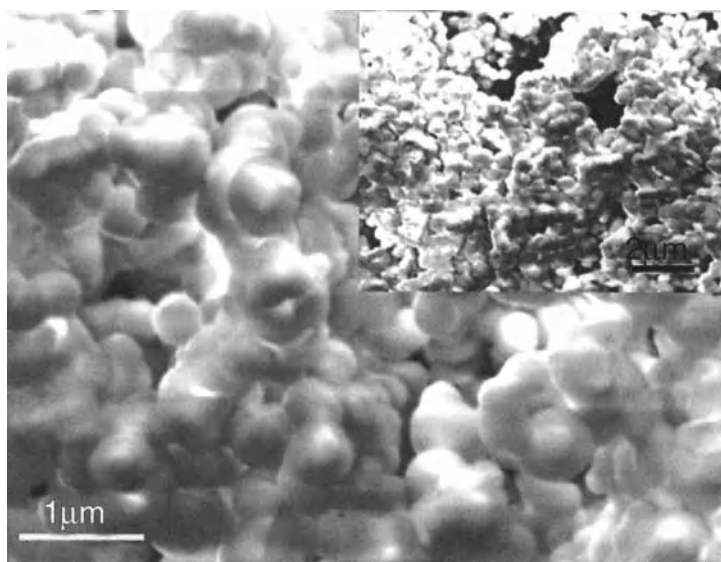


Figure 11.8 SEM micrograph of biosilica-immobilized butyrylcholinesterase [28].

being entrapped inside the particles during the synthesis process. In the case of butyrylcholinesterase (BuChE), the nanostructures were tested for enzyme activity and it was found that more than 95% of the initial free enzyme activity was retained. Only a small portion of it ($5.4\% \pm 4.9$) was lost during immobilization/encapsulation procedures. When compared with the stabilization degree obtained when chemically produced silica (sol gel) is used to encapsulate BuChE, only 10% of the initial enzyme activity was maintained. This is attributed to the mild synthetic conditions required for the biomimetic synthesis of silica, conditions that avoid any enzyme denaturation. The nanostructures constructed using biosilica also show increased enzyme loading capacity (20% w/w) as compared to the sol-gel process (0.1–5% w/w). This can be contributed to the hydrophobicity of alkyl silicates, the denaturing effect of alcohol by-products and also to the protein aggregation at elevated concentrations [61]. Using biosilica can also stabilize the proteins from thermal denaturation. BuChE encapsulated into biosilica and then subjected to thermal stress maintained 100% of its activity even after 30 days at 65°C [61]. The role of biosilica for this exceptional stability is once again clearly demonstrated. At this point the use of biosilica for protein stabilization has been well documented and optimized. It can be utilized effortlessly for the design of new enzyme-based systems, such as biosensors and bioreactors, as we will see in the next section.

11.5.3 Biosensor Development

The formation of silica under physiologically compatible (benign) reaction conditions has many technological applications. One such highly valued system is that of the biosensors [61]. Biosilica entrapped enzymes are actually an ideal substrate for biosensor development since they can produce composite materials with improved stability of the enzyme and no loss of activity. Even though there are not many reports on biosensors making use of the biosilica, due to the novelty of this process, the majority of them are based on electrochemistry. The entrapment of glucose oxidase [63] and acetylcholinesterase [60, 64] into biosilica, in the presence of carbon nanotubes, has been shown to allow the direct electron transfer from the enzyme to the immobilization surface. Biocatalytic precipitation of silica composites was realized with lysozyme providing the scaffold for silica formation in the case of glucose oxidase (GOx), and with poly(L-lysine) polymer in the case of acetylcholinesterase (AChE), both methods using tetramethyl orthosilicate (TMOS) as a silicic acid precursor. The reports published by Prof. Chaniotakis' group are the first ones to use poly(L-lysine) as a silica mediator for the encapsulation of enzymes and nanomaterials for the construction of biosensors. The process resulted in the formation of biocompatible, electrochemically active nanocomposite structures with high enzyme loading levels, with the carbon nanofibers efficiently promoting the electron transfer from the enzyme to the electrode surface leading to biosensors of enhanced analytical characteristics. The high electroactive surface, area of the composite confirms the understanding that intimate contact between the enzyme molecule and electrode surface is a prerequisite for direct electron

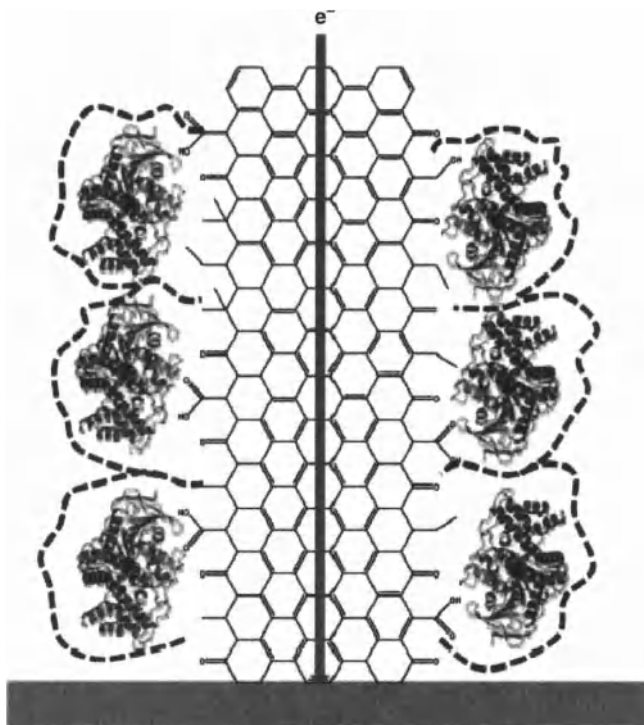


Figure 11.9 Biomimetically synthesized silica-carbon nanofiber architectures for the development of highly stable electrochemical biosensor systems [60].

transfer bioelectrocatalysis. Direct electron transfer between an enzyme and an electrode provides the potential for miniaturization and high power output. In addition, an enzyme-electrode based on direct electron transfer theoretically functions at a potential range that is close to the redox potential of the enzyme itself [63]. The biosilicification process resulted in decreasing both the thermal inactivation of the protein and the degradation from proteases found in the solution [60]. The transduction efficiency of carbon nanofibers in combination with the enhanced stabilization of the used enzyme encapsulated in biomimetically synthesized silica paved the way for a new class of biosensors that can find large-scale application in bioanalytical nanodevices.

More recently, in a 2009 report, β -glucuronidase (GUS) was encapsulated inside biomimetically synthesized silica, using protamine, a natural cationic polypeptide, as initiator of the condensation of sodium silicate. The enzyme loading efficiency was found to be as high as 100% within its solubility limit in aqueous solution. All enzyme molecules were proven to have been tightly entrapped in the biosilica particles, no leaching being registered even under high ionic strength conditions. The catalytic activity of the encapsulated GUS was evaluated by converting its substrate baicalin, into baicalein, both the substrate and the product being important effective components of herbal medicines. A high productivity of baicalein of 75% was obtained, only 5% lower than the case for its free enzyme counterpart. No appreciable loss in activity

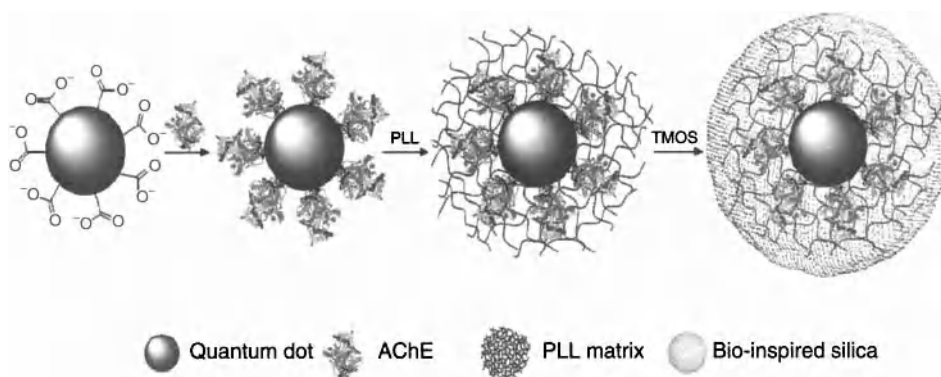


Figure 11.10 Biosilicated CdSe/ZnS quantum dots as photoluminescent transducers for acetylcholinesterase-based biosensors [66].

was observed after 30-days storage, and 80% of the initial activity could be retained after 4 repeated cycles.

The first biosensing system based on the photoluminescent properties of semiconducting CdSe/ZnS quantum dots (QDs) conjugated to AChE and further entrapped into biosilica has been reported recently [66]. Quantum dot-enzyme conjugates have been subjected to biosilicification with the use of poly(L-lysine) and TMOS. The QDs played the double role of immobilization platform for the enzyme and of signal transducer of the system due to their excellent photoluminescence efficiency. The response of the nanobiosensor to the addition of the enzymes' substrate is evaluated by monitoring the changes in the QDs' photoluminescence. These changes are related to the changes in pH of the surrounding environment of the QDs and are induced by the enzymatic reaction, which causes the production of acetic acid. Thus, the changes in the photoluminescence of the QDs can be associated with the analyte concentration. It was shown that the silica shell does not modify the optical properties of the QDs, and that its small pores allow for the free diffusion of the analyte to the active center of the enzyme.

11.5.4 Surface Modification for Medical Applications

There is a great interest in alternative bone-replacement materials in orthopedics, and bone surgery in particular. Bone replacement biotechnology can be aided significantly from on-site fabrication of hard, biocompatible biomaterials. The availability of silicatein catalyzed biosilica synthesis opens new possibilities for these applications, and has started to find its way into biomedical applications. Synthesis of silica-containing bioactive surfaces under ambient conditions that do not damage biomolecules like proteins is ideal for on-site and *in vivo* bone repair. Taking advantage of the strength, custom production, biocompatibility and stability of biosilica, allows for its utilization in this area. The mild conditions required for biosilicification (low temperature and pressure) render this material usable in such medical applications. There are

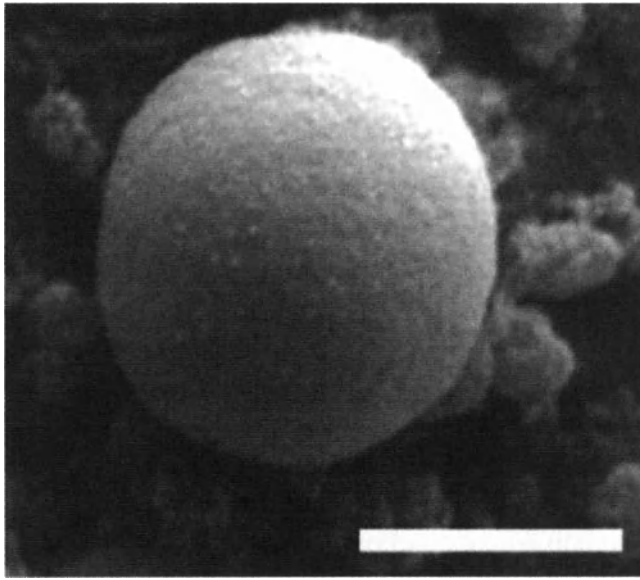


Figure 11.11 SEM ultrastructure of mineralizing SaOS-2 cultures grown on biosilica modified surface. Scale 2 μm [69].

a number of silica-containing materials that can be used as scaffolds in tissue engineering of bone and cartilage [67, 68].

The role of the bioactive materials is to stimulate the calcium phosphate precipitation on the damaged surface and to form a mechanically stable interfacial bone-like layer [67].

The enzymatic modification of the plates by biosilica deposition on the protein-coated surface can aid in the significant increase in calcium phosphate formation of SaOS-2 cells as observed by scanning electron microscopy (Figure 11.11) These results suggest that by supporting calcium-phosphate deposition *in vitro*, biosilica (silicatein)-modified surfaces are potentially bioactive *in vivo*, by stimulating osteoblast mineralization function.

When the osteosarcoma cells were grown in the absence of biosilica, mineralization was observed to a much smaller extent. The obtained biosilica-modified surfaces are shown to present biological activity and can be further used in order to enhance osteoblast function. These types of surfaces are of high interest for the tissue engineering field since they can be used to promote new bone formation [69]. This process can now be performed under physiologically relevant conditions [70]. This was achieved with the modification of the protein with a sequence that renders it binding affinity to hydroxyapatite (HA). Upon modification and expression, the HA-binding capacity of the enzyme is demonstrated in combination with both synthetic and dental HA. Furthermore, the immobilized modified silicatein was able to catalyze the biosilicification of both synthetic HA nanofibrils and dental HA. Due to these properties, this bioengineered silicatein presents important biomedical potential in the development of novel bioactive bone replacement materials or in the regeneration of bone

defects. In addition, tooth surface defects and dentinal tubules could be corrected by the use of immobilized silicatein-synthesized biosilica layers [70].

11.6 Conclusions

It has been only 12 years since the elucidation of the synthesis of the unique nanostructured biosilica. Within this period, the scientific community has understood the technological importance of this nanomaterial. Biosilica structures are being synthesized under very mild, biologically compatible conditions, providing new matrices for biotechnology-relevant applications. Their nanoporous structures allow for the construction of membrane structures for protein stabilization, biosensor development, chemical separations, and energy collection and transformation. Even though biosilica has already found a range of new applications, it is expected that further understanding of the formation mechanism will soon allow for the generation of biosilica structures with well-controlled and predicted morphologies. Such control will enable the custom tailoring of diatom silica structures for a wide variety of highly valued nanotechnological applications. Biomimetically synthesized silica will also find applications very soon in the areas of drug encapsulation, hormones and bioactive molecule stabilization for controlled delivery in organisms, and for the coating of metal implants that would result in a decrease in their cytotoxicity and an increase in their biocompatibility.

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Molecularly Imprinted Nanomaterial-based Highly Sensitive and Selective Medical Devices

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Abstract

Molecular imprinting provides a unique opportunity for the creation of three-dimensional cavities with tailored recognition properties. Over the past decade, the molecularly imprinted polymer (MIP) field has expanded considerably across a variety of disciplines, leading to novel approaches and many potential applications. The application of the imprinting approach to novel polymeric formats offers new insights and attractive methods for the preparation of synthetic receptors. In particular, MIP technology provides a means for ready access to nanostructured polymeric materials of predetermined selectivity. The versatility of the technique has attracted the attention of many workers for developing nanomaterials, with biological or biomimetic properties, for ultimate use in therapeutics and medical devices. The state-of-the art of MIP-based nanomaterials may set a new trend in the development of chemical sensors, and that will have a big impact on the future of nanoscience. In fact, the exotic properties of nanostructured materials, such as high surface/volume ratio, functionalization ability, favorable electronic and thermal features, and electrocatalytic characteristics have paved the way of designing many fabrication strategies for sensors and biosensors. In this monograph, we present a useful guide to all researchers who are interested in exploiting MIP technology for potential applications in the development of nanostructured sensors as highly sensitive and selective medical devices.

Keywords: Molecularly imprinted polymers, nanotechnologies, nanomaterials, nanostructure-based sensors, chemical sensors, medical devices

12.1 Introduction

Since clinical analyses of biomolecules are expensive and time-consuming processes in clinical laboratory settings, more and more measurements of analytes are performed at various locations, by hospital point-of-care settings, caregivers in non-hospital settings, and patients at home. Today one of the main

challenges is the development of suitable methods to perform the rapid *in situ* analyses. These methods must be sensitive and accurate, and able to determine various substances with different properties in “real-life” samples. In the past decade the field of molecular diagnostics has witnessed an explosion of interest in the use of nanomaterials in assays for gases, metal ions, DNA, and protein markers for many diseases. Extensive work has been done on practical, robust, and highly sensitive and selective detection agents that can address the deficiencies of conventional technologies. Chemists are playing a vital role in designing and fabricating new materials for application in diagnostic assays. In certain cases assays based upon nanomaterials have offered significant advantages over conventional diagnostic systems with regard to sensitivity, selectivity, and practicability. Some of these methods have recently been reviewed with a focus on the materials themselves or as sub-classifications in more generalized overviews of biological applications of nanomaterials [1–7].

Nanomaterials themselves can also form a novel platform of chemical/biological detections due to their unique electrical, optical, catalytic or magnetic properties [8, 9]. Moreover, the large surface-to-volume ratio and good dispersivity of nanomaterials provide a huge adsorptive surface for enriching target species [10–12]. In particular, the chemical immobilization of biological receptors such as antibodies and enzymes, or organic ligands at the surface of nanostructures, will further enhance the sensitivity and selectivity of detections [8, 13]. Various nanostructures have been investigated to determine their properties and possible applications in sensors and biosensors. These structures include nanotubes, nanofibers, nanorods, nanoparticles, thin polymeric films, nanocomposites, and metallic nanowires (Table 12.1). The properties of nanostructured materials, such as high surface/volume ratio, their ability to be functionalized, favorable electronic and thermal features, and electrocatalytic effect attracted considerable attention for the assembling of electrochemical enzyme-based sensors and biosensors. Therefore, chemo/biosensors based on functionalized inorganic or organic nanomaterials have widely been explored for the detections of various analytes, such as quantum dots for the fluorescent detection of nitroaromatic explosives [14], DNA [15] and enzyme activity [16, 17], and functionalized carbon nanotubes for the electrochemical detection of various organic/biological analytes [18, 19].

Although biological receptors have specific molecular affinity and have been widely used in diagnostic bioassays and chemo/biosensors, they are often produced via complex protocols with a high cost and require specific handling conditions because of their poor stability, and the natural receptors for many detected analytes don't exist [20–23]. Thus, efforts to develop biomimetic materials with antibody-like recognition characteristics have been driven by a number of factors including fundamental interest, the needs of analytical science, and the desire to establish novel therapies and medical devices. One of the most promising methods for the preparation of biomimetic materials is molecular imprinting, which is a technique for producing highly selective synthetic receptors and can, in principle, be applied to molecular structures spanning from ions to biomacromolecules [24, 21, 22]. The method involves the

Table 12.1 Typical nanomaterials.

Nanomaterials	Size (approx.)	Materials
Nanocrystals and clusters (quantum dots: QDs)	Diameter 1-10 nm	Metals, semiconductors, magnetic materials
Other nanoparticles	Diameter 1-100 nm	Ceramic oxides
Nanowires (NWs)	Diameter 1-100 nm	Metals, semiconductors, oxides, sulfides, nitrides
Nanotubes	Diameter 1-100 nm	Carbon, layered metal chalcogenides
Polymeric nanotubules	Diameter 20-200 nm	PPy; PANY; 1,2-DAB ^a
Nanocomposites	Diameter 20-200 nm	PPy; PANY; 1,2- DAB/nanotubes; nanoparticles ^b
2-dimensional arrays (of nanoparticles)	Several nm ² – μm^2	Metals, semiconductors, magnetic materials
Surfaces and thin films	Thickness 1 – 1000 nm	Various materials
3-dimensional structures (superlattices)	Several nm in all three dimensions	Metals, semiconductors, magnetic materials

^aPPy: poly(pyrrole); PANY: poly(aniline); DAB: (diaminobenzene).

^bNanotubes and nanoparticles as doping agent.

formation of cavities in a synthetic polymer matrix that are of complementary functional and structural character to a template molecule/entity.

The ability of molecularly imprinted polymers (MIPs) to selectively recognize and bind the template structure in the presence of closely related chemical species has made them of interest for use in a range of biomedical and biotechnological applications. Although molecular imprinting is parallel to the immune system's production of antibodies, it also offers significant contrasts and even advantages, such as no requirement for laboratory animals, time efficiency, relatively low cost of production, and no problem of immunogenic substances. Moreover, these materials are stable to extremes of pH, temperature, and organic solvents, unlike their biological counterparts [25]. As antibody-combining sites and receptor mimics, MIPs have demonstrated binding affinities and cross-reactivity profiles comparable to their biological counterparts, and have even been employed as substitutes for biological antibodies in medical diagnostic assays [26–29] and in a wide array of other analytical techniques. Following decades of effort, the first significant examples of MIPs mimicking the catalytic efficiencies and substrate selectivities of enzymes have been reported [30–32], following a strategy similar to that used for the production of catalytic antibodies [33]. Importantly, MIPs are now finding their way

into novel therapeutic tools, or medical devices, e.g., for controlled release and selective adsorption [34–43]. The rapid advancement toward the use of MIPs in medicine is further highlighted by recent reports of MIPs with blood compatibility [44] and of *in vivo* studies describing the fate of MIPs in an animal model [45]. This monograph represents a useful guide for all researchers who are interested in MIP technology, highlighting the potential analytical applications in terms of nanostructured sensors as highly sensitive and selective medical devices.

12.2 Molecular Imprinted Polymer Technology

12.2.1 Introduction of Molecular Recognition

Scientists have long ago discovered that biological recognition is very specific and selective. This specific recognition capability is a key factor for all of life processes, which require molecules to recognize each other and bind each other in order to react. For example, enzymes can bind the proteins in our food and form complexes that catalyze their decomposition [46]. Our biological defense system can form antibodies that can recognize the corresponding antigens and disarm them by forming harmless complexes [47]. These and other significant findings have been inspiring scientists and engineers for decades to mimic the exquisite recognition ability of biological molecules. In one outstanding example, Pederson discovered crown ethers [48] and did groundbreaking work in the creation of artificial compounds. By extending the work of Pederson, Cram designed and synthesized three-dimensional molecules that could mimic the functioning of natural molecules [49]. Lehn synthesized a three-dimensional molecule that combined with neurotransmitter in the brain, opening the possibility of creating artificial enzymes that function more effectively than natural enzymes [50]. For their development and use of molecules with structure-specific interaction of high selectivity, these three individuals shared the Nobel Prize in chemistry in 1987. Despite the successful design and synthesis of those “small-molecules” receptors, there remain challenges for designing and constructing synthetic macromolecular receptors. One promising approach for preparing macromolecular receptor is molecular imprinting.

12.2.2 Molecular Imprinting Polymerization: Background

Molecular imprinting involves the synthesis of highly crosslinked, thermoset polymers in the presence of template molecule(s) [51]. In most cases, the template is a small molecule, biological macromolecules, or a microorganism [52]. By removing the template molecules after polymerization, a 3D cavity remains in the network; the cavity has similar size dimensions and shape to the template, and functional groups are positioned well to complement the template molecule or analogue structure (Figure 12.1). These characteristics enable the network material to selectively recognize or “remember” the template in

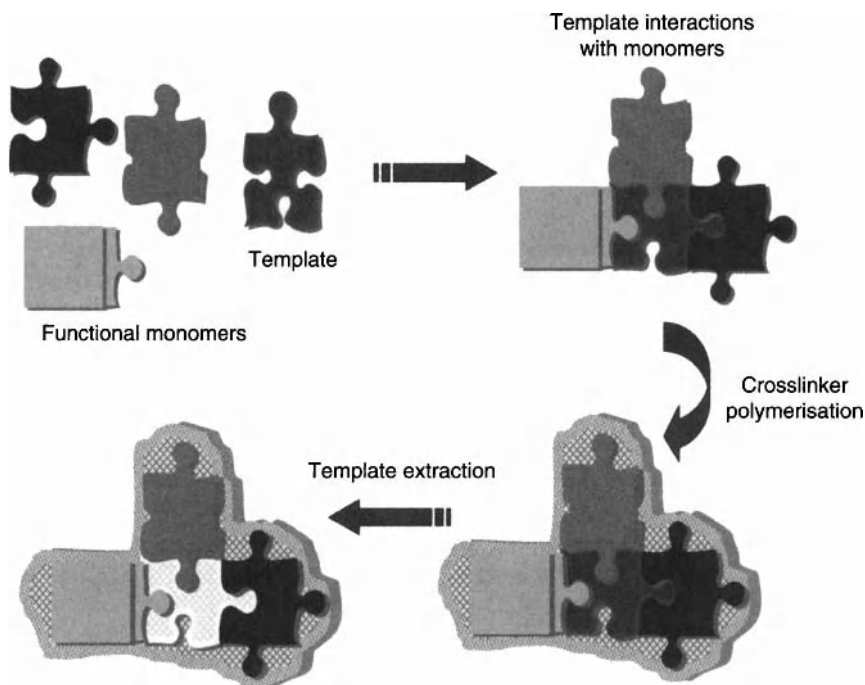


Figure 12.1 Schematic representation of molecular imprinting process.

subsequent applications. An excess of crosslinking monomer is ordinarily used to preserve and stabilize the specific cavities [53]. Before delving into polymerization mechanisms, knowing a brief history of imprinting is valuable.

12.2.3 Contributions of Polyakov, Pauling and Dickey

Polyakov [54] prepared silica for chromatographic applications by acidification of sodium silicate solutions by the addition of benzene, toluene, or xylene as a modifier on the silica gel. In other words, the “modifier” served as the template, which directly affected the surface properties of the resultant silica gel. Nine years later, Pauling proposed the theory of the antibody [55]; he thought that antibody formation occurred in the presence of an antigen, which played a role as template during the antibody formation. Although this antibody-antigen theory was later proven to be incorrect [56], this idea inspired the basic concept of molecular imprinting. Chemists found this idea to be useful by forming a 3D structure around the template to prepare synthetic antibody analogs that recognize the templates with similar selectivity as their biological counterparts. Dickey reported the first experiment to exploit this idea in 1949 [57], and his method described the preparation of silica gel via polymerization of sodium silicate in the presence of dye.

An outstanding breakthrough in molecular imprinting was achieved in 1972 when the group of Takagashi and Klotz [58] and Wulff and Sarhan [59] independently reported the first examples of molecular imprinting in synthetic

organic polymers. Wulff first proposed a “covalent approach” for the synthesis of MIPs. Latter, Mosbach and coworkers [60–62] developed a noncovalent interaction (e.g., hydrogen bonding, dipolar interaction, or electric interaction) approach to construct MIPs that eventually became more popular because of its simplicity.

12.2.4 Approaches Toward Synthesis of MIPs

Covalent imprinting refers to a molecular imprinting strategy whereby the template and monomer units are attached by covalent bonds to form a template-monomer complex after a chemical reaction, independent of polymer formation. Copolymerization of this template-monomer with a high proportion of crosslinker, in a porogenic solvent, results in a polymer which has template covalently bound within the polymer body. Removal of this template and subsequent rebinding step will both involve chemical reactions and the rebound template will be indistinguishable from template immediately following polymerization. MIPs obtained under the covalent approach have some interesting advantages over MIPs obtained under the noncovalent approach because the template molecule is covalently attached to the functional monomer. This means that a nonexcessive amount of functional monomer is required in the synthetic process, diminishing the nonselective interactions during the rebinding process. Homogeneity amongst all the binding sites and well-defined interaction sites are also achieved because they all came from the cleavage of the template molecule. Figure 12.2 depicts the basic

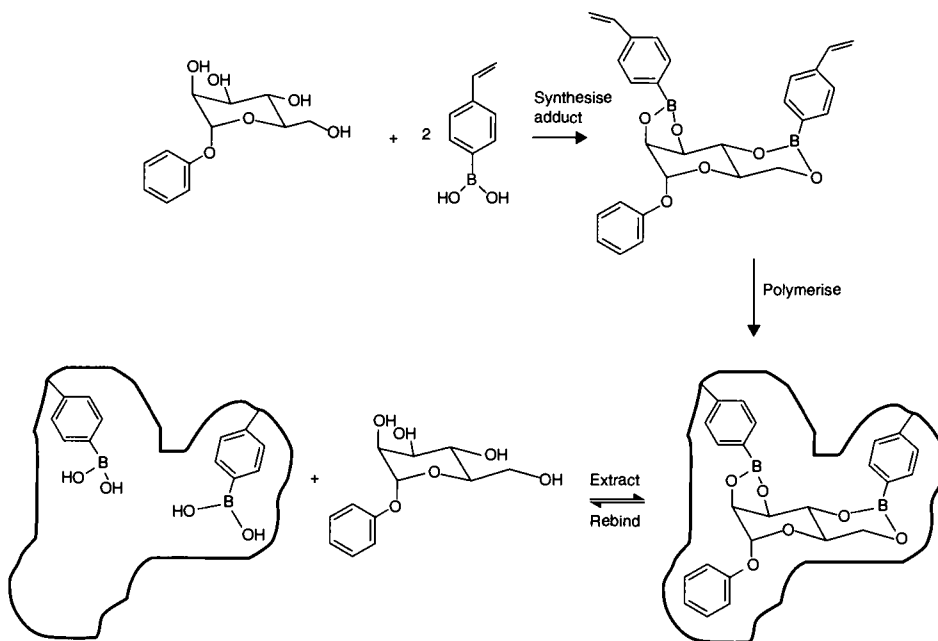


Figure 12.2 Molecular imprinting via covalent approach.

steps involved in producing a MIP under the covalent approach, as well as the rebinding of the target analyte onto the MIP. Although advocates of the covalent approach claim to achieve a more uniform distribution of imprinting sites than can be achieved by the noncovalent method, the limited options for reversible chemical reactions that can be used for covalent molecular imprinting circumscribe the applicability of this method. Also, in principle, some of the reversible reactions are suitable for imprinting and the subsequent rebinding, but the association-dissociation kinetics are too slow for chromatographic applications.

Currently, noncovalent molecular imprinting is the more widely applied technique to create the MIPs. Noncovalent imprinting uses the typical forces of attraction between molecules such as hydrogen bonds, ion-pairs, dipole-dipole interactions, hydrophobic interactions, and van der Waals forces to generate adducts of template (e.g., folic acid, FA) and functional monomers (2,4,6-triacrylamido-1,3,5-triazine, TAT) in solution (Figure 12.3). Compared to

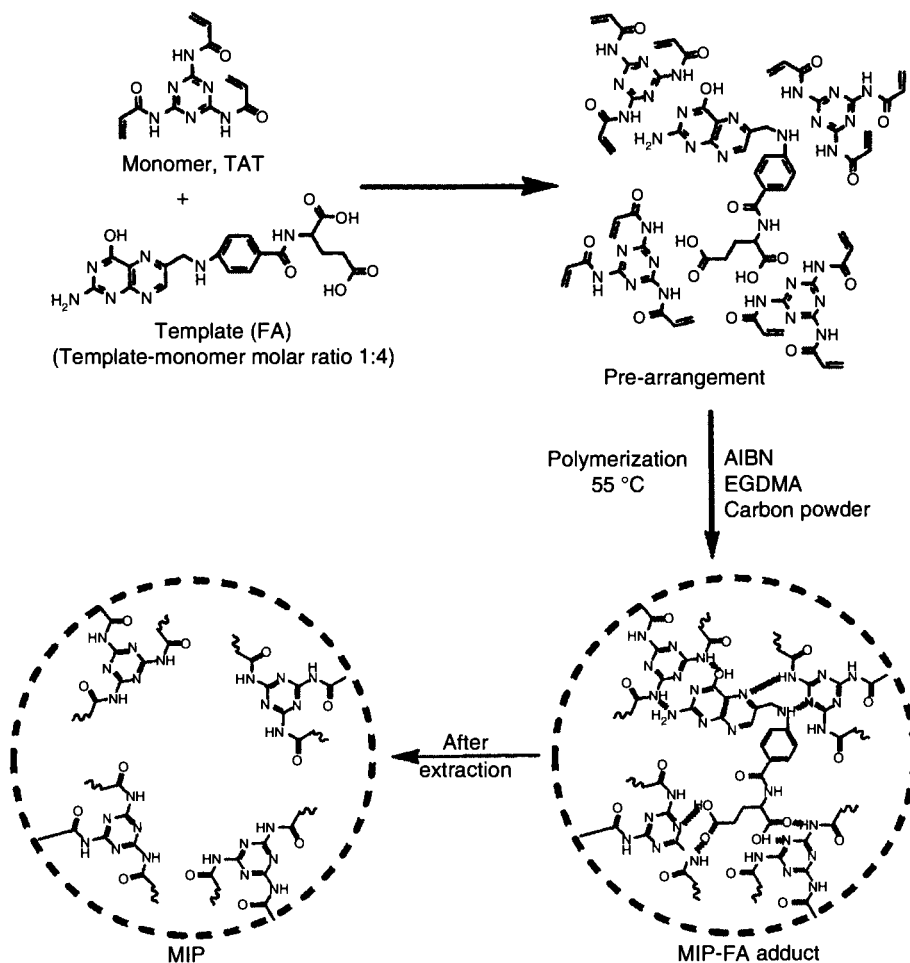


Figure 12.3 Molecular imprinting via noncovalent approach.

covalent bonding, these interactions are weaker and allow rapid and reversible binding, as needed for example in chromatographic separation applications. But at the same time, because of the weaker interaction, usually large excess amounts of functional monomers are required during the polymerization in order to have an effective imprinting. This excess functionality may cause some problems, especially for catalytic or preparative chromatography applications, because the binding sites in the resultant polymer networks are not exclusively located inside the cavities. That is to say those nonspecific binding sites are also present in the resulting polymer. Furthermore, since the interactions of template molecule and functional monomer during the polymerization process are noncovalent and polymerization is an exothermic process, the complex formed can be slightly deformed during the polymerization process. Once the MIP is obtained, the possible deformation of the complex template molecule-functional monomer causes heterogeneity amongst the binding sites. Another common drawback for this synthetic approach is the fact that, once the polymer is obtained, if there is any template molecule still trapped within the matrix, it may elute during the normal use of the polymer as a sorbent, masking the results obtained. This process is known as bleeding of the cartridge. The solution adopted to overcome the bleeding problem is to incorporate an analogue of the target molecule when aiming to detect low concentrations of the analyte of interest [63–65].

In the semicovalent approach, prior to the polymerization process, the template is covalently attached to the functional monomer and, once the polymer is obtained, rebinding of the target molecule is through noncovalent interactions. Therefore, this approach combines advantages from both the covalent and noncovalent approaches (Figure 12.4).

The advantages of the covalent approach are that it tries to keep homogeneity amongst the binding sites, has uniformity on the interaction sites and does not use an excess of functional monomer (therefore diminishing nonspecific interactions). The main advantage of noncovalent interactions is the fast kinetic uptake and release of the target analyte.

However, the semi-covalent approach suffers from the inconvenience that not so many template molecules can be attached to a functional monomer and that it is not as straightforward as the noncovalent approach. Because of the interaction between the functional monomer and the template molecule, there is normally a single interaction point within the cavity using this approach. For this reason, retention of the target analyte on the polymer obtained under this approach is sometimes lower than for polymers obtained under different protocols.

Another drawback of the semi-covalent approach is that, once the template molecule is removed from the MIP, the space generated within the cavity might not be large enough to properly accommodate the target analyte. This situation arises when the template molecule is directly attached to the functional monomer. In this case, to remove the template molecule, a covalent bond must be cleaved and, since rebinding of the target analyte to the MIP is through noncovalent interactions, these interactions need more distance to be established.

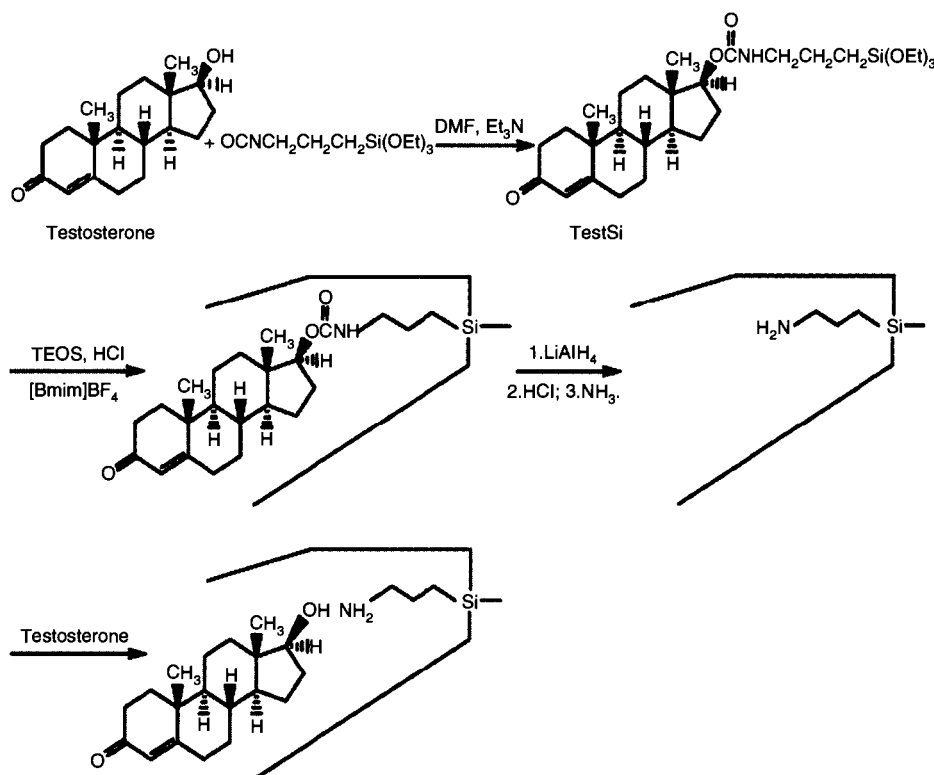


Figure 12.4 A representation of the basic steps involved in the synthesis of a MIP under the semi-covalent approach incorporating a sacrificial spacer [66].

Therefore, the cavity generated by the template molecule covalently bonded to the functional monomer during the synthesis of the MIP might not be able to properly accommodate a hydrogen bond interaction between the target molecule and the functional monomer.

12.2.5 Optimization of the Polymer Structure

Synthesis of MIPs is a chemically complex pursuit and demands a good understanding of chemical equilibrium, molecular recognition theory, thermodynamics, and polymer chemistry in order to ensure a high level of molecular recognition [67]. The polymers should be rather rigid to preserve the structure of the cavity after splitting off the template. On the other hand, a high flexibility of the polymers should be present to facilitate a fast equilibrium between release and reuptake of the template in the cavity. These two properties are contradictory to each other, and a careful optimization became necessary. The challenge of designing and synthesizing an MIP can be a daunting prospect to the uninitiated practitioner, not least because of the sheer number of experimental variables involved, e.g., the nature and levels of template, functional monomer(s), crosslinker(s), solvent(s) and initiator, the method of initiation

and the duration of polymerization. Moreover, optimization of the imprinted products is made more difficult due to the fact that there are many variables to consider, some or all of which can potentially impact upon the chemical, morphological, and molecular recognition properties of the imprinted materials. Fortunately, in some instances it is possible to rationally predict how changing any one such variable, e.g., the crosslinker ratio, is likely to impact upon these properties [68].

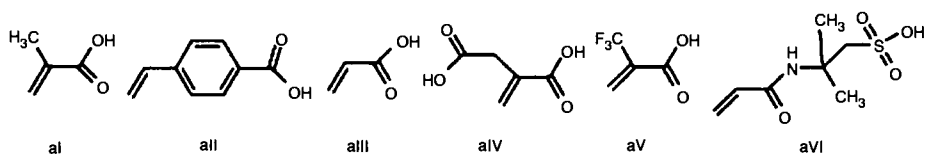
12.2.5.1 *Template*

The template is of central importance and it directs organization of the functional groups pendent to the functional monomers in all molecular imprinting processes. In terms of compatibility with free radical polymerization, templates should ideally be chemically inert under the polymerization conditions, thus alternative imprinting strategies may have to be sought if the template can participate in radical reactions or is for any other reason unstable under the polymerization conditions. The following are legitimate questions to ask of a template: (1) does the template bear any polymerizable groups? (2) does the template bear functionality that could potentially inhibit or retard a free radical polymerization? (3) will the template be stable at moderately elevated temperatures or upon exposure to UV irradiation? The imprinting of small, organic molecules (e.g., pharmaceuticals, pesticides, amino acids and peptides, nucleotide bases, steroids, and sugars) is now well established and considered almost routine. Optically active templates have been used in most cases during optimization. In these cases the accuracy of the structure of the imprint (the cavity with binding sites) could be measured by its ability for racemic resolution, which was tested either in a batch procedure or by using the polymeric materials as chromatographic supports. One of the many attractive features of the molecular imprinting method is that it can be applied to a diverse range of analytes; however, not all templates are directly amenable to molecular imprinting processes. Most routine MIPs were using small organic molecules as template. Although specially adapted protocols have been proposed for larger organic compounds, e.g., proteins, cells, imprinting of much larger structures is still a challenge. The primary reason is the fact that larger templates are less rigid and thus do not facilitate creation of well-defined binding cavities during the imprinting process. Furthermore, the secondary and tertiary structure of large biomolecules such as proteins may be affected when exposed to the thermal or photolytic treatment involved in the synthesis of imprinted polymers. Rebinding is also more difficult, since large molecules such as peptides and proteins do not readily penetrate the polymer network for reoccupation of binding pockets.

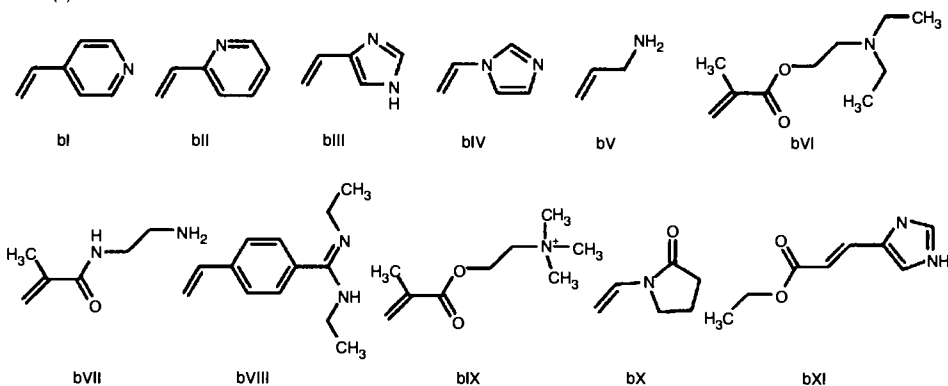
12.2.5.2 *Selection of Monomers*

The careful choice of functional monomer is of the utmost importance to provide complementary interactions with the template and substrates (Figure 12.5). For covalent molecular imprinting, the effects of changing the template

Acidic (a)



Basic (b)



Neutral (n)

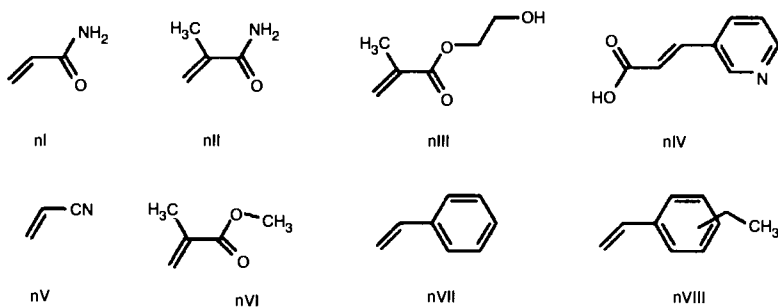


Figure 12.5 Structure of common functional monomers: Acidic; aI: methacrylic acid; aII: p-vinylbenzoic acid; aIII: acrylic acid; aIV: itaconic acid; aV: 2-(trifluoromethyl)-acrylic acid; aVI: acrylamido-(2-methyl)-propane sulfonic acid. Basic; bI: 4-vinylpyridine; bII: 2-vinylpyridine; bIII: 4(5)-vinylimidazole; bIV: 1-vinylimidazole; bV: allylamine; bVI: N, N-diethyl aminoethyl methacrylamide, bVII: N-(2-aminethyl)-methacrylamide; bVIII: N, N-diethyl-4-styrylamidine; bIX: N, N, N-trimethyl aminoethylmethacrylate; bX: N-vinylpyrrolidone; bXI: urocanic ethyl ester. Neutral; nI: acrylamide; nII: methacrylamide; nIII: 2-hydroxyethyl methacrylate; nIV: trans-3-(3-pyridyl)-acrylic acid; nV: acrylonitrile; nVI: methyl methacrylate; nVII: styrene; nVIII: ethylstyrene.

to functional monomer ratio is not necessary because the template dictates the number of functional monomers that can be covalently attached; furthermore, the functional monomers are attached in a stoichiometric manner. For noncovalent imprinting, the optimal template/monomer ratio is achieved empirically by evaluating several polymers made with different formulations with increasing template [69]. The underlying reason for this is thought to originate

with the solution complex between functional monomers and template, which is governed by Le Chatelier's principle. Applying Le Chatelier's principle to the complex formed prior to polymerization, increasing the concentration of components or binding affinity of the complex in the prepolymerization mixture would predict an increase in the prepolymer complex. Correspondingly, there is an increase in the number of final binding sites in the imprinted polymer, resulting in an increased binding or selectivity factor per gram of polymer. From the general mechanism of formation of MIP binding sites, functional monomers are responsible for the binding interactions in the imprinted binding sites, and for noncovalent molecular imprinting protocols, which are normally used in excess relative to the number of moles of template to favor the formation of template-functional monomer assemblies. It is very important to match the functionality of the template with the functionality of the functional monomer in a complementary fashion (e.g., H-bond donor with H-bond acceptor) in order to maximize complex formation and thus the imprinting effect. Higher retention and resolution was found by the two co-monomer imprinting polymer than the single monomer imprinting polymer, which indicated an increase in the affinity of the MIP with the sample as a result of the cooperation effect of the binding sites. However, it's important to bear reactivity ratios of the monomers to ensure copolymerizations to be feasible.

12.2.5.3 *Selection of Crosslinker*

Since a very high degree of crosslinking (70-90%) is required for achieving specificity, only a limited number of crosslinkers have been utilized in the molecular imprinting (Figure 12.6). The solubility of the crosslinker itself in the prepolymerization solution and the solubility of the monomerized template species reduce the number of possible alternatives. Nevertheless, several different crosslinkers have been tried with different degrees of success [70]. Originally, isomers of divinylbenzene were used for crosslinking of styrene and other functional monomers into polystyrenes. Later, it was found that acrylic- or methacrylic-acid-based systems could be prepared with much higher specificity. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are presently commonly employed in several systems [71]. In recent years, several other crosslinkers have been studied. Thus, tri- and tetrafunctional acrylate crosslinkers, such as pentaerythritol triacrylate (PETRA) and pentaerythritol tetraacrylate (PETEA) have been used for the preparation of peptide-selective molecularly imprinted polymers [72-73].

12.2.5.4 *Choice of Porogens*

The solvent (porogen) plays an important role in the outcome of a molecular imprinting process, a role which is particularly pronounced in self-assembly systems [74, 75]. To be a porogen in the imprinted polymerization, the solvent governs the strength of noncovalent interactions in addition to its influence on the polymer morphology. Generally, the more polar the porogen, the weaker the resulting recognition effect becomes, as a consequence of the influence of the

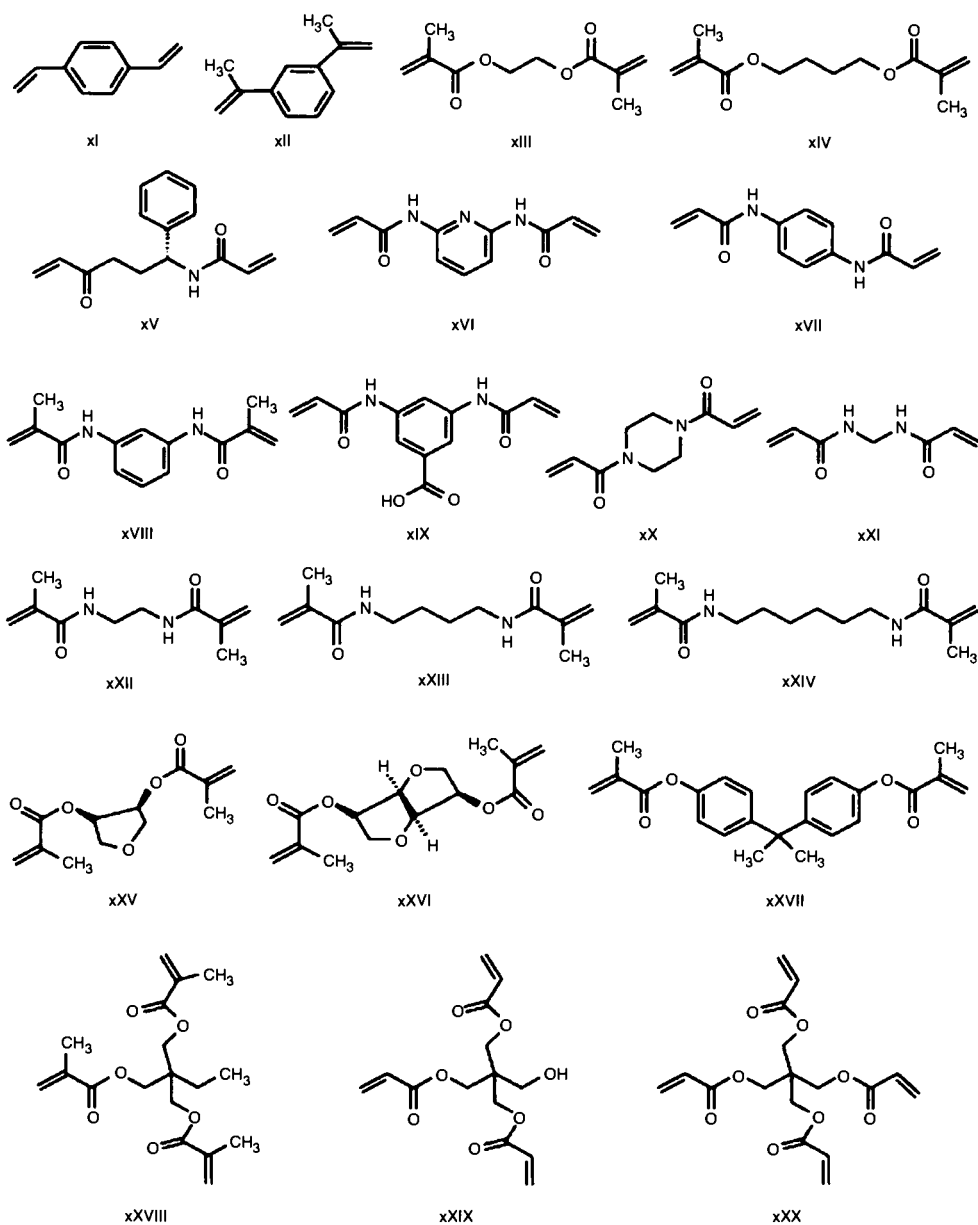


Figure 12.6 Structure of commonly used crosslinkers: xI: p-divinylbenzene; xII: 1, 3-diisopropenyl benzene; xIII: ethylene glycol dimethacrylate; xIV: tetramethylene dimethacrylate; xV: N,O-bisacryloyl-L-phenylalaninol; xVI: 2,6 bisacryloylamido pyridine; xVII: 1, 4-phenylene diacrylamide; xVIII: N,N'-1,3-phenylenebis(2-methyl-2-propenamide); xIX: 3,5-bisacrylamido benzoic acid; xX: 1,4-diacryloyl piperazine; xXI: N,N'-methylene bisacrylamide; xXII: N,N'-ethylene bismethacrylamide; xXIII: N, N'-tetramethylenebismethacrylamide; xXIV: N,N'-hexamethylenebismethacrylamide; xXV: anhydroerythritol dimethacrylate; xXVI: 1,4,3,6-dianhydro-D-sorbitol-2,5-dimethacrylate; xXVII: isopropylenebis(1,4-phenylene)dimethacrylate; xXVIII: trimethyl propane trimethacrylate; xXIX: pentaerythritol triacrylate; xXX: pentaerythritol tetraacrylate.

Table 12.2 Polar and nonpolar solvents as porogens in molecular imprinting.

Name	Boiling Point (°C)	Dielectric constant
High polar solvent		
Water	100	80
N,N-dimethylformamide	153	38.3
Methanol	68	33
Acetonitrile	81	36.6
Dimethylsulfoxide	189	46.7
Low polar solvent		
Dichloromethane	39.8–40	9.1
Tetrahydrofuran	66	7.52
Chloroform	60.5–61.5	4.8
Hexane	69	2.02
Toluene	111	2.4

solvent polarity on noncovalent interactions [76-78]. The best imprinting porogens for accentuating the binding strengths are solvents of very low dielectric constant, such as toluene and dichloromethane. The use of more polar solvents will inevitably weaken the interaction forces formed between the print species and the functional monomers, resulting in poorer recognition. On the other hand, the influence of porogen on the structure of the prepared polymers may compensate for this apparent drawback on the specific surface area, and the mean pore diameter of the polymer structure is dramatically dependent on the type of porogen used. Thus, acetonitrile (ACN) as a fairly polar solvent leads to more macroporous polymers than chloroform (Table 12.2). A lower surface area and a lower macro-porosity may lead to diminished recognition, because of lower accessibility to the sites.

In the recognition step, similar questions about the choice of solvent arise. Since all noncovalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. When applying the polymers to gradually more polar solvents, the recognition is diminished. Also, the morphology is affected since the swelling of the polymers is dependent on the surrounding medium. Thus, swelling is most pronounced in chlorinated solvents, such as chloroform and dichloromethane (DCM), as compared to, e.g., ACN and tetrahydrofuran (THF). This swelling behavior may lead to changes in the three-dimensional configuration of the functional groups taking part in the recognition in the polymer binding sites resulting in poorer binding capability. As a rule of thumb, the best choice of recognition solvent should be more

or less identical to the imprinting porogen in order to avoid any swelling problems, although this is not necessarily a prerequisite. The polymer swelling taking place when polymers are prepared in organic porogens and subsequently used in aqueous phase, is not necessarily a gross obstacle, however. The swelling in water is approximately the same as for many other solvents, such as acetonitrile. Insofar as the selection of porogen is concerned, it is also desirable that the solvent used should have a high boiling point and be inert under polymerization conditions. Further, to support the effective and homogeneous heating of the polymerizing mixture, especially at the final stages of the process when most of the monomer molecules have been polymerized and have limited mobility, the absorbent of the thermal energy recommended should be a polar solvent. Of all polar solvents, dimethylsulfoxide (DMSO) is preferred in the whole work because it is an inert medium with high boiling point and absorbing ability of thermal energy [79].

12.2.5.5 Polymerization Conditions

Several studies have shown that polymerization of MIP at lower temperatures forms polymers with greater selectivity versus polymers made at elevated temperatures. Usually, most people used 60°C as the polymerization temperature. However, the initiation of the polymerization reaction was very fast and therefore hard to control at this temperature, and that resulted in low reproducibility of MIP. Furthermore, the relatively high temperatures have a negative impact on the complex stability, which reduces the reproducibility of the monolithic stationary phases and produced high column pressure drops. Thus, the relatively low temperatures with a prolonged reaction time are selected in order to yield a more reproducible polymerization. Where complexation is driven by hydrogen bonding then lower polymerization temperatures are preferred, and under such circumstances photochemically active initiators may well be preferred as these can operate efficiently at low temperature. For example, Mosbach *et al.* [80] presented a study on enantioselectivity of L-PheNHPh-imprinted polymers, one polymer being thermally polymerized at 60°C, the other photochemically polymerized at 0°C. The results showed that better selectivity is obtained at the lower temperature versus the identical polymers thermally polymerized [81]. The reason for this has again been postulated on the basis of Le Chatelier's principle, which predicts that lower temperatures will drive the prepolymer complex toward complex formation, thus increasing the number and, possibly, the quality of the binding sites formed.

12.2.5.6 Binding Site Heterogeneity

During the formation of noncovalent complexes between the template and functional monomers, the template will compete with the functional monomers to interact with other template molecules; in this case, the polymerization will create a special kind of binding site heterogeneity [82] caused by the imprinting of template aggregates. This phenomenon could be minimized by using a lower concentration of template during molecular imprinting and by

wisely choosing the solvents and functional monomers. Because the template aggregates are not strictly defined, the resultant binding sites may have different affinities with the template.

As polymerization proceeds, there may also be a change in the solution-phase molar ratio of functional monomer to crosslinker if one polymerized more readily. The result is a time-dependent crosslinking density. Polymer produced at low conversions may be more or less rigid than polymer produced at high conversions. As a consequence, different crosslinking densities around the binding sites are expectedly formed [83], thereby resulting in different degrees of accessibility. In general, most MIPs have a wide pore-size distribution associated with various degree of diffusional mass transfer limitation. The sites associated with meso- and macro-pores (>20 Å) are easily accessible compared to the sites located in micro-pores (<20 Å) when diffusion is slow [84].

As mentioned earlier, in the noncovalent approach, excess amounts of functional monomers are used to form prepolymeric complexes between template and functional monomers. These excess amounts will cause a portion of the functional monomer to exist randomly positioned in the system without any association with the template. Thus, various structures of the prepolymerization complexes would be expected, including nonassociated functional monomers. These nonassociated functional monomers will form binding sites with various association constants that are influenced by the local environment.

Finally, some binding sites may collapse or otherwise deform during the extraction processes, and these deformation events also generate variations in the association constants of the binding sites with the template.

When heterogeneous MIPs are applied in chromatographic separations, nonlinear binding isotherms lead to a tailing problem for solute that is retained on the most favorable binding sites. So far, the mechanism of retention of the template on the imprinted polymers is not understood very clearly; although a simple ion-exchange model was found to be effective to describe retention in certain MIPs [85]. A better understanding of the imprinting process and the recognition mechanism will certainly lead to improved imprinting techniques and enhanced chromatographic performance.

12.2.5.7 *Methods for Preparing MIPs*

Most of the literature examples for preparing MIPs use highly crosslinked organic polymers as the imprinting matrix, and most of these are synthesized by free radical copolymerization of functional monomers with a large amount of crosslinking monomer in the presence of solvent. The materials are usually prepared by the so-called monolithic technique, which means that the polymerization builds polymer monoliths that need to be sieved prior to use. The main disadvantages for this technique are the loss of material and disruption of imprint sites during grinding and sieving, the large size distribution of the

ground particles, and the irregular particle shape, all of which contribute to the heterogeneity of binding sites in particles and transport problems in applications. More details for this technique and other surface imprinting approaches are introduced in the following sections.

12.2.5.7.1 Monolith Technique by Free Radical Polymerization

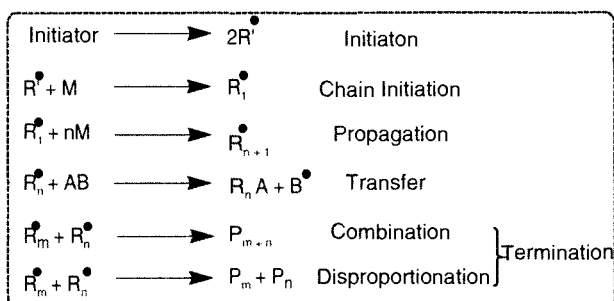
The double bond in most vinylidene monomers reacts with free radicals, radical cations, and radical anions to form a transient radical with a lifetime long enough to react with another double-bond-containing monomer. By repeating this process, a polymer chain forms. Meanwhile, the growth of the polymer chain can be stopped by several termination reactions. The major steps in addition polymerization are initiation, propagation, and termination.

a. Initiation

Due to its electrical neutrality, the free radical is a generally useful initiator compared to cationic and anionic initiators. Free radical polymerizations are affected to a small degree by the presence of acids and bases or high polarity of solvents. These characteristics make free radical polymerization popular for the preparation of MIPs because a wide variety of reaction conditions can be chosen to accommodate imprinting of different templates. Initiation of free radical polymerization can occur by thermal or photochemical means. Azo or peroxide initiators, with bond energy of 105-170 kJ mol⁻¹ [86], are used most commonly in molecular imprinting. Scheme 12.1 shows a general mechanism for free radical polymerization. Since the rate of initiation is much slower than the consumption of the initiator radical, the rate of initiator decomposition can be considered as the rate of initiation, it is written as:

$$V_i = \frac{d[R1^\bullet]}{dt} = 2k_d f[I] \quad (12.1)$$

where the factor 2 indicates that two radicals are generated per initiator. k_d is the decomposition rate constant of initiator,

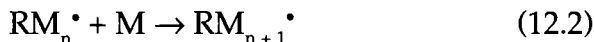


Scheme 12.1 General free radical polymerization mechanism.

f represents the efficiency of a radical to propagate a chain, $[I]$ is the concentration of initiator and $R_1^\bullet$ is the concentration of propagating chains.

b. Propagation

An active chain forms when the initiator free radical reacts with a monomer unit, and propagation occurs when the active chain reacts with additional monomer units.



The rate of propagation is given as:

$$V_p = k_p [M] [RM^\bullet] \quad (12.3)$$

where $[RM^\bullet]$ represents the concentration of reactive chains.

c. Termination

When chain transfer agents are present, or if the concentration of free radicals is too high, the free radicals can react with each other or with the chain transfer agents. These chain transfer agents could be solvent, monomer, and template molecules. Such reactions form an inactive covalent bond and terminate propagation by the reactive chains. The rate of termination is given as:

$$V_t = 2k_t [RM^\bullet]^2 \quad (12.4)$$

d. Steady state

Under conditions of steady state polymerization, the rate of production of free radical equals to the rate of consumption, i.e.,

$$V_i = 2k_d f [I] = V_t = 2k_t [RM^\bullet]^2 \quad (12.5)$$

Solving for $[RM^\bullet]$ in equation 1.5 and substituting the resulting expression into equation 1.3 gives the rate of polymerization:

$$V_p = k_p \{f k_d [I]/k_t\}^{1/2} [M] \quad (12.6)$$

Although this free radical polymerization has been applied in most cases to construct monolith MIPs that need to be ground and sieved prior to application, several new ideas have been proposed recently. In one example, the polymerization mixtures (including template, monomers, initiator, solvents) are filled into the pores of silica gel, then the polymerization is initiated by heating or UV radiation, and finally, the silica gel is dissolved and separated to produce spherical beads consisting of 100% MIP [87]. Figure 12.7a illustrates this

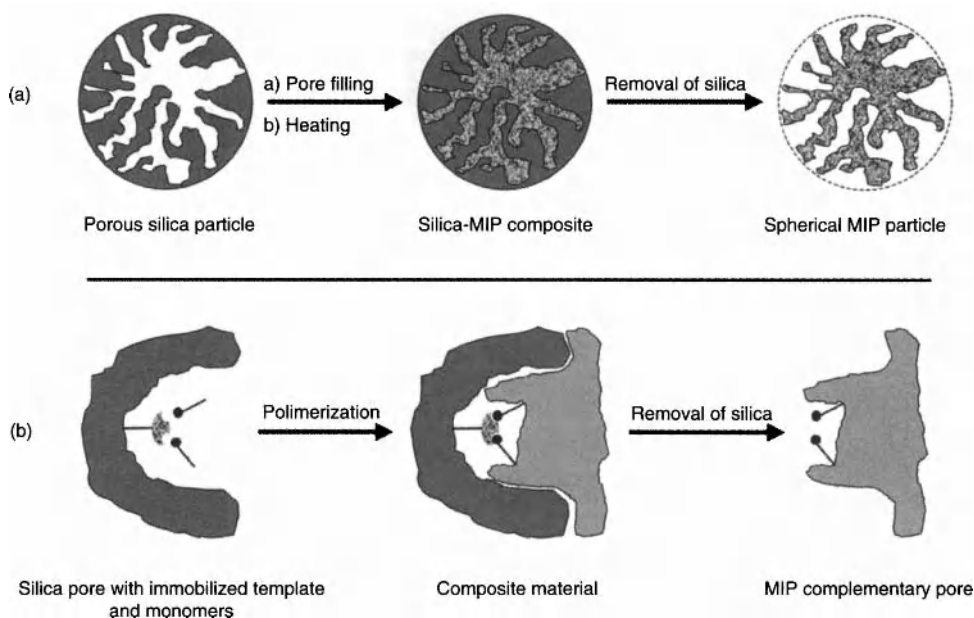


Figure 12.7 New approaches for conventional free radical polymerization [89].

approach. This study concluded that silica-MIP composites and spherical MIPs, when used as chromatographic stationary phase materials, showed higher mass transfer rates and lower back pressures than conventionally prepared MIPs. However, significant peak broadening and tailing were still observed. Another approach has been proposed in order to overcome mass transfer limitations [88]. Figure 12.7b illustrates this approach, whereby the template molecule is immobilized covalently to a silica surface first, then polymerization and silica dissolution are carried out using the above mentioned approach. This approach makes sure that all binding sites are located near the surface and template-template interactions may be avoided during polymerization. A drawback is that loading capacity seems very critical, especially for the separation and recognition of large biomolecules, such as proteins.

12.2.5.7.2 Surface-Confined Polymerization

Although the feasibility of application of MIP materials has been demonstrated, commercial uses are still few. The MIPs prepared by conventional methods (monolithic technique) show high affinity binding sites; and, at the same time, the morphology, pore properties and other structure features of the polymers are nonoptimal for template mass transfer. In order to solve the problem of slow mass transfer, some attempts have been tried to make MIPs in bead form by suspension polymerization [90], precipitation polymerization [91], and emulsion polymerization [92] techniques. Major concerns of the multiphase polymerization techniques include: (a) for suspension imprinting in water, water disrupts most noncovalent imprinting procedures and is most

effective for covalent and metal chelation-based processes, and (b) for suspension imprinting in fluorocarbons the liquid fluorocarbon is very expensive.

Another direct way to prepare spherical imprinted beads involves grafting the MIPs on the surface of a spherical matrix, such as silica gels [93], resins [73], etc. Interestingly, some groups have shown interest in new grafting techniques that can control the thickness of MIP films and maintain the spherical shape of MIP beads. Polymer grafting techniques fall into two general categories: "grafting to" [94] and "grafting from" [95]. The "grafting to" method refers to the attachment of premade polymer chains to reactive sites on suitable substrates via covalent bond. One limitation for this method is that the density of immobilized polymer chains is low for several reasons. When significant numbers of polymers chains are attached on the surface, the concentration of polymer chain near the interface become higher than that in bulk solution. Therefore, it becomes more and more unfavorable for the free polymer chains to diffuse from solution to the surface, and the rate of immobilization of polymer chains becomes slower and slower. Also, when the density of grafted polymer chains on the surface increases, the attached chains become more and more stretched in order to fit more chains on the surface. Stretching is entropically disfavored, i.e., chains lose entropy as they change from coil confirmation in solution to a stretched confirmation on the surface. A second more relevant limitation for using a "grafting to" approach to prepare surface bound MIPs is that MIPs are polymer with a high degree of crosslinking which have low solubility used for the grafting reaction.

In the "grafting from" method, polymer chains are grown by monomer addition from solution to surface-bound initiators on a suitable substrate. Because chain growth occurs by monomer addition, this method can increase the density of polymer chains. Furthermore, polymerization times can be used as an independent variable to control the thickness of the polymer surface films. Some techniques are presented in the following text for grafting MIPs films from spherical substrates via the "grafting from" method.

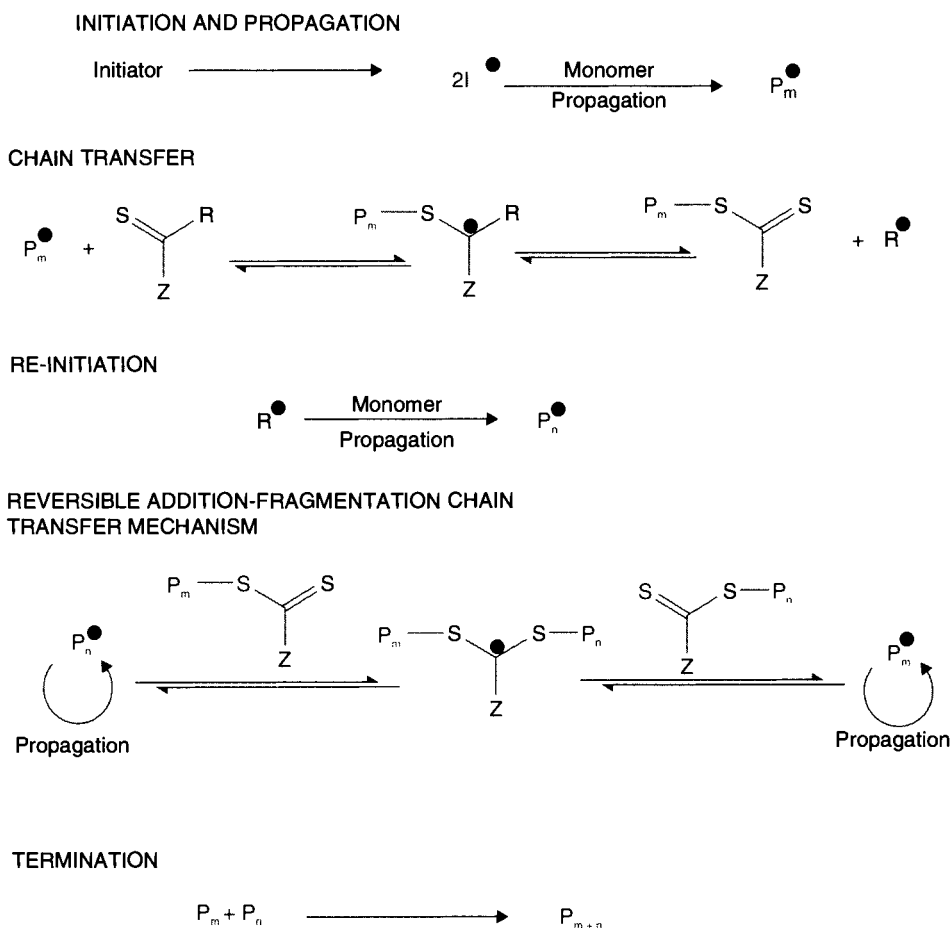
12.2.5.7.3 Grafting MIPs via Azoinitiator-functionalized Substrates

Sellergern and coworkers [93] recently reported the synthesis of surface-confined MIPs using an azoinitiator bound to a silica substrate. In this approach, the azoinitiator was confined to the support surface via a two-step reaction sequence. First, glycidoxyparyltrimethoxysilane or 3-aminoparyltrimethoxysilane was immobilized on the surface by reacting with silanol groups. Secondly, the product in the first step was reacted with 4, 4'-azobis (4-cyanopentanoic acid) at a very low temperature. This initiator-functionalized substrate was suspended in a mixture of monomer(s) and solvent. Just like the conventional monolith method, the polymerization could be initiated by heat or UV radiation. MIPs prepared by these methods were reported to exhibit superior mass transfer properties and saturation capacities when compared to MIPs prepared by the conventional monolith method or compared to MIP films prepared by "grafting to" procedures [93]. Nevertheless, two problems exist for this method. Firstly, it remains confined to the surface and can grow a surface-confined polymer chain. The other radical is released

into solution, where it can form a non-surface-confined polymer chain. Indeed, these authors point out that solution phase polymerization and gelation were hard to avoid [93]. Secondly, the density of free radical cannot be tuned easily, so chain-chain termination cannot be suppressed.

12.2.5.7.4 Grafting MIPs via Initiator Transfer Terminator (Iniferter)-functionalized Substrates

In order to avoid the presence of an active free radical in solution during the decomposition of immobilized initiator, Sellaergren and coworkers [96] investigated the use of benzyl-N, N-diethyldithiocarbamate iniferter species immobilized on silica and polystyrene supports. The advantage of that approach was the formation of a more stable dithiocarbamate radical in solution that did not initiate solution-phase polymerization. This idea was proposed early in 1982 by Otsu and Yoshida [97] who extended the idea of living polymerizations to free radical systems in the use of initiator-transfer-agent-terminators, or iniferters. This method is also called reversible addition fragmentation chain



Scheme 12.2 Schematic representation of the RAFT mechanism.

transfer (RAFT) polymerization (Scheme 12.2), whereby dithio compounds, such as dithiocarbamates, dithioesters, etc., are generally used as efficient RAFT agents (Figure 12.8). The rapid switching mechanism between dormant and active chain ends affords living polymerization character. Such initiators act both as primary radicals to initiate polymerization and as radical terminators, consequently allowing a near linear polymer chain growth with time. However, the iniferter mechanism was also found to generate radicals that can initiate new chains throughout the course of the polymerization; therefore, this system also shows a significant loss of active end groups from the growing chains due to bimolecular termination. Consequently, these systems display relatively large polydispersities.

12.2.5.7.5 Atom Transfer Radical Polymerization (ATRP)

In 1995, Matyjaszewski developed ATRP, one of the most successful methods for controlled/"living" radical polymerization (CRP) [98]. The name ATRP was created on the basis of the atom transfer step, which is the key reaction for controlling the growth of polymer chain, and, for the case of surface-confined polymerization, controlling layer thickness. Scheme 12.3 shows the general mechanism of ATRP.

The radicals are generated through a reversible redox process catalyzed by a complex comprising a transition metal and an organic ligand. A halogen atom X in the dormant species R-X is transferred to the metal center, which undergoes a one-electron oxidation reaction, and a radical, R, is generated. Polymer chains grow by addition of monomer units to the radicals just like in

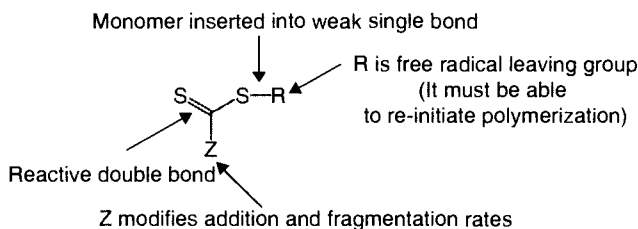
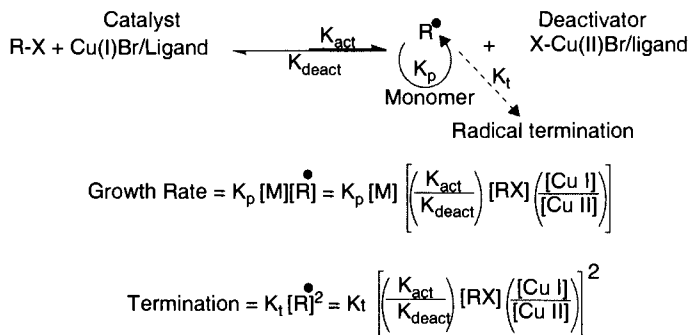


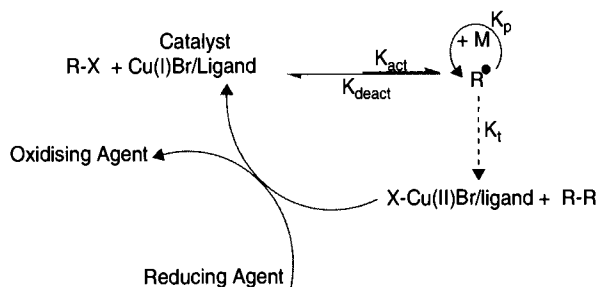
Figure 12.8 Generic RAFT chain transfer agents.



Scheme 12.3 Transition metal-catalyzed ATRP method.

a conventional free radical polymerization. In a well-controlled ATRP system, the dormant species is present in much higher concentration than the active radical species during the polymerization process due to fast initiation and rapid reversible deactivation. This characteristic suppresses chain-chain termination to a very low level, and, therefore, all of the polymer chains grow uniformly. Numerous monomers, including styrenes, (meth)acrylates, (meth)acrylamides, and acrylonitriles, and others [98, 99] have been polymerized successfully by ATRP. The catalyst is the most important component of ATRP because it determines the extent of atom transfer equilibrium and so controls the ratio between the dormant and active species. Among the studied transition metal complexes, those that use copper are superior in terms of cost and versatility in ATRP [98]. Multidentate nitrogen-based ligands, both linear and branched, are used commonly in copper-mediated ATRP. They can be prepared easily and allow further modifications and tuning of the catalyst, they are low cost, and they exhibit higher rates of polymerization while still maintaining good control when compared to complexes with 2,2'-bipyridine and its derivatives [99]. The activity of nitrogen-based ligands increases with the number of coordinating sites and is usually higher for cyclic and bridged systems than for the linear analogues [100]. Alkyl halides are the typical initiators in ATRP, although other initiators have been studied [98]. However, normal ATRP has some limitations such as the catalyst used is sensitive to air and humidity and requires high concentrations of catalyst (usually a copper halide); the catalyst contaminates the polymer, causing discoloration and potentially catalyzing degradation. Removal, for example, by selective polymer precipitation or using ion-exchange resins is difficult and costly. So, several modified ATRP techniques have been developed that require significantly reduced concentrations of copper catalyst. In order to overcome the drawbacks of normal ATRP an improved ATRP technique, activators generated by electron transfer-ATRP (AGET-ATRP) process [101], has more recently been developed. The typical mechanism is shown in Scheme 12.4.

In a typical AGET-ATRP system, a transition-metal complex in its oxidatively stable state such as Cu (II) complex, a reverse ATRP catalyst, is used as catalyst instead of copper (I) complexes for a normal ATRP system, and an alkyl halide for a normal ATRP initiator is also used as the initiator for the AGET-ATRP system [102]. However, the activators, copper (I) complexes, are



Scheme 12.4 Mechanism for AGET-ATRP method.

produced by an *in situ* reduction of the copper (II) complexes with a reducing agent such as ascorbic acid, triethyl amine, tin (II) 2-ethylhexanoate, etc. As normal ATRP, AGET-ATRP is also catalyzed by complexes of transition metals and suitable ligands that mediate a dynamic equilibrium between dormant and active polymer chains. The main role of the ligand in ATRP is to solubilize the transition-metal salt in the organic media and to adjust the redox potential of the metal center for appropriate reactivity and dynamics for the atom transfer [100]. Building on the known use of ligands, nitrogen-based ligands such as 2, 2'-bipyridyl (bpy) and phosphorus-based ligands (e.g., triphenylphosphine) are the most frequently used ones.

12.3 Molecularly Imprinted Nanomaterials

Molecular imprinting can generate recognition cavities with the steric and chemical information of template molecules in a crosslinked polymeric network. However, the effectiveness of molecular imprinting is greatly dependent on the bond nature of template-monomer complex [69, 103–105], the form of imprinted materials [10–12, 106], and the rigidity of polymeric matrix [107–113]. While various imprinted materials were synthesized by different strategies, the imprinted materials ideally suitable for the molecular recognition elements have yet to be explored, because of their small binding capacity and slow binding kinetics. Although the conventional imprinting protocol is simple and effective, there are at least several critical factors to obstruct the applications of molecularly imprinted materials as the artificial receptors in analytical chemistry. Firstly, most of imprinted polymers are highly crosslinked bulky polymers with irregular shape. Although a grinding process is usually used, the extraction of original templates located at the interior area of bulky materials is quite difficult because of the high crosslinking nature of imprinted materials, which reduces the capacity of rebinding target analytes [107–109]. Furthermore, if the generated cavities are not at the surface or in the proximity of the material's surface, the high resistance to mass transfer will still hinder target species from accessing the deep imprinted cavities, thus slowing the kinetics of target analyte binding [110, 111]. Moreover, the rigid polymeric matrix greatly reduces the conformational freedom of molecular recognition by excluding any further chain mobility [112, 113]. Secondly, the uncontrollable random polymerization always suffers from the heterogeneity of the imprinted sites in the formed polymer matrix [114, 115]. One of main reasons is that vinyl functional monomer and divinyl crosslinking agent have essentially different polymerizing abilities, and the used amount of crosslinking agent is much larger than that of functional monomer. Finally, the analyte binding at MIP materials usually lacks signal output due to the poor assembly ability at the surface of transducer, limiting their use in chemical detections or bioassays [116]. Therefore, optimization of the imprinted materials at a molecular level to provide a better antibody/enzyme mimic remains a great challenge.

One of the attempts to address these problems is that the imprinted materials are prepared in an optimizing form that control templates to be situated at the surface or in the proximity of the materials surface [79, 90, 95, 117–124, 125, 126–129]. Mosbach [90] first reported the surface molecular imprinting strategy by covalent immobilization of template molecules at the surface of a solid substrate. After the imprinting polymerization and the removal of substrate, all of the templates were situated at the surface of imprinted materials, providing a complete removal of templates and an excellent accessibility to target species, and conformational flexibility of recognition. The approach is well suited for imprinting of proteins [117–119], cells [120, 121] and virus [122] immobilized onto a flat substrate, because the templates with large sizes are removed with more difficulty in traditional MIPs. However, the surface area of the substrate is very limited, and accordingly the total number of the resultant recognition sites is always very small. Another similar alternative proposed by Sellergren [95, 125, 126] is to prepare the molecularly imprinted films by chemical immobilization of azo-initiators/chain-transfer agents at the surface of substrates, followed by initiating an imprinting polymerization reaction of organic monomers on substrate. Although the immobilization of initiators is often complex and chemically instable, the method should be adaptable to supports with different morphologies. On the other hand, Zimmerman [127, 128] developed a monomolecular dendritic imprinting strategy by polymerization of dendrimer with vinyl end groups. Each polymerizing dendrimer contains only one imprinted site after removal of templates. Recently, the use of natural polymers for preparing molecularly imprinted materials may also provide a potential solution to the above concerns [112, 113]. The types of gel-state materials, such as chitosan hydrogels, are formed with a lower crosslinking density than the traditional imprinted materials synthesized from polymeric monomers, and thus they are more flexible and have a greater conformational freedom of molecular recognition.

Recently, the introduction of novel nanotechnologies and surface chemistry into molecular imprinting strategy has attracted considerable research interest because of the wider applications of imprinted nanomaterials in bioassays and chemosensors [10–12, 130, 131]. In general, the gap between biological and artificial sensors is mostly the usually much higher affinity and thus sensitivity reached with, e.g., antibodies. The molecular imprinting nanotechnologies are expected to greatly enhance the molecular affinity of MIP materials, and thus provide a wider range of applications approaching to biological receptors [132]. MIP nanoparticles have higher surface area-to-volume ratios; thus, imprinted cavities are more easily accessible by the templates and the binding kinetics are improved [12, 133]. This format fits better with surface imprinting strategies [136], facilitating the design of *in vitro* assays with enzyme-conjugated probes, which are usually too bulky to fit into recognition cavities. In addition, because MIP nanoparticles easily remain in solution, it is simpler to dose them precisely for use in assays [134, 135]. MIP nanoparticles have already been used as enzyme substitutes [136, 137], drug delivery systems [138, 139], and antibody

substitutes [12, 140–142], as well as in capillary electrophoresis [143–146] and in sensors [147–149].

Unfortunately, fabrication of MIP nanoparticles is not easy. Manufacturing aspects such as the degree of crosslinking and requirements for strong template-monomer interactions can narrow the choice of protocols suitable for MIP nanoparticle production [150]. The most popular synthetic strategies include precipitation polymerization, mini- and micro-emulsion polymerization, core-shell approaches (with subsequent grafting) and living radical polymerization processes, such as ATRP and RAFT. Each of these procedures has its own set of pros and cons, which have already been emphasized. Consequently we will not repeat a similar discussion on these materials here, but rather try to review literature based on applications of MIP-based nanomaterials for development of highly selective and sensitive devices.

12.4 Molecularly Imprinted Nanomaterial-based Sensing Devices

MIPs have been increasingly used for the development of chemo/biosensors [116, 151, 152–154], because in practical applications as synthetic recognition elements they have many advantages, such as high stability, low cost and ease of preparation. The key to the biomimetic sensors is to establish a reliable link between the target binding event and transducer. Therefore, a major concern for the development of MIP-based sensors is how to measure the analyte binding at MIP materials. Typically, the MIP-based sensors are fabricated by assembling MIP materials onto the surface of transducer, and thus the analyte binding is transformed into a measurable signal. In general, the efficiency of sensors doesn't only depend on the selectivity and sensitivity of MIPs to target species, but also on the approaches of signal output. The optimal transduction approach to a readable signal output can be expected to maximize the selectivity and sensitivity of sensors. In principle, many physical measurements such as electrochemical voltammetry, fluorescence, piezoelectricity and surface plasma resonance can be used for the signal detection in MIP-based sensors [116, 151, 152–154]. One should consider the properties of target analytes and the forms of MIPs to determine what transducer is used. For example, electrochemical voltammetry is an excellent approach for measuring the binding of electroactive analytes at the MIP nanofilms [155]. During the past ten years, the literature on the development of MIP-based sensors, particularly electrochemical [155–176] and optical [177–188] sensors, has been dramatically growing. At the same time, mass-sensitive transducers are increasingly popular since the approach has the advantage, theoretically at least, that it can be universally applied to a broad range of targets [189–217]. With insight into the different types of sensing, we will summarize the recent advances on electrochemical and optical sensors and mass sensitive devices.

12.4.1 Electrochemical Sensors

In contrast with the increasing number of MIP reports on separations and mass-transduction sensors, it is surprising that the design of electrochemical sensors based on molecular imprinting technology is rather scarce. Electrochemical sensing has a number of advantages over other detection techniques in terms of good limits of detection, low cost, easy miniaturization and the potential for automation. MIP-based electrochemical sensors were first reported in the early 1990s by Mosbach's group [155]. They described the integration of a phenylalanine anilide imprinted polymer into a field-effect capacitance sensor and reported a significant reduction in the overall capacitance when the sensor was exposed to the target species. During the past decade, remarkable progress in MIP-based electrochemical sensors has been achieved by the use of conductometric/potentiometric measurements and MIP nanomaterials, greatly extending the range of detected targets and improving the sensitivity, selectivity and simplicity of electrochemical sensors [151, 153, 155–176]. The electrochemical sensors are most commonly fabricated by installing MIP nanomaterials, as recognition elements, onto the surface of electrode. The changes of current and peak voltage at cyclic voltammetry upon the analyte binding can sensitively respond to the concentration and kind of analytes, respectively, because of the oxidation or reduction of analytes at the MIP-modified electrode.

Levon and coworkers [156–158] had developed various interesting potentiometric sensors based on the surface imprinting technique coupled with a nanoscale transducer, indium tin oxide (ITO). An octadecylsiloxane monolayer was covalently bound to ITO-coated glass surface in the presence of templates. After extraction of templates, potentiometric measurements showed selective detections of the imprinted target species, such as methyl phosphonic acid with a detection range of 5.0×10^{-5} to 0.62 M [156], dipicolinic acid with a detection range of 1.5×10^{-6} to 0.0194 M [157], and N-carbobenzoxypartate acid with a detection range of 5.0×10^{-6} to 1.2×10^{-2} M [158]. Moreover, the response times were surprisingly short due to the imprinted ultrathin monolayer, i.e., ~ a few seconds except for the last one which is ~2 min.

Riskin *et al.* [159] demonstrated that polyphenol can be electropolymerized on a Au electrode to fabricate the MIP-modified electrode for the detection of herbicide molecules, N,N'-dimethyl-4,4'-bipyridinium (MV^{2+}). The template-phenol interactions provided a driving force for the formation of MV^{2+} -imprinted sites in the electropolymerized polyphenol film. When the MIP-modified electrode was used to detect the herbicide in solution, the current rapidly leveled off to an equilibrium value after 20 min, and there was a 14-fold increase in the binding affinity of MV^{2+} , as compared to the nonimprinted film. Meanwhile, the current response at the MIP electrode upon the structurally-analogous 1,2-bis-4,4'-methylbipyridinium ethylene was 8-fold lower than that observed with MV^{2+} . Also, they [160] reported another electrochemical sensor for the detection of trinitrotoluene (TNT) with enhanced sensitivities by imprinting of structure-like picric acid as substituting templates in the composite film of Au nanoparticles (AuNPs) and conductive polymers.

The imprinting of molecular recognition sites into the π -donor oligoaniline-crosslinked AuNPs structure greatly enhanced the detection sensitivity. As little as 46 ppt of TNT (200 pM) can be clearly detected by the modified electrode, which was 370-fold the sensitivity of the p-aminothiophenolate monolayer-modified electrode. In contrast to other sensor systems, the imprinted π -donor Au nanoparticle crosslinked array is a highly sensitive electrochemical approach for the ultrasensitive detection of TNT.

Kan *et al.* [161] reported a novel electrochemical sensor to detect the neurotransmitter dopamine by modifying the glassy carbon electrode with a composite of multiwalled carbon nanotubes (MWNTs) and dopamine-imprinted polymers. The MWNT-MIP-modified electrode not only possessed a rapid dynamic binding with an equilibrium period of 30 min, but also exhibited a high selectivity and sensitivity toward dopamine with a linear range of 5.0×10^{-7} to 2.0×10^{-4} M.

Meanwhile, a sensitive molecularly imprinted electrochemical sensor was created for selective detection of a tricyclic antidepressant imipramine by combination of AuNPs with a thin molecularly imprinted film [162]. The sensor was fabricated onto the ITO electrode via stepwise modification of AuNPs by self-assembly and a thin film of MIPs via sol-gel technology. It was observed that the molecularly imprinted film displayed excellent selectivity towards the target molecule imipramine. Meanwhile, the introduced AuNPs exhibited noticeable catalytic activities towards imipramine oxidation, which remarkably enhanced the sensitivity of the imprinted film. Due to such combination, the as-prepared sensor responded quickly to imipramine, within only 1 min of incubation. The differential voltammetric anodic peak current was linear to the logarithm of imipramine concentration in the range from 5.0×10^{-6} to 1.0×10^{-3} mol L⁻¹, and the detection limits obtained was 1.0×10^{-9} mol L⁻¹. This method proposed was successfully applied to the determination of imipramine in drug tablets, and proven to be reliable compared with conventional UV method. These results reveal that such a sensor fulfills the selectivity, sensitivity, speed, and simplicity requirements for imipramine detection, and provides possibilities of clinical application in physiological fluids.

Zhang *et al.* [163] have constructed a novel sensitive molecularly imprinted electrochemical sensor for selective detection of clindamycin by combination of a multiwall carbon nanotubes (MWCNTs) layer with a thin molecularly imprinted sol-gel film. The sensor was fabricated onto Au electrode via stepwise modification of MWCNTs and a thin sol-gel film of MIP by using the electrodeposition method. The molecularly imprinted film displayed excellent selectivity towards clindamycin. Due to such a combination, the sensor responded quickly to clindamycin. The response peak current was linear to the concentration of clindamycin in the range from 5.0×10^{-7} mol L⁻¹ to 8.0×10^{-5} mol L⁻¹, and the detection limit was 2.44×10^{-8} mol L⁻¹. This imprinted sensor was applied to the determination of clindamycin in human urine samples successfully. These results revealed that the imprinted sensor fulfilled the selectivity, sensitivity, speed and simplicity requirements for clindamycin detection, and provided possibilities of clinical application in physiological fluids.

Meanwhile, this group also reported a novel sensitive and selective imprinted electrochemical sensor for the direct detection of L-histidine by combination of a molecular imprinting film and MWCNTs [164]. The sensor was fabricated onto an ITO electrode via stepwise modification of MWCNTs and a thin film of MIPs via sol-gel technology. The proposed imprinted sensor was characterized by using scanning electron microscope (SEM) and electrochemical methods involving cyclic voltammetry (CV), differential pulse voltammetry (DPV) and amperometric i-t curve. A linear ranging from $2.0 \mu\text{mol L}^{-1}$ to 1.0 mmol L^{-1} for the detection of L-histidine was observed with the detection limit of $5.8 \times 10^{-9} \text{ mol L}^{-1}$ for $S/N=3$. This imprinted electrochemical sensor was successfully employed to detect L-histidine in human blood serum.

Berti *et al.* [165] have described for the first time integration between MIPs and polyaniline (PANI) nanostructured electrodes. They explored a simple, inexpensive, and fast route to grow PANI nanotubes, arranged in an ordered structure directly on an electrode surface, by electrochemical polymerization using alumina nanoporous membranes as a "nano mold." The advantages of using nanostructures in this particular biosensing application have been evaluated by comparing the analytical performance of the sensor with an analogous non-nanostructured MIP-sensor for catechol detection that was previously developed. A significantly lower limit of detection for catechol has been obtained (29 nM, one order of magnitude), thus demonstrating that the nanostructures are capable of improving the analytical performance of the sensor.

An electrochemical strategy on the basis of rationally designed molecularly imprinted sol-gel polymer embedded with AuNPs was developed for the specific and sensitive determination of Sudan I [166]. The rationally designed sensing Sudan I imprinted sol-gel was prepared by mixing Sudan I with 3-aminopropyltriethoxysilane, tetraethoxysilane, chitosan, and AuNPs, followed by copolymerization and extraction of the template molecules. The hybrid forming membrane was used for the linear sweep voltammetric (LSV) determination of Sudan I in water/ethanol solutions. The LSV responses exhibited high sensitivity and selectivity, as discriminated from Sudan I analogues. Under optimal experimental conditions, LSV peak currents were linearly proportional to the concentrations of Sudan I in the range from 0.1×10^{-7} to $1.0 \times 10^{-5} \text{ M}$, with a detection limit of $2.0 \times 10^{-9} \text{ M}$. The strategy is generally applicable in developing sensitive, selective, and moreover, reusable electrochemical sensors for quantitative determination of electroactive species.

Recently, Wang *et al.* [167] have developed a novel electrochemical sensor based on MIP film for aspirin detection. The sensitive, conductive hybrid membrane was fabricated at the surface of Au electrode by the copolymerization in the mixing solution containing additional p-aminothiophenol (p-ATP), HAuCl_4 , and acetylsalicylic acid (ASA). The ASA was spontaneously imprinted into the poly-aminothiophenol gold nanoparticles (PATP-AuNPs) complex film. The molecularly imprinted sensor was characterized by electrochemical impedance spectroscopy (EIS), DPV, and CV. The linear relationships between current and logarithmic concentration were obtained in the range from 1 nmol

L^{-1} to $0.1 \mu\text{mol L}^{-1}$ and $0.7 \mu\text{mol L}^{-1}$ to 0.1 mmol L^{-1} . The detection limit of 0.3 nmol L^{-1} was achieved. This molecularly imprinted sensor for the determination of ASA has high sensitivity, good selectivity, and reproducibility, and with the testing in some biological fluids also has good selectivity and recovery.

Yuan *et al.* [168] have constructed an electrochemical sensor for 17β -estradiol (E_2) based on the MIP membranes. For this, 6-mercaptopuronic acid (MNA) and E_2 were first assembled on the surface of platinum nanoparticles-modified glassy carbon electrode (PtNPs/GCE) by the formation of Pt-S bonds and hydrogen-bonding interactions, and subsequently the polymer membranes were formed by electro-polymerization. Finally, a novel molecularly imprinted sensor was obtained after removal of E_2 . A good linearity was obtained in the range of 3.0×10^{-8} – $5.0 \times 10^{-5} \text{ mol L}^{-1}$ ($r = 0.996$) with an estimated detection limit of $1.6 \times 10^{-8} \text{ mol L}^{-1}$. This sensor had successfully been used to analyze E_2 in water samples without complex pretreatment. Meanwhile, the average recoveries were higher than 93.9% with $\text{RSD} < 3.7\%$.

4-Nonylphenol (4-NP) and bisphenol A (BPA) were reported to affect the health of wildlife and humans through altering endocrine function. Huang *et al.* [169] have described a novel electrochemical sensor for sensitive and fast determination of 4-NP. For this, titanium oxide (TiO_2) nanoparticles and gold nanoparticles were introduced for the enhancement of electron conduction and sensitivity. The resulting electrodes were characterized by CV in the linear range from 4.80×10^{-4} to $9.50 \times 10^{-7} \text{ mol L}^{-1}$, with the detection limit of $3.20 \times 10^{-7} \text{ mol L}^{-1}$ ($S/N = 3$). The sensor was successfully employed to detect 4-NP in real samples. Meanwhile, they [170] also developed an electrochemical sensor by introducing MWCNTs and gold nanoparticles in thin film of molecularly imprinted sol-gel polymers, casted on gold electrode by electrochemical deposition. The imprinted sensor was successfully tested to detect BPA in real samples in the linear range from 1.13×10^{-7} to $8.21 \times 10^{-3} \text{ mol L}^{-1}$ with the limit of detection (LOD) of $3.6 \times 10^{-9} \text{ mol L}^{-1}$ ($S/N = 3$).

Recently, our group has developed various interesting electrochemical sensors based on surface imprinting approach [171–176]. Several chronic diseases (for example, gigantocytic anemia, leucopenia, mentality devolution, psychosis, heart attack, and stroke), especially those concerned with malformation during pregnancy and carcinogenic processes, are related to the deficiency of folic acid (FA) which is a water soluble vitamin. So, we have developed an electrochemical sensor for the selective and quantitative recognition of FA, using a preanodized sol-gel coated pencil graphite electrode (PGE) with imprinted polymer immobilized to its exterior surface [171]. The MIP-FA adduct nanofilm on sol-gel modified PGE surface was porous and about 50 nm thick. In the present work, MIP has been prepared by an “initiator-fragment incorporation radical polymerization” technique involving a new trifunctional monomer, TAT (Figure 12.9). The analyte recapture at pH 2.5 in aqueous environment simultaneously involved mixed hydrophobically driven hydrogen bondings and ionic interactions with pteridine substructure and purely hydrogen bonding interactions with glutamic acid residue of FA. The encapsulated analyte was instantly oxidized and then cathodically stripped off responding differential

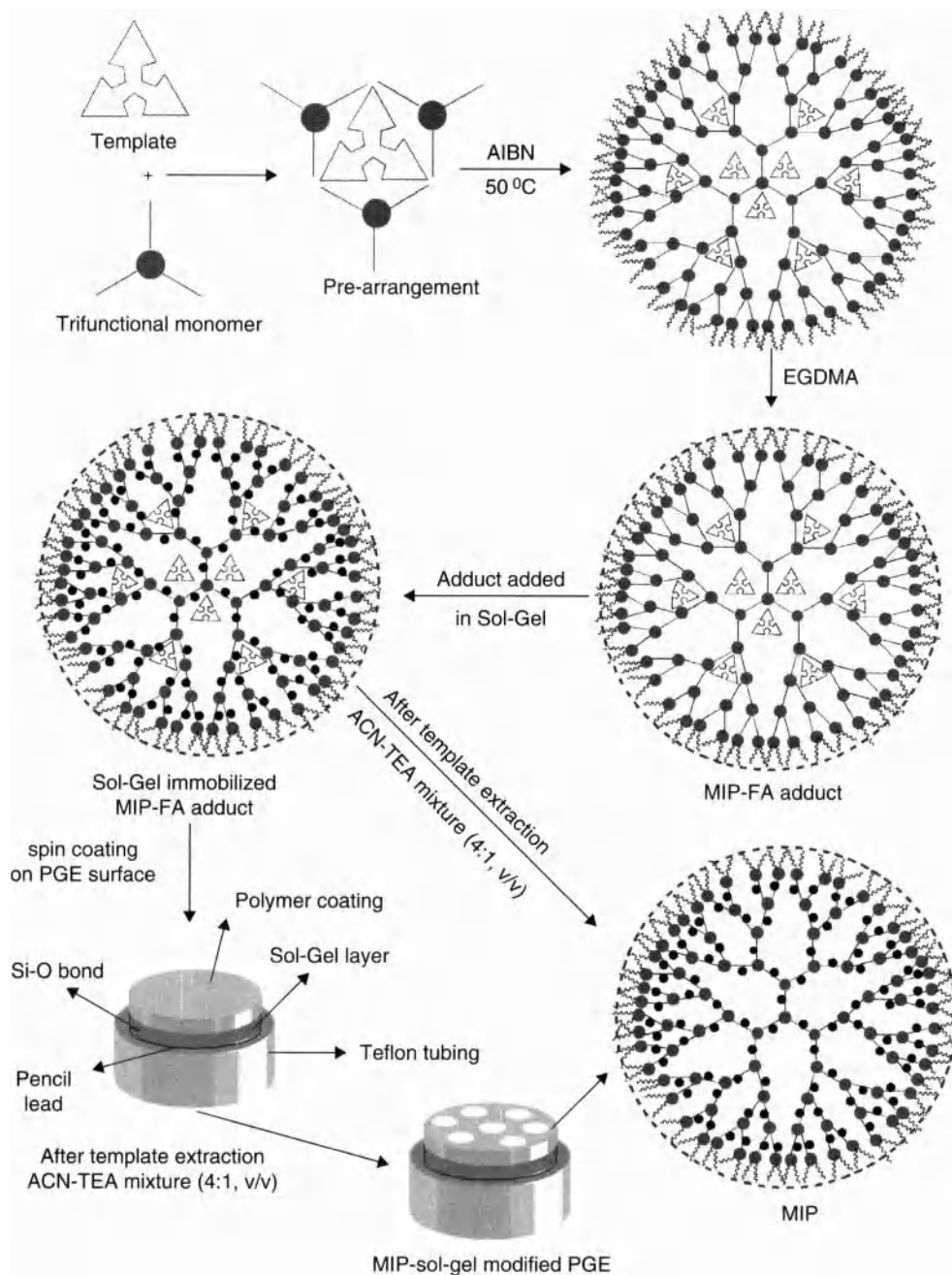


Figure 12.9 (a) Schematic representation of the preparation of the MIP-sol-gel coated PGE. The symbol (•) represents silane binding as Si-O-C-OH at various carbonyl centers of MIP. (b) Recognition mechanism of template (FA) bindings within MIP cavities via noncovalent interactions showing "multi-molecular imprinting" in hyperbranched (dendrimer-like) polymer.

pulse cathodic stripping voltammetric (DPCSV) signal. The FA was selectively detected with a limit of detection of $0.002 \mu\text{g mL}^{-1}$ (3σ , $RSD \leq 3.0\%$), without any cross-reactivities and real matrix complications.

Furthermore, we have described a new, simple, and easy method for the generation of stable molecularly imprinted sites in polymeric nanofilm (192 nm thick), combining self-assembled monolayer and layer-by-layer approaches through thermal cross-linking of the layered structures, onto the surface of silver electrode (Figure 12.10) [172]. Modified silver electrodes demonstrate enantiodifferentiation and sensitive (detection limits $0.0062 \text{ ng mL}^{-1}$ for D- and $0.0060 \text{ ng mL}^{-1}$ for L-tyroxine) determination of D- and L-tyroxine

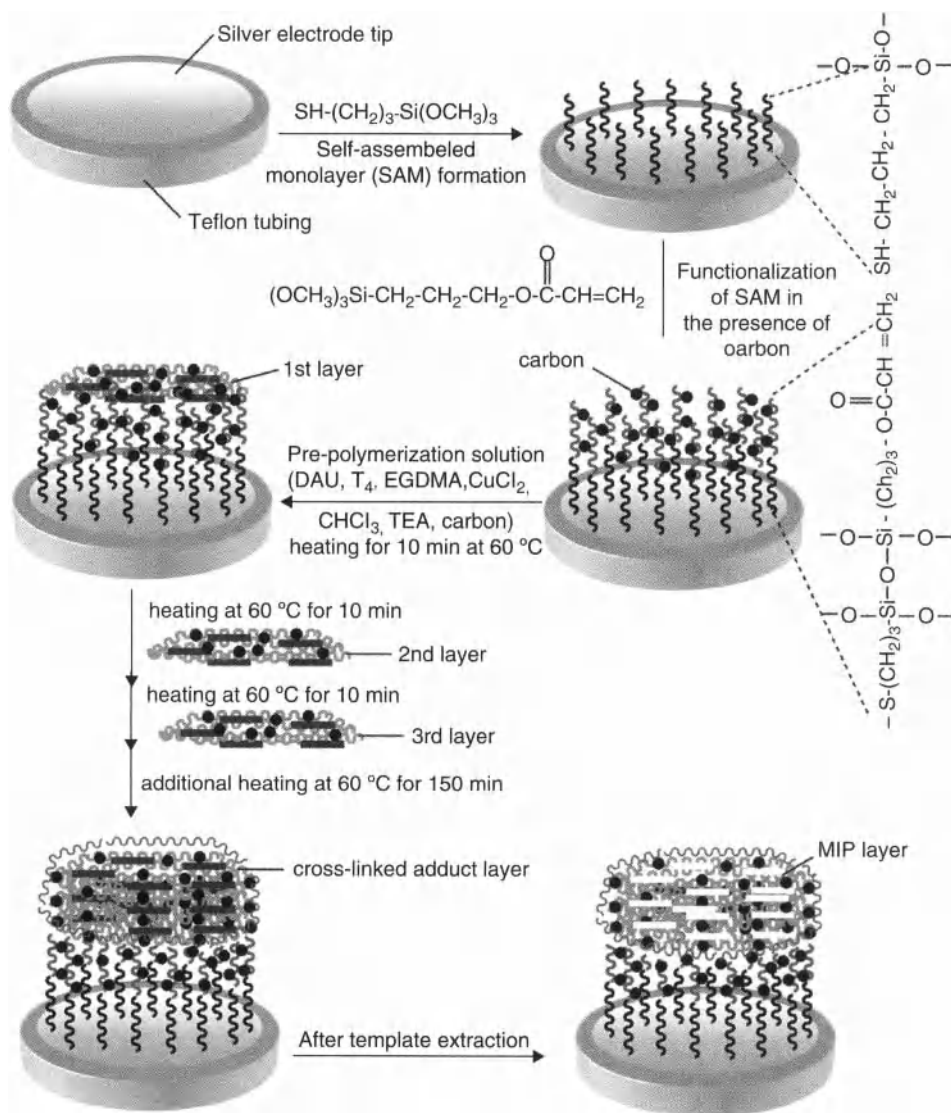


Figure 12.10 Schematic representation of self-assembling and LbL approaches to create imprinted sites on the Ag electrode.

(an essential hormone produced in the thyroid gland) with the help of differential pulse anodic stripping voltammetric (DPASV) technique. The sensor was also validated for D- and L-thyroxine determinations in biological and pharmaceutical samples with quantitative (100%) recoveries and therefore, can be used as practical sensors for biomarking especially thyroid-related diseases caused by thyroxine.

Phosphatidylcholine is a major component of all biological membrane; its incorporation in polymer backbone assures water-compatibility, biocompatibility and specificity to molecularly imprinted nanomaterials, without any cross-reactivity or interferences from biological sample matrices. For the first time, we have developed an ultra-thin layer (22 nm thick) of new MWCNTs-MIP composite onto the PGE surface, to analyze insulin at trace level (range 0.068–5.682 nmol L⁻¹, LOD 0.0183 nmol L⁻¹) [173]. For grafting MIP on MWCNTs, the vinyl group was first introduced on the surface of MWCNTs, which directed the free-radical polymerization of functional monomer [(p-acryloylaminophenyl)-(4-aminophenyl)-diethyl ammonium]-ethylphosphate, AEP] and crosslinker (EGDMA) in the presence of insulin on the MWCNTs surface. The resulting MWCNTsMIP possessed a faster kinetics of electron-transfer reaction than carbon-MIP composite, and revealed higher selectivity and sensitivity for insulin without any false-positive contribution. The specific and quantitative DPASV response of insulin on MWCNTs-MIP modified PGE was obtained, owing to the large surface area of MWCNTs-MIP and the imprinted sites located on the surface of MWCNTs. The most attractive feature with the use of MWCNTs-MIP modified PGE for electro-oxidation of insulin is the easy renewal of surface for the next use by the method of template extraction in real samples. Any single electrode could be used for as many as 35 times with quantitative recoveries and thus can be exploited as a practical sensor for the effective biomarking of insulin in human serum of diabetic patients.

Recently, a new kind of MIP-modified graphite electrode was fabricated by “grafting to” approach, incorporating sol-gel technique, for the detection of acute deficiency in serum ascorbic acid level (SAAL), manifesting hypovitaminosis C in our laboratory [174]. For this, we immobilized an MIP layer firmly onto the graphite electrode surface via a sandwich layer of organosilne. The film thickness of the entire coating on graphite electrode surface was determined to be 46.6 nm using a side imaging by SEM. The modified electrode exhibited ascorbic acid (AA) oxidation at less positive potential (0.0 V) than the earlier reported methods, resulting in a LOD as low as 6.13 ng mL⁻¹ (RSD = 1.2%, S/N = 3). The diffusion coefficient (1.096×10^{-5} cm² s⁻¹), rate constant (7.308 s⁻¹), and Gibbs free energy change (–12.59 kJ mol⁻¹) due to analyte adsorption, were also calculated to explore the kinetics of AA oxidation. The proposed sensor was found to enhance sensitivity substantially so as to detect ultra-trace level of AA in the presence of other biologically important compounds (dopamine, uric acid, etc.), without any cross interference and matrix complications from biological fluids and pharmaceutical samples.

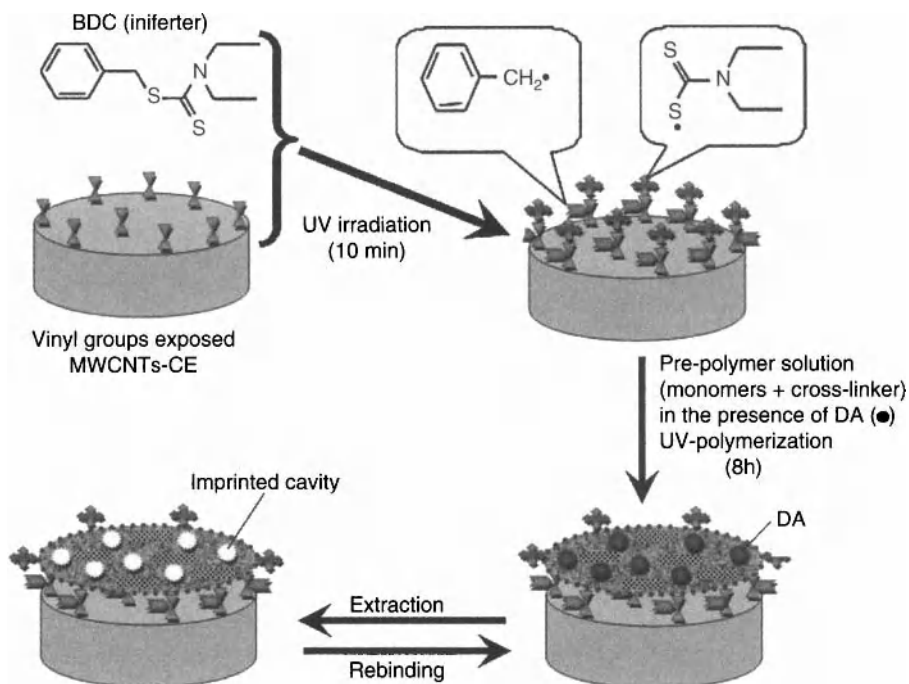


Figure 12.11 Schematic representation of MIP-modified MWCNT-ceramic electrode fabrication for DA.

A new MWCNT-ceramic electrode (MWCNTs-CE) composite with homogeneous MIP outer layer of about 0.22 nm for the selective and sensitive recognition of dopamine (DA) was synthesized [175]. For grafting MIP on MWCNTs-CE, the vinyl groups were introduced through sol-gel modification using silane acrylate, with direct photopolymerization in the presence of an iniferter (benzyl N, N-diethyldithiocarbamate), functional monomer (4-nitrophenyl acrylate), crosslinker (EGDMA), and the print molecule (DA template), on the exterior surface (Figure 12.11). The MIP modification adopting surface “grafting from” approach actually helped in obtaining a larger electro-active surface area with easily accessible sites for achieving highly symmetric and quantitative response of DA as compared to bare MWCNTs-CE/ carbon ceramic electrode (CCE)/MWCNTs-CCEs. It is further concluded that MWCNTs-CE is better than CCE/MWCNTs-CCEs for MIP grafting in terms of DA current response with symmetry. The high sensitivity of MIP-modified MWCNTs-CE sensor along with specific and quantitative DPASV response might allow for its potential use in the clinical settings for the diagnosis of DA-related diseases. MWCNTs-CE possessed a faster electron kinetics than MWCNTs-CCEs, and revealed higher selectivity and sensitivity for DA (detection limit 0.143–0.154 ng mL⁻¹, 3σ), without any matrix interferences and false-positives, in real samples.

Enantioselective trace level sensing of L-histidine (*LOD*, 1.980 ng mL⁻¹, *S/N* = 3) was feasible with the use of a typical, reproducible, and rugged

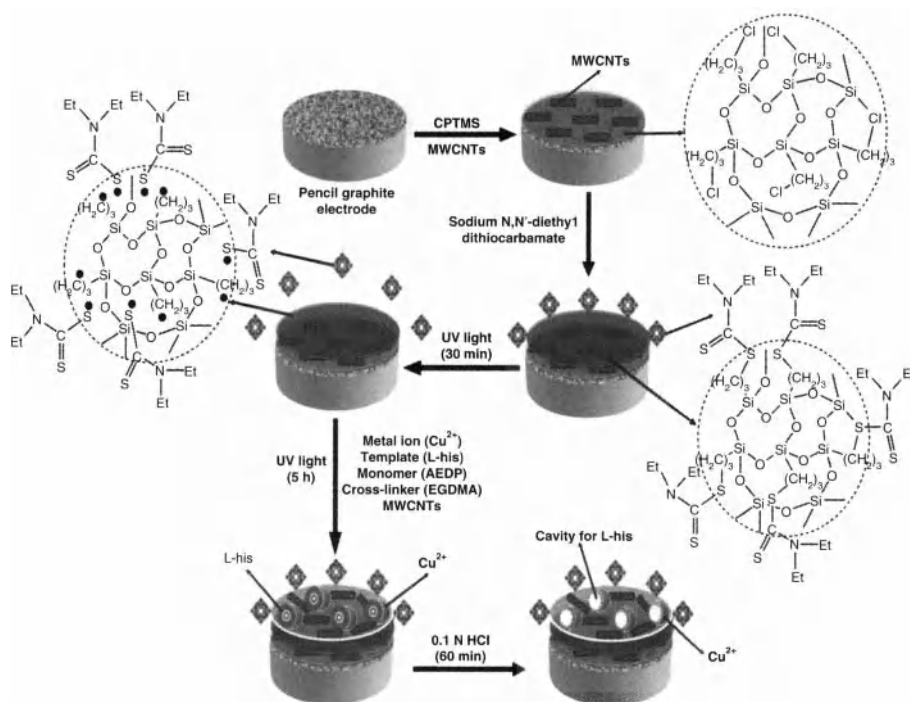


Figure 12.12 Fabrication of CIP-modified PGE.

complex imprinted polymer (CIP)-based PGE, in aqueous samples [176]. For the CIP-modified sensor fabrication, we adopted a surface “grafting from” approach, where an iniferter was grafted onto a solid substrate (ormosil-modified PGE) in order to grow a polymer film of controlled thickness through photo initiated free-radical polymerization (Figure 12.12). In this instance, the Cu^{2+} ion-mediated imprinting of L-histidine in an MIP motif actually helped upbrining electrocatalytic activity to respond an enhanced DPASV oxidation peak of L-histidine, without any cross-reactivity and false-positive, in real samples. The proposed sensor could be considered suitable for the practical applications in biomarking histedinemia, a disease associated with L-histidine metabolic disorders, in clinical settings.

12.4.2 Optical Sensors

As previously stated, when progressing from the macrolevel/microlevel to the nanoscale, materials not only demonstrate advantages such as greater surface area and recognition site accessibility, but also start exhibiting new properties. A number of research groups have taken advantage of these altered properties, an example of which involved the incorporation of quantum dots (QDs, semiconductor nanoparticles that exhibit remarkable photoluminescence behavior) into MIPs. Lin *et al.* [177, 178] have demonstrated that binding to recognition sites in the vicinity of the fluorescent nanoparticles caused quenching of the photoluminescence, presumably owing to the fluorescence

resonance energy transfer between quantum dots and template molecules. In both cases, the artificial receptors of various templates were synthesized with functional monomers such as methacrylic acid (MAA), semiconductor like CdSe/ZnS core-shell derivatized with 4-vinylpyridine and EGDMA as the crosslinker.

Recently, Wang *et al.* [179] have developed a new type of MIP-based room temperature phosphorescence (RTP) optosensor by anchoring the MIP layer on the surface of Mn-doped ZnS QDs via a surface molecular imprinting process. The new MIP-based RTP sensing protocol was applied to detect trace pentachlorophenol (PCP) in water samples without the interference of autofluorescence and scattering light of matrixes. The detection limit for PCP was 86 nM, and the precision for five replicate detections of 0.4 μ M PCP was 2.8% (RSD). The recovery of spiked PCP in river water samples ranged from 93–106%. Lin *et al.* [180] have reported the application of incorporation between QDs and MIPs in biological systems. In this study, poly(ethylene-co-ethylene alcohol) creatinine-, albumin- and lysozyme-imprinted polymers nanoparticles are synthesized via phase inversion of poly(ethylene-co-ethylene alcohol) with various ethylene mole ratios when target molecules and hydrophobic quantum dots are mixed within the polymer solution.

MIPs have long been studied for applications in biomolecule recognition and binding; compared with natural antibodies, they may offer advantages in cost and stability. Lee *et al.* [181] reported the development of MIPs that “self-report” concentrations of bound analytes via fluorescence changes in embedded QDs. Composite QD/MIPs were prepared using phase inversion of poly(ethylene-co-vinyl alcohol) (EVAL) solutions with various ethylene mole ratios in the presence of salivary target molecules (e.g., amylase, lipase, and lysozyme). These major protein components of saliva have been implicated as possible biomarkers for pancreatic cancer.

Optical sensors have proven to be a useful method in identifying explosive devices by recognizing vapors of explosive compounds that become airborne and emanate from the device. To detect high explosive compounds such as TNT, an MIP sensing mechanism was developed by combination of MIP microparticles (prepared using methacrylic acid as functional monomer) with fluorescent quantum dots via a simple crosslinking procedure [182]. This sensing system was capable of functioning in an array of environmental conditions. To study the sensing mechanism’s ability to detect nitroaromatic analytes, the fluorescent-labeled MIP particles were tested for their performance in detecting aqueous 2,4-dinitrotoluene (DNT), a nitroaromatic molecule very similar to TNT, as well as TNT itself. These preliminary data indicate that the system is capable of detecting nitroaromatic compounds in solution with high sensitivity, achieving lower limits of detection of 30.1 and 40.7 μ M for DNT and TNT, respectively.

More recently, a combination of MIP and Mn-doped ZnS QDs for highly selective and sensitive determination of 4-nitrophenol by chemiluminescence (CL) was described by Liu *et al.* [183]. The application of MIP-capped Mn-doped ZnS QDs to CL system was also studied using a KIO_4 - H_2O_2 system.

This application combines the good selectivity of MIP with the high sensitivity of CL. The linear range of this CL system is from 0.1–40 μM , and the detection limit for 4-nitrophenol in the water can reach 76 nM. The method was also used in the real water samples, and the recoveries can fall in the range of 91–96%.

Molecular imprinting is an attractive technique for preparing mimics of natural, biological receptors. Nevertheless, the imprinting of macromolecule remains a challenge due to their bulkiness and sensitivity to denaturation. In this work, Jing *et al.* [184] have presented a method for preparing multifunctional lysozyme-imprinted Fe_3O_4 nanoparticles. These imprinted nanoparticles had high adsorption capacity (0.11 mg mg^{-1}), controlled selectivity, and direct magnetic separation (22.1 emicro g^{-1}) in crude samples. After preconcentration and purification with magnetic MIPs nanoparticles, a sensitive CL method was developed for determination of lysozyme in human serum samples. The results indicated that the spiked recoveries were changed from 92.5 to 113.7%, and the *RSD* was lower than 11.8%.

A core-shell nanostructure was employed in constructing the MIP chemosensor for the improvements of template extraction efficiency and imprinted sites accessibility [185]. Vinyl-substituted zinc (II) protoporphyrin (ZnPP) was used as both fluorescent reporter and functional monomer to synthesize atrazine-imprinted polymer shell at silica nanoparticle cores. These imprinted sites were located in polymer matrix of the thin shells (~8 nm), possessing better accessibility and lower mass-transfer resistance for the target molecules. This MIP probe showed a *LOD* of about 1.8 μM for atrazine detection. The core-shell nanostructured MIP method not only improves the sensitivity, but also shows high selectivity for atrazine detection when compared with the nonmolecular imprinted counterparts.

Li *et al.* [186] have described a general protocol for the making of surface-imprinted magnetic fluorescence beads via RAFT polymerization. The synthesized beads exhibited homogeneous polymer films (thickness of about 5.7 nm), spherical shape, high fluorescence intensity and magnetic property (Magnetization (*Ms*) = 3.67 emu g^{-1}). The hybrids bind the original template 17 β -estradiol with an appreciable selectivity over structurally related compounds. This study therefore demonstrates the potential of MIPs for the recognition and separation of endocrine disrupting chemicals.

A newly designed MIP material was fabricated and successfully utilized as recognition element to develop a QDs based MIP-coated composite for selective recognition of the template cytochrome c (Cyt) [187]. The composites were synthesized by sol-gel reaction (imprinting process). The fluorescence of MIP-coated QDs was stronger quenched by the template *versus* that of non-imprinted polymer (NIP)-coated QDs, which indicated the composites could recognize the corresponding template. Under optimum conditions, the linear range for Cyt is from 0.97–24 μM , and the detection limit is 0.41 μM .

Using a very different approach, Pipemo *et al.* [188] have described the fabrication of polymer nanofibers with entrapped MIP nanoparticles and studied their possible use in a fluorescence-based biosensor application. The MIP was imprinted with the fluorescent amino acid derivative dansyl-L-phenylalanine.

Poly(vinyl alcohol) was used as a support for MIP nanoparticles because it is water-soluble and can be spun into very thin fibers. The fibers exhibited close to 100% recovery upon extraction and rebinding of the target molecule.

12.4.3 Mass Sensitive Devices

In principle, the measurement of mass is the most general method suitable for the detection of any analyte since the mass is a universal property of matter. Piezoelectric devices such as a quartz crystal microbalance (QCM) can provide an extremely sensitive measurement to the mass of the analyte binding at the surface of piezoelectric materials through an accompanying decrease of the oscillation frequency of a piezoelectric crystal. The variation of vibrational frequency (ΔF) can be evaluated from Sauerbrey equation [189]: $\Delta F = -2.3 \times 10^6 \times F_0^2 \times (M/A)$, where F_0 is a fundamental frequency of piezoelectric crystal, M is the mass of absorbed species, and A is the adsorbing surface area. The theoretical detection limit if using quartz crystals is about 10^{-12} g. Although the volume of literature on MIP-based piezoelectric sensors has increased at a relatively slow rate compared with electrochemical and optical sensors, the synergetic advantages of the selectivity provided by MIP with the sensitivity provided by piezoelectric sensing makes the sensors almost universally applicable with good limits of detection, low cost and the possibility of easy miniaturization and automation [153, 190–199]. A recent review has summarized the applications of the piezoelectric sensors in the selective detections of small molecules [190]. Here we mainly review the recently released publications on applications of MIP nanomaterials in piezoelectric sensors and summarize the extension of their applications from small molecules to biomacromolecules and to bulky analytes such as microorganisms and cells.

A MIP-based QCM sensor is usually fabricated by immobilizing a MIP layer onto the surface of QCM as a recognition element. Accordingly, the sensing system can selectively detect the specific binding of target analyte at the MIP materials [157, 190–195].

A FA sensor was prepared via an electropolymerized molecularly imprinted polymer (E-MIP) film of a bis-terthiophene dendron on a QCM using CV electrodeposition [200]. A key component of the E-MIP process is the use of a bifunctional monomer design to precomplex with the template and function as a crosslinker. The predicted geometry of the 1:2 FA/terthiophene complex was obtained through semiempirical AM1 quantum calculations. A relatively good linearity, $R^2 = 0.985$, was obtained within the concentration range 0–100 μM FA with detection limit of 15.4 μM (6.8 μg).

Pietrzyk *et al.* [201] have prepared an adenine-templated MIP film, deposited on a poly(bithiophene) barrier film, served as the recognition element of a piezo-microgravimetric (acoustic) chemosensor. A 10MHz AT-cut shear-thickness-mode bulk-acoustic-wave quartz crystal resonator with Pt film electrode was used as the signal transducer. The MIP film was deposited by electrochemical copolymerization of two functional monomers of bis(bithiophene) derivatives, bearing either the 18-crown-6 or dioxaborinane substituent, in the presence

of the adenine template. The chemosensor performance was evaluated with the piezoelectric microgravimetry detection at QCM under FIA conditions using a carrier acetonitrile-water (1:1, v:v) mixed solvent solution in the linear dynamic concentration range from at 0.1–1 mM. The LOD was as low as 5nM adenine. Meanwhile, a histamine piezoelectric (acoustic) sensor using an electropolymerized MIP film (200 nm thick) has been devised and tested by this group [202]. In this study the sensitivity of the chemosensor (0.33 Hz/mM) was more than twice that of the chemosensor without the poly(bithiophene) barrier film (0.15 Hz/mM).

The routine measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in biological samples is a difficult analytical problem due to the low levels of the analyte and complex matrix. A new 8-OHdG imprinted QCM sensor has been developed for selective determination of 8-OHdG in serum samples using methacryloyl aminoantipyrine-Fe(III) [MAAP-Fe(III)] and methacryloyl histidine-Pt(II) [MAH-Pt(II)] as metal-chelating monomers via double metal coordination-chelation interactions [203]. The obtained results showed that the application of double metal-chelate monomer systems has been more effective than single metal-chelate monomer systems. In this study, the detection limit and the linear working range were found to be 0.0075 and 0.0100–3.5 μM , respectively. Furthermore, 8-OHdG level in blood serum which belongs to a intestinal cancer patient was determined by the prepared QCM sensor.

Krozer [157] reported the QCM sensor with dissipation (QCM-D) by coating the sensor surface with premade molecularly imprinted nanoparticles. The nanoparticles were physically entrapped into a thin poly(ethylene terephthalate) (PET) layer spin-coated on the transducer surface. By controlling the deposition conditions, a high nanoparticle loading can be gained in the stable PET layer, allowing the recognition sites in nanoparticles to be easily accessed by the test analytes. The highest uptake of the nanoparticle film to propranolol corresponded to approximately 2 nmol cm^{-2} or about 1×10^{15} molecules cm^{-2} . The detection limit of the MIP-QCM sensor was about 10 μM , and the chiral recognition and discrimination between R- and S-propranolol can also be achieved. Similarly, Chan [191] described the use of the thin permeable films of MIPs as biomimetic recognition materials on QCM surface. The sensor can provide a high enantioselectivity and sensitivity for the discrimination of D- and L-tryptophan enantiomers with a detection limit of 8.8 μM . MIP was synthesized using acrylamide (AM) as monomer and trimethylpropane trimethacrylate (TRIM) as a crosslinking agent. The signal obtained by the binding of L-tryptophan was 3–4 fold higher than that obtained from D-tryptophan with the enantiomeric selectivity coefficient of 6.4.

On the other hand, the direct surface polymerization on a self-assembled alkanethiol monolayer of a gold-coated QCM resonator can also produce the thin MIP coating layer to build MIP-QCM sensors [192, 193]. In one approach [204], MIP was bound to the self-assembled monolayer (SAM)-coated QCM Au electrode for the detection of BPA. SAM, composed of 2-aminoethanethiol and 11-mercaptoundecanoic acid, had ion-pair interactions with the functional monomers in MIP to form proper adhesions. MIP contained specific cavities by

self-organized structure of functional monomers around a template. The sensor can detect BPA selectively in the liquid phase with a sensitivity of 100 ppb. It was observed that polymerization solution without solvent had better sensor performance, MIP-QCM sensor with SAM was better in terms of stability than that without SAM; greater weight of membrane improved the sensitivity of the sensor, and the optimized material type and its composition can lead to improved sensor.

Piacham *et al.* [193] studied the preparation of thin MIP nanofilms on the surface of QCM. The thickness of the MIP film was below 50 nm. The selective recognition of target analytes can be easily detected by underlying quartz crystal resonator. When used in a flow injection analysis system, the assembled QCM sensor generated a large frequency change (>30 Hz) upon encountering a small amount of analyte (0.19 mM), and had a very short response time (<1 min), and displayed certain chiral selectivity towards the original template, S-propranolol, at a concentration higher than 0.38 mM. Haupt *et al.* [194] reported another enantioselective QCM-MIP sensor. Using S-propranolol as a template, a poly(trimethylolpropane trimethacrylate-co-methacrylic acid) membrane was prepared by sandwiching the prepolymerization solution between a gold-coated QCM chip and the surface of a UV lamp. The resultant sensor showed good enantioselectivity for the "S" enantiomer when compared to the "R" enantiomer. In addition to imprinted polymer films, inorganic composite nanofilms have recently been prepared and used in the QCM sensors. Yang *et al.* [145] fabricated the glucose-sensitive TiO_2 ultrathin composite films by molecular imprinting on a gold electrode of QCM for the detection of monosaccharides. The largest binding capacity was 2.3-fold that of the corresponding nonimprinted films.

The determination of proteins is very important in quick medical diagnostics and modern drug discovery [205, 206]. The traditional detection approaches such as dye labeling and electrophoresis usually suffer from poor selectivity and sensitivity when the specimen is a protein mixture. The MIP film coupled with QCM may be a potential analytical method for the detection of proteins. In the case of protein imprinting, MIP film is a most desirable form of material since the removal of high-molecular protein template becomes much easier than in normal bulky MIP monoliths [90, 189, 205, 206].

Recently, a mixed salivary protein and poly-EVAL solution was coated onto a QCM chip and a molecularly imprinted EVAL thin film formed by thermally induced phase separation (TIPS) [207]. The optimal ethylene mole ratios of the commercially available EVALs for the imprinting of amylase, lipase and lysozyme were found to be 32, 38, and 44 mol%, respectively. Finally, the salivary protein-imprinted EVAL-based QCM sensors were used to detect amylase, lipase and lysozyme in real samples (saliva) and their effectiveness was compared with that of a commercial ARCHITECT ci 8200 chemical analysis system. The limits of detection (LOD) for those salivary proteins were as low as $\sim$ pM.

Yang *et al.* [208] have prepared a titania film imprinted by bilirubin molecule at the surface of quartz crystal using molecular imprinting and surface sol-gel

process. The interaction between bilirubin and imprinted film was investigated using QCM technique. Compared with pure titania film, the molecularly imprinted titania film exhibits a much higher adsorption capacity for the target molecule, and the adsorption kinetic parameter estimated from the *in situ* frequency measurement is about $1.6 \times 10^8 \text{ M}^{-1}$, which is ten times higher than that obtained on pure titania film. Moreover, our study indicates that the molecularly imprinted titania film possesses a better stability and reusability.

MIP-coated QCM sensor was developed for the determination of albumin concentration [189]. Albumin was imprinted with 3-dimethylaminopropyl methacrylamideacrylate. The albumin MIP was then coated on QCM Au electrode. In the tetraethylene glycol dimethacrylate-crosslinked polymer system, the adsorption capacity of different Au electrodes was in the decreasing order of $\text{Au-OH} > \text{Au-COOH} > \text{Au-NH}_2 > \text{Au}$, whereas the time taken to achieve a steady-state frequency was in the decreasing order of $\text{Au-NH}_2 < \text{Au-OH} < \text{Au-COOH} < \text{Au}$. However, in the trimethylolpropane trimethacrylate cross-linking system, the adsorption capacity of different electrodes was in the decreasing order of $\text{Au} > \text{Au-NH}_2 > \text{Au-OH} > \text{Au-COOH}$. The developed albumin MIP-QCM sensor had higher response to albumin than non-MIP-QCM sensor. The adsorption ratios of cytochrome c : lysozyme : albumin : myoglobin were 160 : 1 : 1942 : 30 and 13 : 1 : 249 : 86 for albumin MIP-QCM and non-MIP-QCM, respectively. The developed sensor had a linear range from 60–150 ppm for albumin. The clinical application of the developed sensor was tested by determining the albumin concentration in human serum. The values obtained by MIP-QCM and clinical assay were 4.80 g dL^{-1} and 4.60 g dL^{-1} , respectively, which showed good correlation and, thereby, demonstrate the potential of the developed sensor for clinical applications.

Zhou *et al.* [209] have described a simple, inexpensive and fast method for the generation of MIP film on QCM crystals using mussel-inspired polymer using dopamine as adhesive proteins of mussels. Polymerization of dopamine in the presence of template molecule (1,3,5-pentanetricarboxylic acid, an analogue of domoic acid, in this case) could produce an adherent molecularly imprinted polydopamine film coating on QCM crystals. Advantages, such as high hydrophilicity, high biocompatibility and controllable thickness, make this molecularly imprinted polydopamine film an attractive recognition element for sensors. The quantitation limit of domoic acid was 5 ppb with the linear range of 0–100 ppb. The QCM sensor has high selectivity and was able to distinguish domoic acid from its analogous p-phthalic acid and o-phthalic acid owing to the molecular imprinting effect. In addition, the practical analytical performance of the sensor was examined by evaluating the detection of domoic acid in mussel extracts with satisfactory results.

Furthermore, the rapid and inexpensive screening of viral and bacterial infections is urgently needed for preventing the spread of infection in animal and plant populations [210–213]. However, the detections of viruses and bacteria are generally time-consuming and expensive tasks because the biological detection needs a complex process including incubation, separation, dying and microscopy [210–213]. The bioimprinted QCM sensors may

provide a fast and selective biodetection by the combination of bioimprinting and QCM. Hayden recently developed the surface imprinting techniques on polymer-coated QCM to detect tobacco mosaic viruses (TMV) [214, 215] and living yeasts [216] in aqueous media. The imprinting cavities or trenches on the polymer surface mimicking the shape and surface functionality of the viruses and bacteria served as recognition sites for their rebinding. The sensors were applicable to TMV detection ranging from 100 ng mL^{-1} to 1 mg mL^{-1} [215], and allowed a selective on-line monitoring of the yeast cell concentrations in water over 5 orders of magnitude [216]. Moreover, they continued to expend the bioimprinting concept to the recognition of mammalian cells, which is a greater challenge because of their lower mechanical stability compared to microorganisms [117, 216, 217]. Erythrocyte-specific interactions with recognition sites on surface imprinted polyurethane were applicable for blood-group typing of the main ABO antigens [117]. The interesting finding in MIP-QCM sensor was highly relevant for clinical applications in serology.

In addition to the above mentioned mass-sensitive sensors, some MIP-nanomaterial-based surface Plasmon resonance (SPR) sensors have also been reported. For example, MIP gel with embedded gold nanoparticle was prepared on a gold substrate of a chip for fabricating an SPR sensor sensitive to a low molecular weight analyte [218]. The polymer synthesis was conducted by radical polymerization of a mixture of acrylic acid, N-isopropylacrylamide, N,N-methylenebisacrylamide, and gold nanoparticles in the presence of dopamine as model template species on a sensor chip coated with allyl mercaptan. The modified sensor chip showed an increasing SPR angle in response to dopamine concentration, which agrees with the expected sensing mechanism. Furthermore, the gold nanoparticles were shown to be effective for enhancing the signal intensity (the change of SPR angle) by comparison with a sensor chip immobilizing no gold nanoparticles. The analyte binding process and the consequent swelling appeared to be reversible, allowing one the repeated use of the presented sensor chip. Matsui *et al.* [219] have developed a SPR sensor highly sensitive and selective for the herbicide atrazine, which was composed by immobilizing atrazine-imprinted polymer with gold nanoparticles on a gold thin film as a sensor chip. In the detection, the atrazine-imprinted polymer was expected to work as synthetic receptor for selectively capturing atrazine in organic solvent, and the gold nanoparticles were expected to exhibit a coupling effect with the gold thin film to enhance the local electromagnetic field between the nanoparticles and the gold film, making the sensor chip highly sensitive for changes in micro-environmental polarity. This sensor was able to detect 5 pM atrazine in acetonitrile.

Frasconi *et al.* [220] have described a SPR sensor by functionalizing gold nanoparticles (AuNPs) with thioaniline electropolymerizable groups and (mercaptophenyl)boronic acid. The antibiotic substrates neomycin (NE), kanamycin (KA), and streptomycin (ST) include vicinal diol functionalities and, thus, bind to the boronic acid ligands. The electropolymerization of the functionalized AuNPs in the presence of NE, KA, or ST onto Au surfaces yields bisaniline-crosslinked AuNP composites that, after removal of the ligated antibiotics, provide molecularly imprinted matrixes which reveal high sensitivities

toward the sensing of the imprinted antibiotic analytes (detection limits for analyzing NE, KA, and ST correspond to 2.00 ± 0.21 pM, 1.00 ± 0.10 pM, and 200 ± 30 fM, respectively). The imprinted AuNP composites were used to analyze the antibiotics in milk samples. Meanwhile, they also fabricated SPR sensor on Au surface for amino acids (for example, L-glutamic acid, L-aspartic acid, L-histidine, and L-phenylalanine) by creating imprinted sites in the electropolymerized AuNPs composites [221]. Au nanoparticles functionalized with thioaniline and cysteine were used to assemble bis-aniline-bridged AuNPs composites. Recently, a SPR sensor was developed for the synthesis of molecularly imprinted Au nanoparticle composites on electrodes by electrochemical means [222]. The resulting composites include specific recognition sites for mono- or disaccharides. The method is based on the formation of a boronate complex between the respective saccharide and the boronic acid ligands associated with the AuNPs. The electropolymerization of the AuNPs leads, after cleavage of the respective boronate esters, and removal of the saccharide, to specific recognition sites for the association of the imprinted monosaccharides or disaccharides. The binding of the saccharides to the imprinted sites is followed by SPR. The changes in the refractive index of the AuNP composites upon the binding of the saccharides to the imprinted sites are amplified by the coupling between the localized plasmon associated with the nanoparticles and the surface plasmon wave propagating on the Au surface. This leads to the highly sensitive stereoselective and chiroselective detection of monosaccharides and disaccharides.

12.5 Conclusion

The research described in this monograph has clearly shown that various nanotechnologies could be used in the preparation of MIP nanomaterials and the fabrication of MIP-based nanosensors. MIP-nanomaterials in different format such as nanoparticles, nanotubes, and nanofilms can be synthesized in a controlled manner with the help of the surface topography and existing nanotechnologies. Although MIP-nanomaterials provide fast ingress and egress of analyte diffusion enhancing sensitivity remarkably, the future development of such materials in terms of high molecular affinity, less nonspecific adsorption, multiplexing capabilities, and the development of MIP-nanoarrays is greatly warranted for real applications in biomedical and industrial fields.

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PART III

DRUG DELIVERY AND THERAPEUTICS

Ground-Breaking Changes in Mimetic and Novel Nanostructured Composites for Intelligent-, Adaptive- and *In vivo*-responsive Drug Delivery Therapies

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Abstract

A series of strategies are currently proposed for more effective prophylactic therapy of acute and chronic ailments and palliative medicinal care. These resilient illnesses include cancers, tissue degeneration (e.g., macular, mesenchymal, vascular, epithelial, hemopoietal and parenchymal cells), immune system failure, systemic infections and neurological conditions. Treatments use solid drug and porous drug implants, gel, liquid (semisolids) and solid dosages and drug-bearing tissue mimetics. Such mimetics may be used after surgical intervention. The platform technologies, “molecular sculpture” and application chemistry use various polymer (natural, chemically modified and biocompatible novel/synthetic) and amphiphile self-assembly forms for a visionary nanofabrication, and surface or bulk nanoengineering- or nanolithographic-type patterning of nanomedicines. Smart methodologies of this type are frequently stimuli-responsive (e.g., redox state, enzyme, cation and pH), and use biodegradable materials. Some difficulties with biocompatibility and toxicology are, and continue to be, omnipresent.

Keywords: Polymer, conjugate, composite, conformation, lipid capsule, stealth, nanoparticle, biocompatibility

13.1 Introduction

A valid range of medicines or therapeutics is most likely to function best when attributed the technological know-how or resources required to create a given fit-for purpose standard, and thus, an improved quality of a specific intervention [1]. In this way, drugs (therapeutics; drug products) or hybrid therapeutic-diagnostics, which are broad-spectrum in nature but specific in what they do, are no less valuable than targeted therapeutic moieties. This approach lies

Table 13.1 A survey sampling of the range (indicative) of “biotargets” in contemporary medicine and means of therapeutic delivery.

Disease/illness	Drug/class	Usual route(s) of delivery
Malaria	Quinines	Tablet
Neural (e.g., MS, ME)	Interferon	IV
Viral (e.g., HIV, hepatitis)	Vaccine	IV
Neurodegenerative (e.g., Alzheimer's)	Bioactive	Tablet/IV
Immune (arthritis, eczema, dermatitis)	Steroid/NSAID	Tablet/topical
Hypertension	Diuretic/ACE	Tablet
Heart failure	Beta-blocker/ACE Statins	Tablet Tablet
Lung (e.g., asthma)	SLABA/steroid	Pulmonary
Diabetes (type II)	Insulin	Tablet/IV
Cancer*	Cytotoxic (lethal)	IV
General infection	Antibiotic	Tablet/IV/topical
Endocrinal	Hormone	IV/tablet/transdermal patch
Smoking replacement	Nicotine	Transdermal patch

Key: *- see tabled (Table 13.2) cancer data. NSAID – non-steroidal anti-inflammatory drug; MS and ME are multiple sclerosis and myalgic encephalomyelitis; HIV – is human immunodeficiency virus; ACE -angiotensin-converting enzyme inhibitors; SLABA is short and long-acting beta₂-adrenoceptor agonist used for asthma. IV - represents intravenous (injection form primarily).

behind a new type of insightful molecularly engineered product that traverses contemporary research, permitted products and recent patenting [2, 3]. The ingenuity of the drug product designer and associated potent drugs lies at the very center of contemporary advances in new products, drugs and drug cocktails (refer to Table 13.1).

Areas of significant growth and expansion are indicated in Table 13.2. This tends to be exacerbated because of difficulties in the route of administration in which, opportunities are severely curtailed (Table 13.1). In turn, the appropriate form is often achieved by encapsulation of the drug in a solid, lipid or oily capsule. One of the key issues concerning fabrication of new therapeutics is maintaining the chemical and physical integrity of the drug/product, and this extends to a plethora of cytotoxic drugs (cytotoxics), pulmonary and topical drug devices. Nanoparticle technologies are ubiquitous in therapeutic

Table 13.2 The table is divided into three sections. Oncology: survival stats and etiology, values change between studies but the trends are consistent [23, 24]. Cancer therapy is particularly highlighted since both its therapeutic success and the choice of therapeutic model available is at present rather limited. However, innovation in therapeutic models via selection of drug targets are giving way to more meaningful and actively targeted chemotherapy [5, 25].

Survival† statistics and etiology			
Cancer	% Incidence (approx.)	% Mortality (approx.)	Male: female ratio
Lung	20	25	3.5:1
Bowel	10	13	1.3:1
Breast	10	10	0.01:1
Stomach	7	9	2:1
Pancreas/	3-5	3-5	1.7:1
Prostate*/			
Bladder			
Cervix**	2.5	1.5	Female
Ovary**	2	3	Female
Leukemia	2	2.5	1.5:1
Brain	2	2	1.5:1
Skin	0.8	0.6	0.6:1
Testis**	0.4	0.2	Male
Liver	0.3	0.7	2.1:1
Bone	0.3	0.3	1.7:1
Types and nomenclature			
Tissue affected	Name of cancer	Metastatic† variety	Benign variety
General mesenchymal/epithelial cell	Carcinoma Sarcoma Melanoma§	Yes Yes Yes	
Blood	Leukemia†	Yes	
Lymphatic	Lymphoma		Yes
Young nerve	Blastoma	Yes	
Mixed tissues	Teratoma		Yes
	Fibroma		Yes
	Adenoma		Yes

(Continued)

Table 13.2 (*Continued*)

Innovations in therapeutic models‡		
"Drug"	Target/strategy	Preventative mechanism in the therapy
C/B/V-raf (raf) gene inhibitor e.g., PLX4032	Balk gene expression	Blockage of protein kinases (gene expression)
Methotrexate	Anti-metabolite	Anti-folate, DNA replication
5-fluorouracil	Anti-metabolite	D/RNA intercalator (cell cycle arrest)
Macugon	PEG-aptamer	Blockage of base pair transcription
Imatinib	Protein kinase activity in tumor promotion	Enzyme inhibitor
Nutlin, Tenovin-6 CP31398 (Pfizer)	p53 protein pathway activator	"Guardian angel"

‡ - cure is not always possible* (palliative treatment may represent only option), ** - refers only to male or female specific cancer. † - e.g., Hodgkin's disease, §e.g., Kaposi's sarcoma (etiology Human Herpes Virus 8 (HHV8)). PLX4032 also known as RG7204 or RO5185426 (Plexxikon).

medicine and pharmacy [4–7]. These involve particles of about 100–200 nm diameter that transport and hold the therapeutic molecule.

Rather unfortunately, a variety of common diseases, ranging from dermatitis to pancreas carcinoma, feature regularly in the list of illnesses that are both difficult to treat and hugely prevalent, and with at times a low efficaciousness of the drug delivery system (DDS) dosage form. However, they are featured in new innovation [8], and present a good deal of hope for disease therapy. Much of this poor uptake and targeting is based on large droplet/particle sizes in topical medicines and circulatory exclusion in the case of parenteral and ocular therapeutics (Table 13.1). Strategies to render the particles more transmissible, yet retentive and persistent within the body circulation have had considerable success over the last decade [9]. Further adaptation based on size reduction and ingenious design and engineering on a molecular scale can be used to further enhance drug encapsulation, control over release, biocompatibility, reduced toxicity and potency of the drug delivery system. Much of the resistance to technological advancement of a therapeutic entity lies in negating epithelial and circulatory barriers, and more systematic design given what drug developers know about the monocyte phagocyte (macrophage) system. The aim of a new wave of medicines is to reduce the nonspecificity in apoptosis within targeted tissues. Here, the developments and future advances in the more pragmatic and focused delivery of troublesome therapeutics drugs using

lipidic nanoaggregates is considered in "spectrum mode" and also in terms of key refinements.

A recent focus of chemical pharmacy has shifted emphasis to biological functions. These might include cells (that are particularly beneficial or detrimental to bodily functions), most notably plaque formation or neoplastic tumor tissue. The discordant research also looks at bioactives and their physiological role, or quite commonly now, scaffolds or matrices, which repair faults or permit interim integrative materials to assist in repair. These materials most notably are usually natural gums (polysaccharides) and polymers that are invoked for use in regenerative medicine specifically and are routinely used, for example, in the replacement of cells with new tissue; assisted by these types of polymeric matrices. Functional materials of this ilk frequently use sophisticated polymer chemistry [10, 11] and a host of biomaterials, but might also use biosurfactants and even incorporate exogenous factors such as the lipids and proteins found in the *in vivo* lung surfactant mixes (Curosurf, Survanta, Surfaxin, ALEC, etc.) that are now used to treat respiratory distress syndrome (RDS) in neonates.

These types of natural or "contrived" biomimetics work by developing responsive polymeric biodegradable scaffolds/matrices/particles using a system of ingenious design and intelligent formulation (e.g., for use in the treatment of burns and lesions). Such devices with a range of geometries may operate by stimulation of the extracellular matrix (ECM), e.g., nanoparticulate materials for drug delivery to articular cartilage using colloidal peptide or antibody ligands with specific binding capability. Here, application is only limited by one of imagination and innovation, and there seem to be as many applications as there are forms. Since the number of forms is huge, it suggests medical application is largely unlimited. In numerous instances nanoscopic constitution influences mesoscale assembly that results in specific macroscopic character (e.g., texture and rheological profile). Structural composition and particle size is thus important. In the case of intravenous (IV) DDS nanoparticles, continued circulation in the blood is facilitated by an optimal size, e.g., 40 nm-diameter nanoparticles enable stronger binding of opsonin and plasma proteins than 100 nm and therefore, macrophage clearance from the blood compartment [9]. Consequently, ideally one chooses 100 nm particles to maintain the nanoparticle DDS in the blood, increasing its therapeutic index.

Material platforms can be produced by a range of methodologies. In one currently favored approach, synthetic 20 nm diameter solids can be synthesized by inverse emulsion polymerization, where the particles are very uniformly sized and form within an emulsion droplet. It is then possible to add functional groups to the particle either by co-formation, complex synthetic chemistry or physisorption to produce incorporated polymer, e.g., polyethylene glycol (PEG) as the surface hydrophile. PEG has the advantage of rendering the particle invisible to the blood, liver and splenic macrophages aiding retention in the diseased organ or blood [12]. Carriers can be used best for diseases such as cancer by the long circulatory lifetime of the particle, and by its retention or entrapment in the leaky vasculature of fenestrated tissue found, for example, in a tumor. In cleverly placing a disulfide link between the PEG

and nanomoiety, polymers can be rendered reduction sensitive (for drug or gene release) in the endosome within the cytoplasm of the cell [13].

One is able to use ultra-small nanoparticles (25 nm) to target the lymph nodes, for example, by simply modifying the surface of the particle. A pancake/mushroom configuration of surface trapped or tagged polymer on the nanoparticles activates the complement system (opsonization) and macrophage uptake (14, 15). Injectable biodegradable polymer hydrogels can now be formulated and used to deliver therapeutics *in vivo*. The science can be limited though, since over-complication might involve use of bifunctionalizing (toxic crosslinking agents or media) agents that prove detrimental to the cells (by leaching and leachates) or bioactive proteins (via denaturation) when delivered. Newly recognized platforms include polymers such as polylactic acid (PLA), polycaprolactone (PCL) or PEG. Associated beads or "elastic" hydrogels with slow degradation (1–3 weeks) can be fabricated to fall apart with delay using derivatized functionalized natural gums and plastics (acrylates) to permit cellular reintegration in the case of wound or bone repair management [16–20]. Strategies routinely used are self-assembly, nanofabrication and stimuli-responsive biodegradable materials.

Temperature-sensitive polymers, especially poly(N-isopropylacrylamide), at elevated high critical solution temperature (HCST), such as body temperature (37°C), can be suitably stimulated so the polymer chains are present as contracted hydrophobic aggregates of 20 nm length (10 kDa) or so. Yet, at low critical solution temperatures (LCST) down to room temperature (20°C), the polymer has extended polymer chains of 60-nm length favoring cell attachment [14, 21]. This can be used for *ex vivo* cell cultivation, cornea, bladder and myocardial usage but equally can be used where the inverse of the former size change is true to maintain the particle in the blood in a parenteral (IV) drug product. In an equal way, one can use peptide molecules (Figures 13.1 and 13.2) and build nanostructures by giving the appropriate cue to self-assembly of a nanoparticle so that the hydrophilic end, structure-inducing (signaling) center, and hydrophobic tail structure preference can also be induced by adding polyions to favor cellular uptake. Time and again, the current stumbling block appears at the preclinical testing of biomaterials, its value and its validity when extended to first-in-man clinical trials, which is the point at which many innovations fail.

13.1.1 Diseases of Major Importance in Society

The ubiquitous nature of the major prevalent diseases [22], such as cancers, circulatory-metabolic ailments and organ failure, and those of the central nervous system (CNS), skin and infection, means platform technology has manifold applications. Commonly featured cancers tend to dominate the new product pipeline and are typically those of the endocrine systems, brain, kidney, lungs, bowel, cervix, breast and skin. These diseases require a more focused approach to getting the drug into the body in a usable form

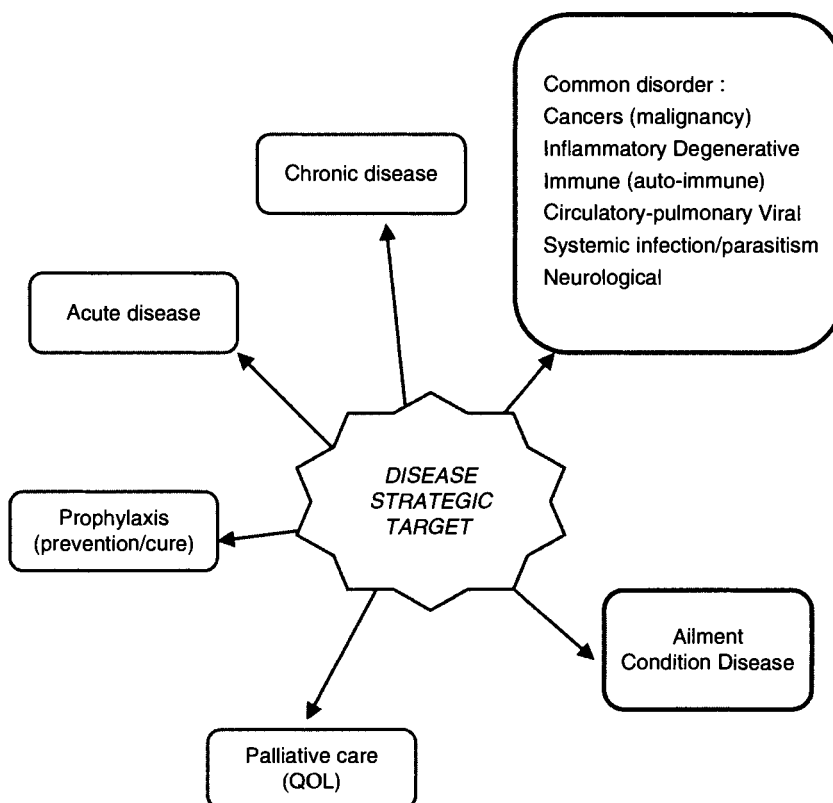


Figure 13.1 Principal considerations and motivations in the strategically planned fight against lethal and nonlethal diseases. The fight can involve a prophylactic or palliative (quality-of-life; QOL enhancement) approach to care.

(see Fig. 13.2) Common circulatory disorders are most frequently based around cardiovascular degeneration, involving asthma or heart and lung failures. The routinely found treatable metabolic disorders typically involve the liver, spleen, lymphatic system and pancreas. In some cases central CNS disorders (multiple sclerosis), skin diseases (eczema, psoriasis), and systemic pathogen infection (immuno-deficiency viruses) result in diseases of the liver, blood and brain.

Using the UK as a prime example, prevalence of diseases diminishes in the order of circulatory-stroke-heart failure (10–25% of all deaths), cancers (15%), respiratory (6%), and then diabetes and finally infectious diseases. Heart diseases and cancers outstrip the next killer diseases fivefold. Drug companies driven by competitive advantage and profit to plough back into R&D, target highlighted diseases such as cancers, atherosclerosis, hypercholesterolemia, type II diabetes, hypertension and other diseases associated with “obesity and a high stress and poor diet lifestyle.” This indicates [22, 26, 27] where drug and biotech companies will target their research effort and is exemplified by the data in the list of newly approved drugs [28]. These drugs include therapeutics

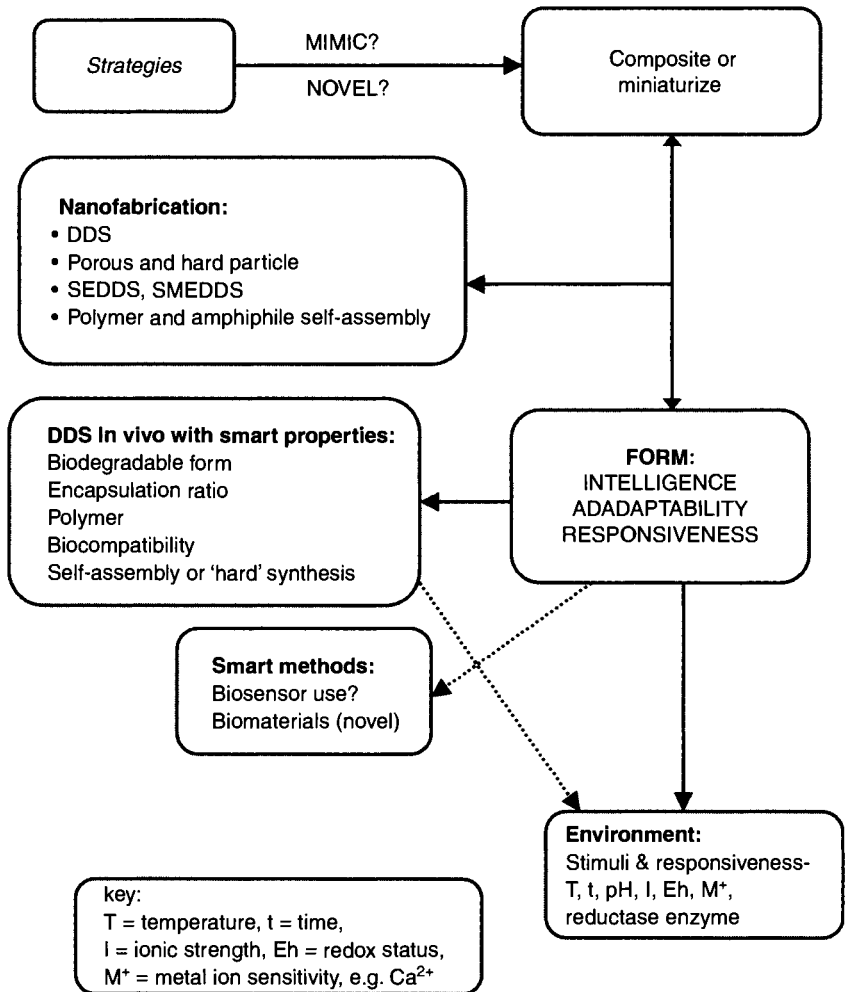


Figure 13.2 Phenomenological representation of bottom-up drug product (drug) design considerations. The form, environment and nanofabrication method are important factors in production of a smart technology that is variable in structure and its use. DDS is drug delivery system, where SEDDS and SMEDDS are self-emulsifying and surfactant-mediated emulsified forms, respectively.

frequently designed as an antibody or stealth format for specific targeting and increased circulatory persistence, respectively. Products of this type include antibody-conjugated drugs such as, Herceptin (breast cancer), Mylotarg (myelogenous leukemia), Avastin (soft tissue cancer), Rituxan/MabThera (lymphomas, leukemias, transplant rejection and several autoimmune disorders) and Bexxar (non-Hodgkin's Lymphoma). Other products could include Glivec/Gleevec (small molecule for treating chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors), Abraxane (albumin-drug conjugate for cancer), Doxil/Caelyx (stealth liposome form for cancer) and Taxol (cancer) (also see Table 13.2).

13.1.2 Types of Cancers and Diseases Requiring Specific Dosage Delivery

The search for cancer therapeutics sits at the very forefront of modern research [24]. This is frequently because these disorders in particular, are generally poorly responsive, tenacious and resistant to treatment/intervention, frequently reoccurrent and relatively poorly targeted at present, even given the best medical advances. Thus, the search for more efficacious and less deleterious, i.e., better anti-cancer drugs, features very prominently in contemporary drug company and academic research. Significantly, of the cancers seen by medics there are habitually predominant forms. Across the sexes these are lung (approximately 5.4% of cases), breast (4.8%), colorectal (4.1%), stomach (4.1%) and liver (2.2%) (see Table 13.2). Some of the more difficult to treat and particularly tenacious and aggressive cancers may include, prostate, pancreas [11] and breast varieties. The latter are on the increase globally [10–12, 25, 29], and this points for the need for a concerted effort for better products. In the more economically advanced nations (developed world) for men the most frequent cancers are lung, prostate and colon, whereas, for women it appears to be breast cancer that predominates, by far and above, that of the next form which is cancer of the colon. Breast cancer [22] occurs, notably more in females, and more than any cancer in men and in any part of the world.

Cancer (neoplasm, carcinoma, sarcoma, etc.) affects approximately 33% of people in their lifetime (Table 13.2). Cancerous tumors (malignant neoplasia) can be considered to be related to a cellular disorder resulting from a whole series of changes in response to an array of environmental, social, genetic and possibly lifestyle factors. Principal carcinogens include radiation (UV, X-rays), chromosomal influences (chromosomes, genetic mutation, oncogenes and proto-oncogenes), chemostimulants (radon, nickel, asbestos-crocidolite minerals, polyaromatic hydrocarbons), biological factors (human papilloma viruses, Hepatitis B virus, Epstein-Barr virus, human immunodeficiency virus (HIV) and bacterial infection) and may extend to natural promoting agents such as hormones. The tumor itself is considered to be a result of unregulated and abnormal growth, and an initial (quick rate) lack of responsiveness to the precision mechanisms of the cell concerned.

What do we mean by the expression “tenacious cancer” and why is this such a key issue? Tenacious cancers are actually really difficult to treat in a variety of senses. Fortunately, not all cancers are of the tenacious variety [28, 30, 31]. Tenacity is not a simple concept, however, and can involve many criteria. The criteria that are usually indicative of tenacious cancers involve disproportionate prevalence, difficulty in therapeutic treatment, difficulty in surgical removal, an aggressive encroachment on tissues, a rich blood supply and high flow within the tissue flow or internal pressure, high cell turnover and inefficient nonideal “make-do” therapies, which are only available at present. However, they are not simply confined to these features alone and might also involve metastases (secondary growths), inaccessibility of the tumor, the presence of sanctuary sites (in the brain, for example, via blood-brain-barrier; BBB)

and physical lack of penetration (e.g., bone). Additionally, tenacity may include essential organ tumors (heart, lung, liver and brain), a high rejection/expulsion rate of therapeutic, loss of responsiveness, cellular ecology and alternative metabolic pathways. Asymptomatic cancers are particularly troublesome, e.g., cervix, bladder, (unlike conventional symptomatic types seen with brain and prostate), and do not always indicate the need for therapeutic intervention.

There are three demographic subdivisions where cancers are observed. First, embryonic cancers (*in utero*) largely of the nerve cells, kidneys, and a range of leukemias, etc. Secondly, leukemias, testicular cancers, bone cancers, etc., which largely affect the young. Thirdly, cancers which are predominant among the elderly population, such as prostate cancers, colon cancers, bladder cancers and types of skin cancer, etc. [23, 32]. To compound this complexity there are essentially four different affected systems [33]. According to this classification – mesenchymal tissues, typically, tumors of blood vessels [34], connective tissues, musculature and lymphatic structures occur most frequently. The second type is cancer of specific tissues and the epithelia such as the endocrine system [35], liver [31], skin, intestine, etc. A third variety involves neoplasia of defense structures and supporting tissues, such as those of the monocyte phagocyte systems (MPS) or reticulo-endothelial system (RES), which include the spleen, the liver and bone marrow. The final variety include cancers of the brain, central nervous system (CNS) [36], spinal cord and associated coverings. Tumorigenic growth also can be found in the form of benign tumors (e.g., lipoma), malignant tumors (invasive cancers) and metastatic tumors (secondary cancers, which arise from the original cancer). Benign cancers are categorized by effectively resembling their parent tissue/cells. On the other hand, malignant cancers show pronounced cellular abnormality and are associated with specific tissue invasion and destruction of the mesenchyme. After a relatively rapid change in response to environmental conditions the growth rate can be slower. The spread of cancer within the body to produce metastases (secondaries) is dependent on the circulation and arrest of the cell [23]. In this case, tenacious cancers of this variety are very problematic indeed, and constitute the cancer that drug developers seek to destroy. Growth rate is correlated with cancer cell differentiation (see Table 13.2). Death is usually a result of indirect means such as organ failure or secondary infection. Where the differentiation is high, tumor growth rate is also high, and where a lower cell differentiation is observed a slower tumor growth rate is observed. Progression of the cancer from stage 1 (small and treatable) to stage 4 (large and unresectable) is generally cell type dependent.

13.2 Obstacles to the Clinician

Using cancers as a most fitting example of difficult to treat ailments it is possible to rate current innovations. The main point of the technological “push” to produce better medicines is balancing therapeutic good versus efficacy versus potency (and thus lethality) of the therapeutic agent (see Fig. 13.1). Practically

speaking, cost is also highly significant, as is the ideal route of administration. At the top of the “needs” list is ease and efficacy, so intravenous (IV) and high encapsulation of highly potent drug is favored. However, there are also significant toxicological drawbacks to this approach if the therapeutic agent is not targeted in an exacting fashion to the intended tissue. The scope of targeted [3] organs or tissues can include paracellular or transcellular delivery [3, 4, 7]. Alternatively, other mechanisms of drug molecule delivery (drug delivery system; DDS) may principally involve the blood [10] and associated pulmonary system [37]. In this case, the organs concerned are most likely to be the brain [37], lungs [11], heart [26], intestines [38], liver [10, 12], skin [39], pancreas [12], cervix or ovaries and testis [40], kidneys [38] and stomach [23], etc., in a purpose-built DDS (see Table 13.2).

Following to some extent societal trends [41], the range of available or current in clinical trial (CT) products is actually rather limited. With a startling mere 6% of new drugs actually reaching the final stage III of clinical trials, one feels drug development is awkward because it is a very inefficient process. The attrition rate of developed drugs is exceptionally high and this can be compounded by lack of imagination in drug development and by inherent difficulties seen with any new therapeutic, such as toxic response or serious side effect. Three recent patents [42–44] show the way by means of new forms of drug development that use nanostructured particle encapsulation for adaptive DDSs. Again, cost is an important consideration for any drug used routinely. Antibody-based marketed prodrugs, for example, Remcade® (Johnson & Johnson) and Herceptin® (Genentech) for arthritis and breast cancer; respectively cost a conservatively estimated \$1000/100 mg and \$70,000 for a full course of treatment, respectively. It goes without saying that national budgets for health in most countries will not permit routine use of such a product, irrespective of how therapeutically appealing they are. Consequently, good-product but high-cost can be utterly inhibitory to routine use.

Certain cancers such as prostate and breast types are significant [24] and are recognized to be on the increase globally. Consequently, the clinician needs to evaluate whether the disease is relatively common or indeed impractically rare, harsh though it may seem, to necessitate significant investment in new drug development. However, other considerations for the product do not necessarily involve life-threatening disorders but ones which may be debilitating and either acute or chronic, and that require appropriate intervention (see Figure 13.1). Degenerative diseases, which may in fact not always be life-threatening but certainly influence the quality-of-life (QOL) of the patient, are typified by diseases such as acne, arthritis or psoriasis. This then starts the debate as to the need and societal value of prophylactic or palliative treatments, and again, the cost in terms of investment in research and the economic feasibility of prescribing these medicines.

The clinician is not just faced with burdensome issues of cost of the treatment but also some diseases which are exceptionally difficult to access due to physiological barriers. Consequently, drug administration, its form, and focus needs to consider a range of parameters highlighted in Figure 13.3. One major

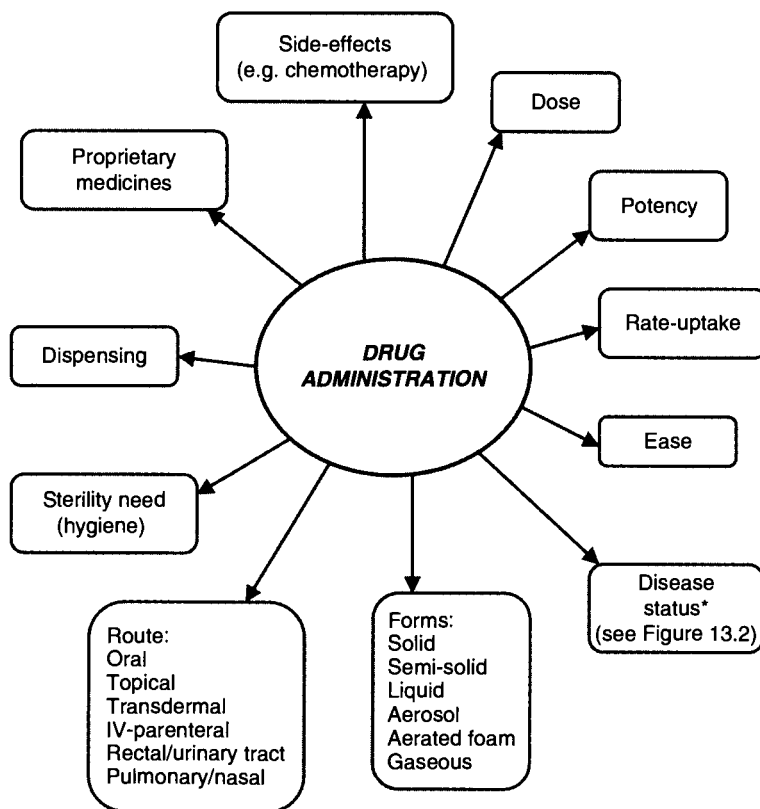


Figure 13.3 *In situ* administration of intelligent, adaptive and responsive drug delivery therapeutics and the obstacles to the clinical team referred to in Figure 13.1.

inhibitory issue in the development of new products for disorders of the central nervous system (e.g., Alzheimer's disease) has been circumventing resistivity to dosing blood-brain-barrier (BBB) because of its intrinsic structural barrier properties [24] or mediated complement activation within the blood compartment [46]. In this case the dual barrier of physical structure and defying the RES/MPS macrophages becomes very telling. Clearly, one coherent standard approach does not work particularly well across the range of diseases.

Everyday concerns to the clinician (Fig. 13.3) such as side effects (necrosis, nausea, bleeding) and delivery route (IV, pulmonary, vaginal, gastrointestinal tract [GIT; oral] and dermal) are central to efficacious use. Dispensing of the drug, its ease and adaptability (for example in "cocktailing" or mixing of drugs) for proprietary dosage forms is also based on product adaptability. The drug dosage, potency, rate of uptake (again, related to site of administration), ease of use, lethal nature of the disorder and therefore, urgency in treatment that relates to short or long-term use are important additional considerations. For some medicines, notably colloidal samples of peptides, protein or nanoparticles dispersed in isotonic buffered saline for IV injection; these have additional prerequisites in terms of product sterility, for example, since the products are usually used for patients with systemic infection or for anticancer

chemotherapies. Although ubiquitous in variety, a series of cancers, such as pancreas, liver, brain and skin and ovarian varieties currently remain, at this point in time, exceptionally difficult to treat. The array of cytotoxic drugs, such as cisplatin and paclitaxel or doxorubicin and the currently most favored modes of administration using IV preparations, solutions and colloids (Figure 13.4) are areas for further clinical exploration [11, 12, 29–31, 33, 35]. Part of the difficulty, at least for the clinician, lies in overcoming particular epithelial and circulatory barriers and, more pragmatically, the nonspecificity in programmed death within targeted organs. This is shown phenomenologically in a cartoon in Figure 13.4; where the cancer is effectively bombarded by a number of strategic approaches (torpedoes 1–5) but can only have success if these defy barriers, which are dosage regime, drug toxicity and patient vulnerability. The focus of this chapter, and a significant key step forward, are developments and future advances in the systematic and focused delivery of noxious drugs via medicinal nanostructured particles that are fabricated in a way to use biocompatible block copolymers and lipids.

The staging (ability to respond to the disease progression) of various cancer's such as those of the lung (poorer success rates), liver (difficult to treat), pancreas (difficult to treat), brain, gonad, lymph node (non-Hodgkin's), bowel and breast (see Table 13.1 and Table 13.2), which are among the most tenacious is divided into four stages. Stage 1 usually refers to tumors that are small and localized (treatable); stage 2 cancers are fully operable; stage 3 cancers are large but in other senses similar to stage 2, and; stage 4 cancers are usually incurable (tenacious, spread, multi-organ), typically involving well spread-out metastases [28, 47]. This means most of the DDS therapies are aimed at use in stage 1 and 2 (Figure 13.4), or essentially for tumor shrinkage or keeping in abeyance in the latter stages of 3 and 4. This is because of the complexity of cancer (see Section 13.1.2).

There are a number of therapeutic (drug) classes (Table 13.3) used in routine chemotherapy [28]. These classes include alkylating agents such as cyclophosphamide (for lymphoma) and antimetabolites such as paclitaxel (Taxol®, used at a frequency of about 20% of all chemotherapeutics for numerous types of resilient cancers) or methotrexate. It also includes plant alkaloids such as vinblastine (Hodgkin's lymphoma and leukemias) and a much larger class of antibiotic chemotherapeutics used with huge inventive success (Table 13.3), which include actinomycin D, doxorubicin (Doxil® liposomes) and daunorubicin (DaunoXome® liposome). Additionally, it can also extend to enzymes (e.g., metalloproteinase, L-asparaginase) for use in chemotherapy. Finally, it can include a miscellaneous group of chemical drugs that includes inorganic chemicals such as cis-platinum diammine dichloride (testicular cancers) and synthetic heterocyclic organic molecules such as procarbazine and hydroxyurea. Frequently now, these molecules are linked to antibodies or polymers to enhance targeting efficiency and circulatory longevity, and this often uses monoclonal antibody or "mab" technology (e.g., tositumomab, etc.). Lastly, in some cases hormones and steroidal drugs can be used to indirectly influence the cancer. Such estrogens and androgens can be used to treat certain cancers

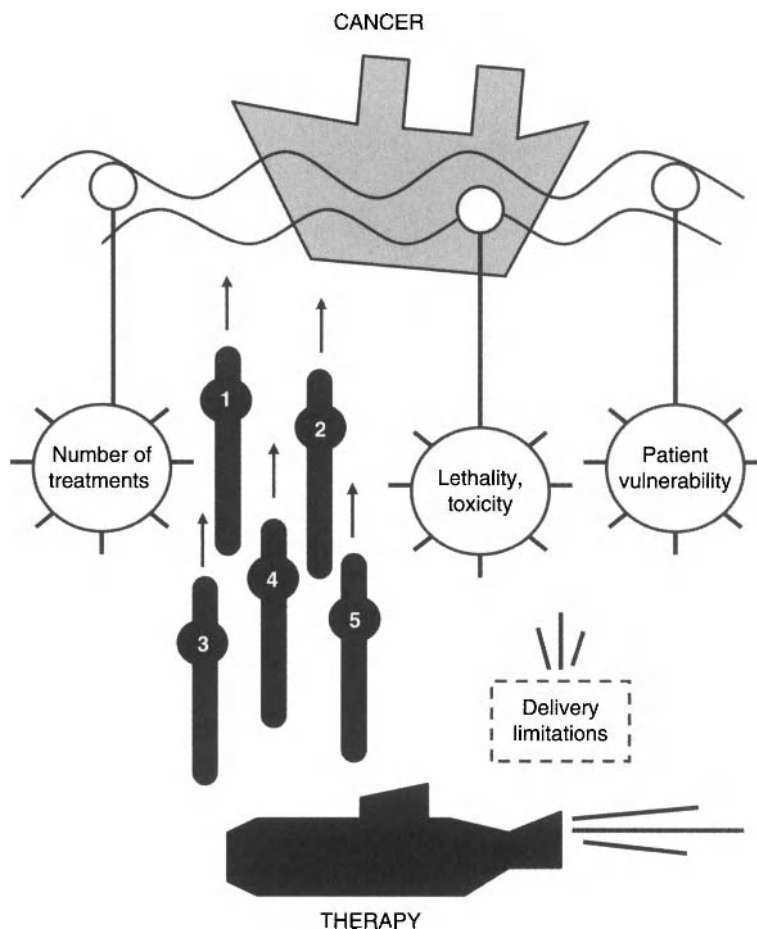


Figure 13.4 Simplified phenomenological model of therapeutic targets and hindrances for tenacious cancers (by way of an example). Cancer is represented by a “floating vessel,” whilst obstacles to uptake and efficaciousness of the therapy are represented by “mines.” Constraints to the medical therapy itself are indicated by the “depth-charge” of delivery limitations and the numbers corresponding to different forms of therapeutics as indicated by a “torpedo” heading for the vessel from the “submarine vessel” representing generic notions of therapy are as follows. Number 1 represents “traditional” chemotherapy (highest importance); 2 represents radiotherapy; 3 represents immunotherapy; 4 represents gene therapy, and; 5 represents possible multistrategy approaches, such as conjugation of an antibody to radioisotope bearing particles. There is a “stochastic” element to avoiding collision between torpedo and mine.

(breast and testicular, respectively) and corticosteroids (such as prednisolone) can also be used to impact on tumor growth by use of a cocktail of specific drugs. In this and many cases use of cocktails and, for example, quadrupling of the drugs used may reduce reappearance of cancers by about 40% in the case of less tenacious cancers such as Hodgkin’s lymphoma [1, 47].

All therapeutics as indicated in Figures 13.4 and 13.5 face difficulties in terms of tissue penetration and the failed access to diseased tissue conferred by this poor penetration, limitations of the delivery vehicle and active targeting. Targeting of the cancer cell without excessive damage to surrounding tissues

Table 13.3 Innovations in active targeting of tissues and the range of technologies that may be used. Lipophilic drugs are difficult to formulate out of a lipidic medium (liposomes, lipid emulsions and lipidic nanocapsules) and thus deliver and this is exacerbated since approximately 30–50% of drugs are “poorly” water soluble.

Moiety	Innovation	Used for
Prodrugs : Renitec (enalapril→ <i>esterase</i> → enalaprilat) Deltasone (prednisone→ <i>liver</i> <i>bioactivation</i> → prednisolone) Valtrex (valacyclovir→ <i>esterase</i> →acyclovir)	Bioconversion <i>in situ</i>	Active/specific targeting hypertension immuno-suppressant <i>Herpes simplex/zoster</i>
Nanotechnology devices: <i>See below</i>	Encapsulation (a range of recent patents (42-45))	Stealth form; active targeting
Recombinant technology: Remicade (infliximab) Herceptin (trastuzumab) Simulect (basiliximab) Bexxar (tositumomab)	Antibody conjugation Iodine ¹³¹ isotope conjugate	Active targeting anti-inflammatory breast CA organ rejection aid follicular lymphoma
Peptides: Levemir (insulin prodrug+albumin) AB101 (insulin PEGylation) Forteo (teriparatib) Lupron (luprolide) Velcade (bortezomib)	Purity/functionality Boron-peptide conjugate	Active targeting diabetes type-II diabetes type-II osteoporosis (PTH hormone) prostate/breast CA myeloma/lymphoma
Nanotechnology devices		
Active (e.g., commercial dosage)	Used for	Formulation/ considerations
Skin cell matrix (Apligraf)	Foot ulceration	Buffered media with bovine type-I collagen
Poly(anhydroglucuronic acid)-cellulose (Surgicel)	Oral surgery	Aqueous. Gives low pH
Propofol (Diprivan)	Anaesthesia	Lipophilic medium (e.g., oil)
Ritonavir (Norvir)	HIV/AIDS	Lipophilic medium (e.g., oil)

(Continued)

Table 13.3 (Continued)

Nanotechnology devices		
Active (e.g., commercial dosage)	Used for	Formulation/ considerations
Lopinavir/ritonavir (Kaletra)	HIV	Lipophile - HPC/hypromellose polymer melt
Paclitaxel (Taxol)	CA	Liposome
Doxorubicin (Doxil)	CA	Liposome
Finasteride (Propecia)	Prostate CA	Lipophilic medium (e.g., oil)
Betamethasone (Celestone)**	Eczema	Lipophilic medium (e.g., oil)
Mometosone furoate (Elocon)**	Psoriasis	Lipophilic medium (e.g., oil)
Tacrolimus (Protopic)	Transplants	Lipophilic medium (e.g., oil)
AmphotericinB (AmBisome)	Anti-fungal	Liposome/acid and base drug
Acyclovir (Zovirax)	Anti-viral	Lipophilic medium (e.g., oil)
Terbinafine HCl (Lamisil)*	Anti-fungal	Lipophilic medium (e.g., oil)
Doxycycline (Vibramycin)	Malaria/ filarial	Lipophilic medium (e.g., oil)

Key: CA – is cancer. HIV/AIDS are human immunodeficiency virus/acquired immune deficiency syndrome.

*- very lipophilic (very hydrophobic) - $\log P \sim 5.4$. ** - corticosteroids tend to be lipophilic (Mometosone furoate $\log P \sim 4.7$).

Doxycycline has a weaker lipophilicity with a $\log P$ value of ~ 1.0 .

AmphotericinB $\log P$ value of ~ 0.8 .

AB101 is a poly(ethyleneglycol)-insulin (PEGylated insulin) IV drug from AntriaBio.

is of primary concern [23]. The cartoon in Figure 13.4 indicates that “fired” therapeutics do not always reach their target and must circumvent delivery limitations (widespread delivery and bioconversion), but also may lose focus over a number of prolonged courses of treatment [28]. Currently, about ten or so antibody-drug conjugates are routinely used for the active targeting of cancers (Herceptin, Avastin, Bexxar, Mylotarg, etc.) with some considerable

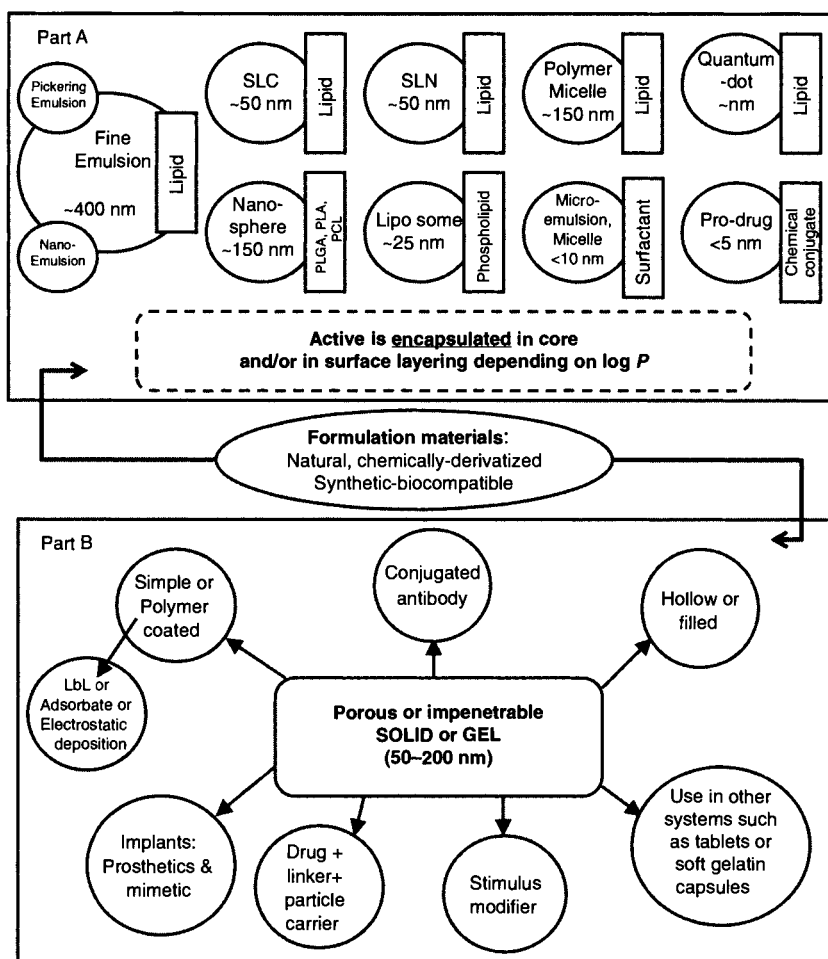


Figure 13.5 Notions of polymers, particle surfaces and interfaces, soft nanotechnology products and materials self-assembly in drug delivery system (DDS) science. Part A shows the forms such as solid lipid nanoparticles (SLNs) and solid lipid capsules (SLCs) available as DDSs, their size range and key excipient type (PLGA is poly(lactate-glycolate), PLA is poly(lactate) and PCL is poly(caprolactone) and Part B indicates the subtlety of modification that is open to drug delivery scientists. The link technology between A and B is indicated.

success. It is also possible to use specific ligands in order to identify and highlight the cell of interest. Again, for cancer cells some success has been achieved by using a drug (e.g., liposomal doxorubicin) conjugated to folic acid which is needed in significant supply by the cancer cell [40]. Other likely candidates that might be adopted to facilitate active targeting include an integrin-conjugated "raf" gene for use in liposomes and the disaccharide galactose bearing (retinoic acid conjugate) polylactate nanoparticles again taken up by certain cells preferentially [28].

A range of therapeutic approaches ranging from radiotherapy to gene therapy can make significant use of such clever conjugation chemistry linked to

intelligent encapsulation of the drug in nanoparticles. Radiotherapy at present largely involves coating of particles bearing the radioisotope [36, 48]. Particles carrying isotopes (Figure 13.5) of radium, uranium are used for skin or surface treatment (α -particles). Whereas more significantly, strontium, iron, iodine, yttrium are used for deeper penetrating β -particle emissions and selenium, iodine, technetium and indium which give yet further penetrating power (i.e., 2–6 mm of tissue) are used for γ -radiation. An antibody-yttrium⁹⁰ conjugate (Zevalin®) and ones containing gadolinium [48] are now used for radiotherapy. Photodynamic therapy (PDT) makes use of quantum dots or nanocrystals of inorganic and organic materials [39] that respond to an external stimulus to give off light. This light can be in the form of heat (infrared) or ultraviolet to aid destruction of the tumor cell *in situ*. When linked to an antibody this technology becomes very powerful.

Gene therapy is centered on the insertion of genes into individual cells, and thus tissues, to treat disease. It works by insertion of a “normal gene” and thus replacement/repair of the abnormal gene. This branch of drug delivery science is still in its infancy with no currently approved products on the market, although many products are the subject of academic research and preliminary clinical trials. Potential success within this area lies in a form of cellular modification, usually meaning modification of genes obtained with viral vectors, plasmids and oligonucleotide-bearing nanocapsules [25]. DNA plasmids and viral vectors such as retroviruses, adenovirus and adeno-associated viruses (AAV) are able to “infect,” in directed fashion a relatively broad range of cell types quite efficiently. Their size itself can be rather inhibitory in terms of payload (drug) carriage within a nanoparticle. Cationic polymers and lipids in particle or capsule form with a positive surface charge represent one form of carrier. There are significant issues and practical concerns with this technology at present regarding toxicity and lethality of the carrier and efficacy of the drug form because if misdirected to the wrong organ, it can instigate tumor mutagenesis or has resulted in death in animal models.

13.3 Hurdles for the Pharmaceuticist

The habitual problem for the scientist that manufactures and formulates a drug product (pharmaceuticist) lies in the nature of the drug itself. Many drugs do not possess ideal hydrophilicities to be solubilized in aqueous systems (see Table 13.4), yet incongruously are insufficiently lipophilic to be encapsulated efficiently in oil or apolar capsules, such as those made from solid fat, emulsifiers or polymers. Additionally, the particle that houses the drug has to be engineered so that it releases, or is made to release, all the cargo of drug in depot fashion at the site of interest (Fig. 13.2 and Table 13.3). Some strategies do exist that make use of prodrugs, where the drug is bound to a polymer or particle so that it is not metabolized and removed from the body. By careful preparation it is indeed possible to fabricate nanodrugs (nanotechnology devices),

Table 13.4 Preformulation and formulation and impact on product efficacy.

Aspect of drug moiety	Relative importance or significance
Solubility, ionisation and pK_a -pH	Affects bioavailability of drug
Compatibility <i>vs</i> excipients use	Affects bioavailability of drug
Penetration aids e.g., DMSO/Ethanol	Affects uptake of drug
Shape/size/texture/consistency	Affects bioavailability of drug
Strength/brittleness of form	Affects drug product (QC)
Porosity of form	Affects drug product (QC)
Polydispersity of excipients	Affects bioavailability of drug
Dose-potency-polymorphism	Affects bioavailability of drug
Ideal delivery route	Affects uptake of drug
Lipinski's rule-of-five (size-composition-polarity)	Affects bioavailability of drug
DLVO flocculation (charge <i>vs</i> coating)	Affects drug product (QC)
Co-solvent	Affects bioavailability of drug

using antibody conjugation or target-specific peptides or oligopeptides that facilitate better specific uptake of the drug moiety. These are frequently in the form of micellar, liposomal, niosomal, or lipid particle DDSs (Figure 13.5). A list of example particulate DDSs that house lipophilic drugs is provided in Table 13.3. The scope of application of these products traverses transdermal to injectable drug products [3, 6, 49].

Since drugs are frequently hydrolyzed *in vitro* or *in vivo*, are acid-labile in respect of their structural conformation, are photo-labile on contact with strong light and also show a tendency to produce multiple polymorphic crystalline forms (with differing solubilities and shapes); the job of a pharmacist is not an easy one. Additionally, since the carrier (nanoparticle/encapsulant) must be both biocompatible and bioavailable when it might be employed as a carrier, this severely curtails the choice of polymers, surfactants and oils or fats that might be used. A range of preformulation considerations is presented in Table 13.4. Issues that habitually arise to make the pharmaceutical formulation more difficult are those of a change in the drug product, which influence the bioavailability of the drug moiety. Typically, these are its species solubility ($\log P$), species pK_a , covalent bonding and oxidation of the drug as a result of interaction with excipient in the drug formulation. For the product itself, issues that influence either the product quality [4, 50] and consistency or bioavailability include flocculation of particles, porosity of the solid and polydispersity

of size and the products mechanical behavior, such as hardness or textural consistency.

New drug products require precise and thoughtful [7] synthesis and careful biological modeling for the specific engineering of "smartness" into fully responsive systems [2]. Surface structure, its ordering and function [14], including adsorption of polymers or use of conjugation chemistry, allows the specific fabrication of intelligent and adaptive materials [3, 6]. Most potent pharmaceuticals of the type used for chemotherapy are administered principally by the intravenous route (occasionally through the skin). Here the longevity of circulation of prodrugs in systemic circulation of the parenteral medicines is crucial [21, 51]. The efficiency of treatment with newer forms of drug system and cytoplasmic uptake are crucial for better efficacy [3, 21] and efficient encapsulation. The failure of many DDS is based on use of passive (chance) targeting rather than active (determined) drug delivery, this means the therapeutic is not sent to where it is required. Cell apoptosis, tissue necrosis and organ failure are often the consequences of accumulated toxicity because of sporadic delivery. In contrast, metabolism, excretion, and loss of therapeutic effect or potency may produce an unsuitable product *in vivo*.

Novel biomimetic and nanostructured composites (Fig. 13.5) include a new generation of novel nanopharmaceuticals [26] and nanodevices. Stealth liposomes [3] and the newer solid lipid nanoparticles (SLNs) [52] or solid lipid nanocapsules (LNCs) with an oily core, represent a new generation of more efficacious DDS [2, 5, 6, 53, 54]. Therapeutic use of block polymers, such as with Sporonox®, Genoxol PM®, Myocet® and Rective® and a range of isotope bearing nanosolids (such as developmental products SPI-77, CYT-6091 Aurimmune, etc.) is increasingly significant [27]. In most cases, the issues that dictate the quality of the product relate to the stability of the drug in its encapsulated form (see Table 13.5). These differ between soft and hard colloidal particles and organic or inorganic particles. Aggregation, denaturation of biopolymers, flocculation, hydrolysis and photodegradation feature routinely in the list of criteria which render the product unsuitable.

The pharmacist must ensure the drug is presented in a manner that disguises the active drug. Certain changes can be avoided by the use of stealthifying the nanoparticle. This technology is employed quite routinely now since it provides better solubility, confers a degree of chemical protection, and can be used to prolong the DDS in the blood compartment avoiding macrophage clearance. Stealthifying with poly(ethylene glycol) or PEG [9] ensures the drug is protected (in a capsule) but does not encroach on the therapeutic active. For nanodrugs physico-chemical features such as aggregation, binding and solubility also feature strongly in preformulation considerations in the design of a new product. The range of considerations also varies according to the chemical nature of the therapeutic molecule. Proteins and peptides, for example, can lose their conformation, irreversibly crosslink, and are subject to the processes of denaturation and flocculation.

Table 13.5 Chemical, biological and physical degeneration reactions. Product "reactions" involving organic (inorganic) – small molecule and peptide or protein drugs.

Degradation	Example	consequences	
Microbiological	fermentation	loss of potency, enzymic change, toxicity	
Chemical	photo or chemo-catalysis*	hydrolysis, autoxidation, ligation, solubility loss, denaturation, substitution	
Physical	polymorphism, foaming	denaturation, precipitation hypo-dosage	
Reactive change		Control of quality issue	Drugs
Aggregation		yes	P, I
Translational modification		yes	P
Denaturation (heat, mechanical)		yes	P
Oxidation-reduction (SS to SH)		yes	O, P
Photodegradation		yes	O
Hydrolysis		yes	O, P
Flocculation		yes	I, O, P
Covalent reaction (amino-carbonyl-type)		yes	O, P
Fouling-binding		yes	P
Prodrug-solubilisation (ester, amide, disulphide)		yes	O, P
Stealthifying (PEGylation, POE-POP)		yes	P

Relevant to organic (O), inorganic (I) – small molecule drugs and polymer/peptides/proteins (P). *Due to pH or temperature fluctuation, long storage times in the presence of environmental perturbation and ionic strength change.

13.4 Nanostructures

Colloids, dispersions and polymer aggregates are used universally in medicine and pharmacy. The size of these structures is typically less than 300 nm in diameter. Dispersions that are either constituted oil dispersed in water (OW) or water dispersed in oil (WO) are referred to as emulsions and constitute the vast majority of products such as creams, lotions and ointments [6, 7]. Refinements

to emulsions which are at best micron-sized, are nanoemulsions (100–200 nm) or solid or other lipid nanoparticles (~100 nm). In addition to nanodrugs made from emulsions it is now possible to encapsulate drugs in polymer micelles or polymeric nanosolids. A further development is to mix lipid and polymer to give a hybrid polymer lipid nanoparticle. These types of products are now finding application for a variety of diseases ranging from dermatitis to cancer, and thereby feature regularly in the list of products for new patents and scientific research [45]. Reducing the size of the DDS has a huge bearing in improving product efficacy and drug bioavailability (see Fig. 13.5), drug encapsulation, control over release, biocompatibility, reduced toxicity and the potency of the drug delivery system.

Conventional nanostructures traverse the size range of many angstroms to microns, include liposomes, niosomes (widely used in cosmetology) [8, 42, 55, 56], polymer micelles [10, 46], nanogels [57], micelles, micro- and nanoemulsions, polymer nanobeads, SLNs and LNCs and various combination “fabrics and scaffolds.” The shape of dispersed solids and self-assembled structures can be ellipsoidal, globular, spherical, disc-like, rod-like, cage-like or tube-like. Liposomes [25, 38, 49, 58] are well known to the drug delivery community as commercialized products such as Doxil®, Myocet/Caelyx®, Taxol®, AmBisome® and DaunoXome®. There are also a whole host of newer products that are currently in clinical trials. Newer forms of lipid-based particles include products such as Surfanta®, Exosurf®, and Surfaxin® which are used for respiratory distress syndrome in neonates. Other nanostructured products include Sporonox® and Visudyne® and a large list of radioisotope and PDT-bearing nanoparticles currently in clinical trials phase II and III, such as Gadomer-17 and Ferglose (39, 59). Alternatively, small cationic liposomes, at about 25 nm can be used to carry DNA (25) through pores in the nuclear membrane to the nucleus itself for gene therapy applications. Prodrug use is one strategy that is gaining ground in pharmaceutical circles this is seen in terms of PEGylation of a plethora of drugs such as Doxil, but also extends to linking molecules like serum albumin to paclitaxel to give Abraxane®.

Polymer micelles such as Genoxol PM®, which uses PEG conjugated to phospholipids, or the micellar systems of DermaVir® transdermal patches and a ranges of products seen in a new patent [42], represent forms of micellar drug delivery. The main consideration with this type of product is the polymer biodegradability [15]. Micellar self-assemblies (e.g., MediCelle™ from Debiopharm, an oncology drug nanocarrier platform) using small molecule amphiphiles include NBI-29, a product that is currently in trial, which uses radioactive gold for prostate tumor therapy. Temperature, irradiative energy and aseptic filtration may be used to produce a sterile injectable product, but temperature may be involved in initiating flocculation and other destabilization processes. SoluMatrix™ nanogel or nanobead technology (in phase II of clinical trials) uses hydrophilic polymer networks to trap diclofenac drug in an aggregate suspension. There is currently a wealth of research [8, 60] looking at encapsulation of drug in polyvinyl pyrrolidone (PVP), polycaprolactone (PCL), polylactic acid (PLA), polylactate-glycolic acid (PLGA), and gelatin

beads (see Fig. 13.5). This extends further to look at 100 nm or so particles made from these polymers, which have an additional sheath of poly(ethyleneglycol) (PEG) that gives the nanoparticle a certain circulatory stealth and persistence. Microemulsions (swollen micelles) and nanoemulsions can be used to give a range of products, such as Nanostat™ NB001, an antiviral product in phase III of clinical trials, and Rective®, which is based on nitroglycerine encapsulation for colonic therapies. There is also an extensive list of Food and Drug Administration (FDA) approved products that use the basic Intralipid® parenteral emulsion formulation devised by Kabi/Pharmacia to produce Diprivan® (a propofol anesthetic product), and Limathason®, which contains dexamethasone palmitate for arthritis and other inflammatory disorders [31].

Solid lipid nanoparticles (SLNs) represent a form of nanoparticle in which the solid spheroid is surrounded by a layer of surfactant remaining from its liquid synthesis, or which may be covered by an extensive layer of hydrophilic polymer. This has the capability to be linked to an antibody to elicit more active targeting of diseased tissues. The polymorphic character of the crystalline fat, sample porosity, and the prevalence of faults in the crystalline body have a bearing on the efflux of entrapped drug. Solid fats generally move in a monotropic fashion from α to β' to β polymorph forms. The former polymorphs having the smallest crystallite size (5 μ m) and lowest number of crystal faults and the latter having the largest (100 μ m) diameter and imperfections. More imperfections mean a better efflux of drug from the lipid matrix and solubility issues associated with varying melting points. Rapid efflux may not be desirable and so aging of the fat particle can influence solubilization of the drug and its release, and thus its bioavailability. For core and shell particles such as SLCs the temperature under which the shell or core are solid is an important feature of formulation and constitution [25] of the crystal form and the drug release profile [55].

A range of fabrics are also possible, these may involve polymer (e.g., polybutylene terephthalate) or polymer bead-coated networks or weaves. The fabric support may be made of alginate, gelatin, soya protein, cellulose, chitosan [8, 29, 57] or other biocompatible support matrix. These can be used for integrative skin implants, for medical dressings, wound management and osteointegrative therapies. Such scaffolds and three-dimensional networks have many obvious medical applications. One such product is Lazanda®, which is based on the PecSys™ pectin dispersant formulation and has been used to great effect with narcotic analgesic drugs such as fentanyl citrate for relief of severe pain or surgical intervention.

In the near future it is likely that both use of poloxamer nanogels [46, 48] and liposomes [49] (and use of PEG chains on liposomes [61]) will be invoked for use in long circulating nanoparticle DDSs. The development is unlikely to stop there with many new formulations involving self-assembling drug conjugates [7], nanotherapeutics [2] of varying form and complexity, nanoscale polymer films [62], and a new wave of medical nanoparticles and nanodevices [14, 63], such as those involving dendritic multishell architectures [64] and polymer conjugates [56].

13.4.1 Key Current Know-how

Drug delivery system science has used nanotechnology in the form of soft matter self-assembled structures for over half a century. The sophistication of design is very impressive, since each particle can be used to create a protective “womb” that surrounds the drug (organic molecule, peptide, etc.). Here, surfactants and polymers (amphiphiles) are forced due to entropic considerations to associate or change compatibility to create a range of supramolecular architectures and structures. These devices now range from polymeric micelles to lipid particles with trapped polymer (nanoemulsions, niosomes, liposomes, microemulsions, SLNs and SLCs, etc.) to nanogels and biodegradable polymer beads. Solids and crystalline corpuscular semi-solids, as in the case of the former and latter, include crude emulsions of the Intralipid®-type and a new generation of poly(caprolactone) or polylactic acid beads. The vast majority of this type of vehicle is used in the form of a spheroid or condensed globule.

Investigative study now recognizes that emulsions exist in a number of forms [4–6]; spanning the size array from nano- (50–100 nm) to fine (<1 μm) to coarse ($\geq 1 \mu\text{m}$) form; each having subtle differences in their structure. These range from the simple covered droplets to more complex solids, coated gels or solid-phase lipid or polymer-lipid mixtures [5–7]. These pharmacy products are administered in aqueous media in topical [15, 26] and parenteral [10, 14, 21, 24, 52, 53] medicines carrying a range of therapeutic agents, but are generally needed for the drugs that have a lipophilic character (Table 13.3). Recent innovations (patented and in development or trial) use SLNs or lipid nanoparticles (LNPs) or LNCs with solid body (hollow core) or liquid cores (lipid nano-shell; LNS) [52, 53, 54, 65, 66]. These can be used as a colloidal suspension or imbedded in a polyolefin or other polymer matrix (e.g., poly(butylene terephthalate), poly(isobutylene) or acrylate) such as a transdermal patch (e.g., Qutenza®). Forms of drug product with this composition offer greater flexibility in terms of the severity of processing conditions that may be used to produce the uniform final product. They provide more possibilities for fabrication and formulation and an increase in the loading efficiency in DDSs and product efficacy. Drug-loaded polymeric triblock copolymer micelles [42] represent one very useful form for solubilizing hydrophobes (see Table 13.3), which have a hydrophilic biocompatible shell and can be used for drugs such as finasteride, paclitaxel, doxorubicin, etc., for treatment of poorly resectable solid tumors.

SLNs, LNPs and LNCs (Fig. 13.5) are extremely utilitarian DDS platform technologies with liquid lipid or aqueous core possibilities, permitting specific loading that is able to encapsulate water or lipid-dispersible ingredients or drug, in their lipid or aqueous core. The active agent itself can be further protected as it may be present in the form of a normal/reverse micellar system within the liquid core. These micelles may then house radionuclides such as iron or iodine, or organo-metallic molecules such as cisplatin for cancer, or other quantum dots/nanocrystals [5, 49] for PDT therapy [39]. This degree of design flexibility or modification has been needed for a long time. Control over the long-term (days) release of the drug provides an ability to encapsulate very

noxious drugs. This then provides an ability to trap hydrophobic drugs in a manner that allows harsher processing to provide better product uniformity. A physical barrier of the type seen with biomimetic LNP and SLN structures is ideal in permitting fabrication of a less vulnerable product. This has to be used in a manner that matches its composition with suitable compounding of a composite that must mimic, or better still, surpass current DDSs. At present a number of products of this type are awaiting acceptance in early-stage clinical trials (Surfaxin® pulmonary solid aerosol for RDS in neonates is a FDA new drug application product awaiting approval, and NanoStat® TM NB001 nanoemulsion (antiviral) is currently in phase III of clinical trials in the US). Rective®, an ointment in nanoemulsion form containing nitroglycerine, was FDA approved in 2011 along with approved SLN products such as Qutenza®, Duragesic®, Depocyt® and Nicoderm®.

A drug delivery system based on a Pickering emulsion, which uses solid drug-bearing nanoparticles to emulsify drug-bearing nanoemulsions has been described in early research studies [5]. This two-tier DDS could also involve nanoscale polymer films [62] and layer built on top of previous layers to provide a composite construction, or may involve coated polystyrene nanoparticles [14] and liposomal particles [49] to provide complex supramolecular assemblies. This can also be extended to use natural gums and other biocompatible excipients (Fig. 13.5), such as polysaccharides or poloxamers. Complex assemblies of this type are rendered reduction or environmentally sensitive by using ion or redox responsive chemically conjugated polymers [21, 67] that make use of lysosomal or cytoplasmic conditions for “in cell” release [38] of the active form of the therapeutic agent. Novel nanodrug products are important to the efficacy (Fig. 13.5) of active tumor targeting [29] and new vectors for passive tumor targeting [25], making use of, for example natural cationic lipids to bind DNA as in the case of cationic lipid SLNs and vesicles for gene therapy. Solid lipid nanoparticles [55] are particularly well suited to drug delivery [52] since the SLN is generally made from biocompatible materials [16,17, 19, 29] that include a diverse array of natural fats, oils and waxes and which are modifiable for controlled drug delivery.

13.5 Surface Coating

Surface coating is used for nanoparticles (NPs) but also for efficacious “fabrics,” implants and scaffolds (Fig. 13.5, part b). The coating may assist in the acceptance and release of the therapeutic agent, but also help to disguise the material (stealth form for NPs) to help retain the implant or material *in vivo*. Trapped therapeutics introduced into the body in various forms promote opsonization and macrophage signaling. Therefore, a biocompatible coating renders the biomimetic nanocomposite invisible to the RES/MPS system and inflammation or edema. A number of coating strategies are possible [2, 44, 51, 67] and these include deposition from solvent (favored for small molecules), electrostatic association (charge compatibility used with polymers), coacervation of

particles in the form of a nanogel (each particle being microheterogeneous) and flocculation of polymers. Techniques can also extend to immersion coating, which uniformly covers the surface or involve production of an adsorbate. The latter tends to produce variable depth since the adsorption process is stochastic to some extent and therefore, the finished product can appear to have varying surface topology. In some instances this varying surface topology can have a detrimental effect for an implant in terms of surface fouling with plasma serum proteins.

Drug release (e.g., dexamethasone) for insertion into tissues from vehicles can be irregular if microstructure varies and therefore, needs careful structuring [16, 17, 19, 20] or defining to give products that restrict plasma protein adsorption and biofilm formation [18]. Biofilm formation in the extra-cellular matrix lying outside the tissues themselves can be a precursor to implant rejection. Natural composites and mimetics like hyaluronic acid/chitosan are now used to great effect [29, 57]. A superiority of physiological performance with these polymers is driven by both the chemical inertness of the material and the large hydrated cushion of water that covers the laid down layer of polymer; that avoids adsorption. Products such as PoroGen® in the form of discs, tubes and layered products, based on polylactic acid (PLA), and polyglycolic acid (PGA) are most commonly used in a commercial setting but may also include polylactic-glycolic acid (PLGA), polycaprolactone (PCL) [8] or PEG in newer developments.

13.6 Cell Promoting, Toxicity and Clearance

The physiological drawbacks (see Figs. 13.2 and 13.6) of new medical products, and designing purpose-integrative or responsive materials for therapy and for what clinicians and pharmaceutical nanotechnologists desire from them is complex. These are captured for strategic targeting succinctly in Figure 13.2. The assault on diseases (see Fig. 13.1) and specific organ targeting (see Fig. 13.3) is captured in clever use of nano-medicinal particles (nanodrugs) that are selectively biodegradable [8, 29] and disguised *in vivo*, and this provides opportunities for avoiding opsonization, defying the serum complement activation system [14, 66] and effective removal from the body by the body's defenses (see Fig. 13.6). From a drug delivery perspective drug nanoparticles are viewed as "foreign matter and infective agents" and are selectively removed by macrophages. Adding a stealth coverage to particles can also permit prolongation of contact with difficult to access areas of the body, for example, those of the blood-brain barrier [37].

The complex pathway and the macrophage activation with the drug being deactivated and destroyed are represented in Figure 13.6. This can take place in the blood compartment and the Kupffer cells of the liver, and macrophages of the spleen and lung. The epithelium is one of several types of cellular assembly, such as connective tissue, muscles and nerves, but is unique in that it covers the cavities and surfaces of structures throughout the body. Functions of epithelial

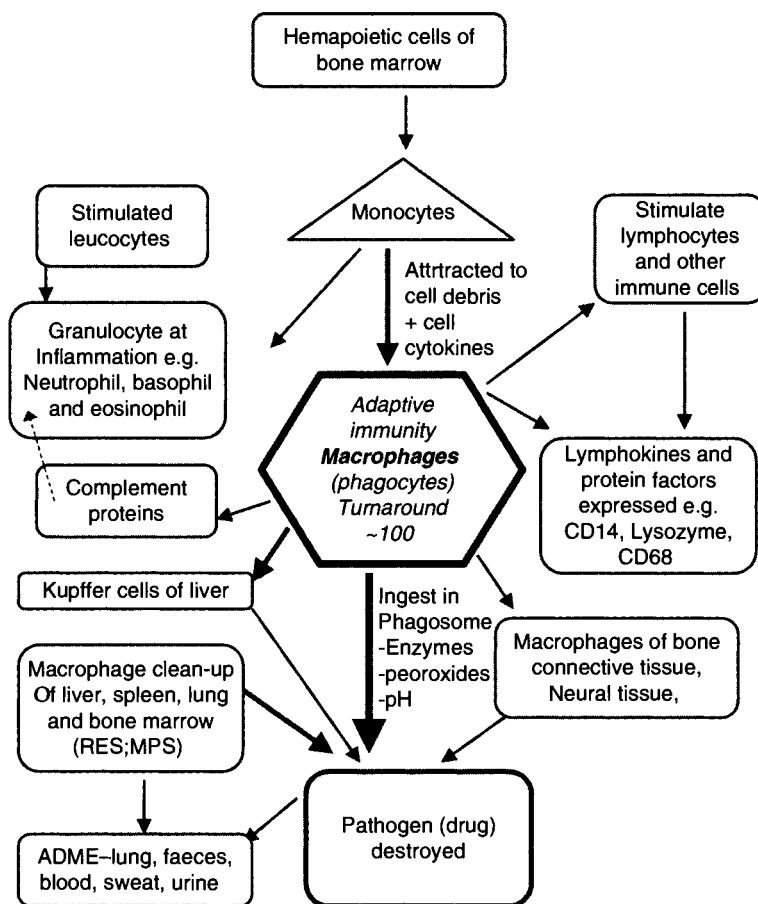


Figure 13.6 The key features and determinants of the monocyte-phagocyte system (MPS) system function and drug absorption, distribution, metabolism and excretion (ADME). The diagram shows key issues encountered in the administration of drugs that may facilitate or hinder drug uptake. The optimum particle size to prevent MPS (reticulo-endothelial system; RES) capture and expulsion is approximately 40–60 nm and this is customarily the size of encapsulating particles used in nanodrug form.

cells include secretion, selective absorption, protection, and transcellular transport. In this manner they become physiologically significant to the biopharmaceuticalist in respect of drug uptake. The epithelial cells constitute among other types of cells the extracellular matrix secretory cells, barrier function cells of the lung, specialized blood cells [23] such as lymphocytes (producing antibodies) and hematopoietic stem cell in bone marrow that make monocytes, which go on to become Kupffer macrophages in liver. Macrophages in the lungs, liver, neural tissue, bone and spleen produce complement proteins [3, 9], and they carry receptors for lymphokines that allow them to be activated into single-minded pursuit of invasive particles [23]. Monocytes and macrophages are phagocytes that act to help initiate specific adaptive immunity in which their role is to phagocytose (engulf and then digest) debris (drug nanoparticles) either as mobile or as stationary cells and stimulate lymphocytes (antibody

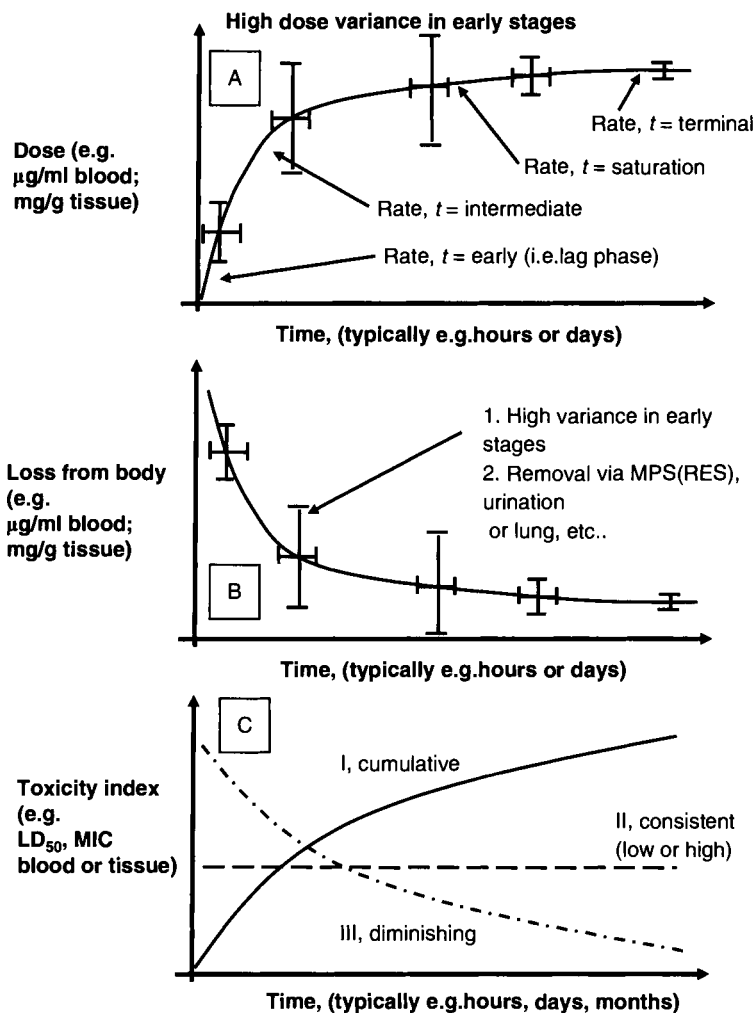


Figure 13.7 Generalized presentation of key features and determinants of drug product efficaciousness. Presented are the pharmacokinetics of drug-to-blood-to-target tissue/target (graph A), *in vivo* expulsion-exclusion-removal losses (graph B) and scaled toxicity *in vivo* (graph C). The drug absorption, distribution, metabolism and excretion (ADME) determine the duration and form of the therapeutic dose.

production). When a macrophage ingests a nanoparticle, the particle becomes trapped in a phagosome (Fig. 13.6), which then fuses with a lysosome. Once inside the phago-lysosome, enzymes and powerful peroxides can digest the particle and destroy its drug contents (Figure 13.7).

For this reason stealthifying drug nanoparticles by covering them with hydrophilic polymers is pivotal to their therapeutic success. In general, hydrophilic polymers in a "brush" conformation [14] rather than a "pancake" conformation, with the former having associated "watery" cushioning are recognized less well by opsonins and thus cleared less efficiently (Fig. 13.7) by the bodies macrophages. In other words, they persist longer in the blood

compartment and targeted organs. This can be used to great success in covering cationic lipids to create DNA-LNC conjugates and for encapsulating nanoparticles for routine therapeutic delivery [25] or other more lethal therapeutic particles for phototherapy [39]. Structural patterning [5, 6] can thus be used to house the therapeutic moiety via the molecular engineering of nanoemulsions (also SLNs, LNCs) and lipid or polymer nanoparticles [42]. Environmentally-responsive nanoparticles [36, 56] using chemo-labile conjugated polymers are on the increase, and this can be seen in the number of new concept products which allude to this technology [9, 42]. The environmental sensitivity of composite materials made of all these hydrocolloids is created by use of insightful inter-molecule chemistry. These can include disulphide (reduction sensitive) "bridges" or enzyme-labile ester, amide and ether functional groups, or the use of lipid phases, which respond to Ca^{2+} ions, such as phosphatidyl serine (PS); to change packing array to aid vesicle cell fusion. In some cases, use of high melting point or low melting temperature lipid eutectic mixtures can also be invoked to [39] permit drug cargo release in therapeutic niosomes and phospholipid vesicles. Finally, PEG and poloxamer (poly(ethylene oxide)-poly(propylene oxide)) polymers can be used based on thermal transitions in form, which negate macrophage clearance.

13.7 Formulation Conditions and Parameters

There is an express need for characterizing both the DDS itself and for defining the DDS product quality in terms of safety, dosage, consistency, hygiene, purity and reproducibility *in vivo* [10, 50]. This is itself initially modeled via a range of stringent regulatory procedures and quality control measures (Fig. 13.3 and 13.7). In the new generation of drug delivery colloids there are varying degrees of "smartness" engineered into the DDS product. A pharmacist must consider polymorphic performance of the drug and of the excipient fats used to make SLNs or LNCs. Polymorphs have widely differing solubilities, hardnesses, melting temperatures, packing densities and dispersibilities. In this way, change in composition is likely to directly influence the bioavailability (bioequivalence) of the drug (Fig. 13.7, graphs a, c). The drug molecule in a particular environment will be solubilized or distributed (in the nanoparticle composite and *in vivo*) according to its ease of partitioning [6, 62] and thus its $\log P$ or $\log D$ and pK_a values (Tables 13.4, 13.5). Due to possible unfavorable association between excipients (covalent bonding) and incompatibilities that arise for time-related chemical aging of the sample, this may then modify the polarity or hydrophile-lipophile balance (HLB) properties of the therapeutic moiety. Low molecular weight species (<500 Da) and molecules with a low number of hydrogen-bond donor functional groups (<5) are more readily taken up by target cells and have more favorable absorption, distribution, reduced metabolism and excretion (ADME) in the body.

Polydispersity of the nanoparticle composite in terms of its size, shape, porosity, mechanical texture (viscosity) and tensile strength also would have

a significant bearing on the rate and amount of drug which leaves a soft-self assembly (liposome, niosome), hard or semi-solid lipid particle (SLN), and thus enters the blood (Figure 13.7, graphs a, c). In addition, the clearance from the blood compartment by the MPS macrophages, drug (Fig. 13.7, graph b) would also be influenced by the surface chemistry of the composite nanoparticle. Ideally monodispersed in nature, such a product would permit more appropriate estimation and consistency with *in vivo* performance.

Amphiphile (polymer and emulsifier) self-assembly and surface nanopatterning can use covalent conjugates to produce product with superior biomimetic properties [40, 68]. These mimetic composites of lipid-polymer drug materials form a plethora of new drug products [21, 29, 51, 65, 67]. Smart methodology of this type is often biodegradable and uses materials from natural or less harsh synthetic routes to provide built-for-purpose biomedical materials and specific drug delivery systems. These nanostructures can be used routinely in parenteral [9, 66] (IV, intramuscular) products (see Table 13.3) but also in transdermal patches, topical creams/gels [52, 54], and even specific vaginal, nasal, rectal, ocular, pulmonary or solid dosage forms (Fig. 13.5).

13.8 Delivery Systems

There are, and continue to be, significant advances in drug delivery [68], particularly with respect to cancer [11, 12] and gene therapy [25] research. An approach which considers toxicity, side-effects and physicochemical or pharmacological properties as paramount usually sits at the forefront of technological success. The major challenges for targeting aggressive and tenacious ailments such as pancreatic cancers [12] are the “benchmark criteria” of specific and targeted delivery of the DDS moiety [34]. Other aspects include the pH, or chemo-lability and sensitivity of the therapeutic (Figs. 13.2, 13.5, 13.6, 13.7) requiring some form of encapsulation (Tables 13.4, 13.5) that can be found with liposomes, niosomes, LNPs or SLNs (see section on nanostructures, 13.4).

Due to varying toxicity, the physicochemical properties of the active pharmaceutical ingredient (API; active; drug) [11] and its integrity need to be carefully protected, contained and encapsulated to reduce side effects. Newer approaches [15, 21] use long-circulating nanocapsule (LNP, LNC) particles [25] with intracellular effects that kill the cell (instigating spontaneous apoptosis or tissue necrosis) [39], and frequently also involve biodegradable nanospheres (Figure 13.5) based on amphiphilic polymers [8]. For administration to the patient these habitually take the form of solids or semi-solids, porous implants and tissue mimetics that are used after surgical interventions, topical creams/ointments and soaks, parenteral liquids, oral dosages, pulmonary sprays, rectal suppositories, vaginal pessaries and transdermal patches. This type of product formulated from a composite material can, and now routinely does, incorporate SLNs, SLCs and metal quantum dots (nanocrystals; Q-dots) [69]. Clever encapsulation of the drug can also make use of the former but may

Table 13.6 New “hot” areas in the nano-medicinal sciences.

Theme	Scope
Using stem cells and biopolymers Scaffolds and matrices	Integrative medicine for new tissue and tissue repair Composites of inorganic and/or carbon-based and/or polymer materials
Regenerative medicine	Tissue replacement using integrative polymer or bead Networks
Functional materials	Biodegradable polymers based on modification of surface chemistry or porosity
Mimetic materials (biomimics)	Provision of extra-cellular “environmental” stimulation and biocompatibility
New routes to synthesis	WO emulsion for reproducible monodispersed size that is based on PLA, PMMA, PLGA, PCL, PAA, p-NIPAAM polymers that aid DDS reproducibility
Hydrogels for injection	PLA gels and nanogels demonstrating elasticity, slow degradation or specific gelation due to HCST-LCST transition of polymer

Key: PLA - poly(lactic acid); PMMA – poly(methylmethacrylate); PLGA – poly(lactic-glycolic acid), PCL – poly(capro-lactone), PAA – poly(acrylic acid), p-NIPAAM - poly(N-isopropylacrylamide). HCST-LCST – higher/lower critical solution temperature (a conformational change in polymer form).

also additionally use PLGA, PLA, PCL and polymethylmethacrylate (PMMA) nanogel beads on their own or associated with SLNs and SLCs [4, 10].

Table 13.6 shows a nonexhaustive list of the currently well-researched areas in DDS science. These can involve, but are by no means merely restricted to, lipidic carriers [38], self-assembled hyaluronic acid nanoparticles [29] for tumor targeting, organo-metallic bearing nanoparticles [31, 48] for neoplasia, enhanced active targeting using tumor metabolites [40], polymeric surfactant-based drug delivery systems [15] and long-circulating DNA-cationic lipid nanocapsules [25]. These can improve drug transport by using a variety of mechanisms to aid passage through the cell. New technologies that may in the future yield sound therapeutic products include Photofrin II®, Foscan® [59], or silica nanoparticles for PDT envisaging use with metastatic melanoma and laryngeal cancers which yields light or reactive singlet oxygen, respectively, that kills cancer cells [39],; and the use of chitosan-conjugated docetaxel [57]. A revolutionary new approach uses polyfunctional and stimuli-responsive nanovehicles [13, 21, 48, 69] for various strategies targeting lymphomas and leukemias.

A new range of nanomaterials for intelligent medical devices (Table 13.6) uses novel nanostructured composites to give better *in vivo* response. Such

materials can mix stem cells and biopolymers for use with integrative medicine and new tissue generation, use scaffolds and 3D-matrices (using a range of materials), or facilitate tissue replacement via the use of polymer beads and biodegradable polymers [70]. Functional materials of this form have carefully crafted internal structure that allows better functionalization for *in vivo* use or extracellular and mimetic use or biocompatibility. Newer forms of fabrication [8, 11, 25, 40] such as emulsion-based synthesis can be used to generate monodispersed consistent PLA, PLGA, PCL or PMMA, etc., nanoparticles for use in a range of therapies. There is also the capability to cover particles with polymer or generate hydrogel matrices using polymers such as PEG and poloxamers to create temperature or environment “swellable or responsive” hydrogels (Table 13.6) or hydro-nanogels. A change of polymer conformation at a critical temperature, for example, changes the form of the nanostructure and elicits a texture or water binding change on a macroscopic level.

13.8.1 State-of-the-Art Technological Innovation

A new array of composite nanostructures that aim for specific engineering (Fig. 13.5, part a) of the dosage form, seek to have systematic (Fig. 13.5, part b) and focused delivery (Fig. 13.7, graph c) and permit passive or active targeting (Fig. 13.2). This mean the drug developer is provided with many opportunities. State-of-the-art approaches make use of targeting aids and ligands such as tissue specific monoclonal antibodies (-mab drugs), translocation aids [34] and monomer mosaic or motifs and patches that are cellular translocation aids. These can include amino acids combinations and RGD (e.g., arginine, glycine, and aspartate motifs) mimicry peptides or incorporated patches within therapeutic peptides such as, e.g., KDEL (lysine, aspartate, glutamate and leucine) which aid membrane, cytoplasmic and cellular translocation [60]. This is now often linked to methods for gene therapy [25]. Loss of normal gene function (e.g., RAS/KRAS/BRAS genes [33]) in many tumors means a suitable tumor suppressor protein (e.g., p53) is either not expressed or not allowed to be expressed. Such factors encourage protection of the genome by inducing cell cycle arrest. These therapeutic proteins can now be chemically stabilized by a range of methods, such as targeting “pathway interferents” via small molecule inhibitor use. It can also extend to safeguarding of an isolated protein in a particular drug delivery vehicle [71]. In this instance a DDS can be linked to newer technologies that make use of nonviral [10] or viral vectors for targeting specific cells. One case of using nonviral vectoring is in the use of folate-mediated targeting of albumin conjugates of paclitaxel [40] for cancer therapies.

The challenge to the medicinal chemist in terms of drug development lies in the accounting for and dismissal of DDS toxicity, significant side effects and unfavorable properties [11]. Lipid nanocarriers and block copolymer-based composite materials [5, 38] of the types listed in Section 13.4 [38] can be used with considerable success for entrapment of apolar drugs in a sufficient way to give a degree of control over egress and to reduce their toxic impact throughout the body. This is particularly the case for a whole list of antineoplastic

drugs in Tables 13.2 and 13.3. Passive targeting of tumors has been investigated by using cationic lipids [25] to house DNA in fat-based LNCs for gene therapy, in addition to targeted nanoparticles for use in PDT of melanoma cells and oesophageal cancers [59] using near-infrared light to produce lethal [39] singlet oxygen. The tumor cell “weakens” its position since it has a typical, if rather irregular, surface structure with particular altered carbohydrate chemistry and mutant proteins, oncoantigens and proliferation-linked determinants. On a cellular level these are entirely predictable and can be used to direct the therapeutic composite particle in a manner that mimics conventional circulatory mobilized particles.

A new wave of specific targeting technologies is now being employed with great success to access specific cells. Much of the headway has been made in cancer therapeutics [48]. Examples of this clever use of medicinal chemistry include “stealthification” of nanoparticles using biocompatible long-circulating chitosan-conjugates of docetaxel [57], nanobeads based on poly(ϵ -caprolactone)-*block*-poly(ethyl ethylene phosphate) [8] or tumor epithelium targeting using RGD-grafted PLGA-nanoparticles [60] that are carrying paclitaxel. In currently developed preclinical trial models, much interest has been generated by (Fig. 13.2) the use of multifunctional and stimuli-sensitive pharmaceutical nanocarriers [13, 21]. Materials usually key-off changes to high (or specific) ion concentration or redox potential (e.g., lysosomes), enzymes (e.g., cytoplasm) or time/temperature profiles, such as the higher temperature of the blood compared to the skin, or ambient sample storage. The currently employed state-of-the-art research methods use lipid-based nanotechnologies [48, 58] and dendrimers [69] for the delivery of fixed-dose anticancer drug combinations.

13.9 Evaluation

A series of groundbreaking novel technologies have revolutionized the current state of affairs for the clinician in terms of therapeutics and their efficacy. This is not however, universally true. In many cases one can regard the available therapeutics for many types of tenacious cancer as being woefully inadequate. Clinicians face the dilemma of delivering large doses so a fraction of the drug reaches the tumor (Fig. 13.8), and in this approach rely on poor recovery of the cancer cell to remove the malignancy. However, medicinal chemists and pharmaceuticalists hope to produce a new generation of drug delivery systems that have greater efficacy and are much less toxic (i.e., either carries less drug and targets a cell or has lower lethality throughout the body). Additionally, such particles need to be biochemically “tuned” to persist in the circulation for varying times depending on surface chemistry and physical size. A number of strategies are currently employed to aid persistence and facilitate a better ADME profile, and these include chemical conjugation (pro-drugging) or stealthification with the use of a hydrophilic polymer coverage [46]. In a number of cases PEG coating, use of biocompatible polymers (Fig. 13.8), or the frequently

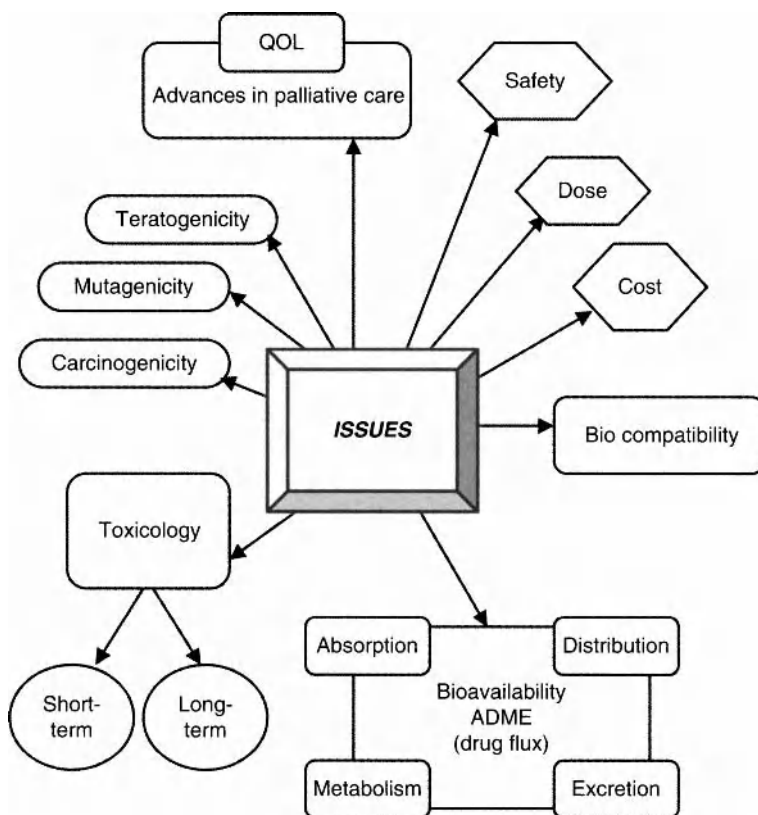


Figure 13.8 Effective prophylactic drugs for acute and chronic diseases and for palliative (QOL assisting) care. The figure takes into account the issues of toxicity and mutagenicity but also whether the dosage form has a likely short- or long-term use and its bioavailability and biocompatibility to render the product both responsive and adaptive *in vivo*.

used niosomal or liposomal encapsulation and labeling with a tissue specific antibody can drive better uptake. This has the added advantage of permitting a larger dose to be focused toward specific diseased tissues and thus avoid a “hit or miss” approach to drug delivery. The physical form, intraparticle diffusion, solubilization of the drug, encapsulation ratio and thus, potency, have an impact as to whether to have hollow or filled nanoparticles or whether to use a solid or gel-phase material (e.g., lipid or polymer or lipid-polymer matrix).

Issues that concern the drug designer include what end purpose the drug product is intended for. This obviously has a bearing on the acceptable cost of the end product and will also have an impact on the likely sophistication permissible in the product (Fig. 13.8). Consistently, throughout the global healthcare systems of a range of countries, issues concerning the relative cost of effective prophylactic therapy (Fig. 13.1) of acute and chronic ailments (Fig. 13.8) or for use in end-of-life palliative care, become quite weighted. For prophylactic therapies a range of newer antibody-based therapies are currently used. Products such as Herceptin (trastuzumab), Avastin (bevacizumab), Bexxar (tositumomab) and Mylotarg (gemtuzumab) currently use antibody

conjugation chemistry for treatment of breast, colorectal and lung, non-Hodgkin and myelogenous leukemia cancer, respectively. The limitation with such technologies can lie in terms of their cost for routine use, which can often be inhibitory for clinicians with limited budgets (Fig. 13.8). Products such as Doxil (doxorubicin), Taxol (paclitaxel), DaunoXome (daunorubicin) and Abraxane (paclitaxel-albumin prodrug conjugate) are more appealing in many senses to clinicians, as their cost is lower and thus their clinical utility is better. The latter, of course, are long circulating systems [8, 48] but not targeted to the tumor as in the case of the former, consequently there is a trade-off. Specificity means a lower general toxicity, active targeting of the tumor but at a higher cost. However, all is not lost, as various regimes of therapy mean it is not essential to use only one type of product and cocktailing of anticancer drugs is used quite frequently.

For a new generation of products using cationic lipids or dendrimers, self-assemblies of polymers [70] and composites mixtures of lipids and hydrophilic polymers, key issues that arise concerning their use are the product physiological biocompatibility, its safety, bioavailability of the drug molecule and its bioequivalence in human models. Cancer therapeutic use, because of its acute rather than chronic nature and current high failure rates (see Table 13.2, mortalities) and poor choice of therapeutics, mean much attention is focused on either high loading (Fig. 4.8) or a better, low loading with prolonged circulation time. This means the dose can be modified and side effects reduced leading to a better chance of rapid recovery or preparedness for a second round of chemotherapy. The issue of quality-of-life (Figs. 13.1, 13.8) is particularly important in respect to many late-stage cancers [24] which are inoperable but that can be maintained, "capsulated" or restricted in terms of metastasis or continued growth. Some cancers are routinely difficult to treat and these include lung, pancreas, ovary, breast, liver and colorectal forms [12, 31, 32, 35].

For this very reason, there has been a recent predominance of nanotechnology-based drug delivery systems as part of a concerted effort to produce less systemically catastrophic parenteral chemotherapies. This routinely involves a range of lipid nanosolids, lipid nanocapsules, polymer-based nanogels, nonviral vectors, dendrimer capsules and protein-based targeting for greater specificity in treating neoplasia, lymphomas, and a plethora of other diseases such as multiple sclerosis, arthritis and other inflammatory disorders such as Crohn's disease [10]. With many products at the point of patent application and early clinical trials, the future for a new breed of composite nanoparticle species looks very promising. Sadly, it is likely to be 5–10 years before the first of these new lipid or polymer nanoparticles exits the rigorous clinical trials and regulatory approval cycle and becomes accessible to clinicians and patients alike.

13.9.1 Future Scientific Direction

The perils of contemporary drug delivery mean that frequently with passive DDSs little of the drug actually reaches the target (Fig. 13.4). One way to fool the ADME expulsion of drug from the body is to use the biomimetic technologies

listed in Table 13.6 and to maintain a longer circulatory lifetime. The essential considerations of better-fitting DDS are shown in Figures 13.3 and 13.8, where the form and type of administration and physiological issues relating to the product suitability are given more emphasis. Better quality (more efficacious) medicines are ones that allow us to use a progeny of stimuli-sensitive media [21, 51, 65], specify a regularity of form and design a molecular-sculpture [14] that permits the pharmacist or clinician to better predict *in vivo* performance [2, 5, 49]. What the patient needs from company scientists and the research community is more effective disease targeting [25] in the specific form of targeted drug delivery [12] with the appropriate weighting and focus on clinical use [10].

A lot of products currently exist (Doxil/Myocet, Taxol, Abraxane), but to some extent we have seen [11, 12, 28, 40] better targeting and negation of side effects and toxicity by using biocompatible polymer-based systems that use antibody technology conjugated to the drug bearing composite. Examples of current drug products where an antibody is chemically conjugated to a drug or drug-bearing species include commercial products such as, Avastin, Mylotarg, Bexxar, Herceptin, etc. The future may indeed lie in use of polyplexes (polymer complexes), lipoplexes (stealth liposomes, SLNs, LNPs, LNCs, etc.), virosomes (viral vectoring liposomes) and niosomes (surfactant liposomes) (Figs. 13.3, 13.5, 13.8). Rendering of drug-bearing nanoparticles as “invisible” to the immune system [9, 21, 23, 29] macrophages using hydrophilic coatings maintains a constant “stream” of drug. When coupled with tissue specific antibody conjugation this creates a nanoparticle that can carry a lower content of cytotoxic drug. Lower dosing and greater targeting (Fig. 13.7, graph a) produce a persistent drug *in vivo* (Fig. 13.7, graph b), a higher degree of efficacy and reduced toxicity (Fig. 13.7, graph c). For potent therapeutics usually delivered by parenteral forms, this means that high dose variances in the early stages post-injection (lag phase) compounded with drug (nanoparticle) removal via MPS macrophages (Fig. 13.6) juxtapositioned against possible rising toxicity of metabolites or drug itself, directly influence the likelihood of drug success.

The invention and careful design of any new innovative products means crucial consideration needs to be given to drug ADME and pharmacodynamic (PD) or pharmacokinetic (PK) modeling. This relates to the evaluation of drug uptake [9, 55, 63] and should take into account drug lethality (LD_{50}) in respect to *in vitro-in vivo*, *ex vivo-in vivo*, bioequivalence (BE) and bioavailability (BA), intercorrelation and drug throughput screening. Awareness and standardization of BE, BA, PK, PD and biomimicry or biocompatibility data (Table 13.6) mean screening regimes (Fig. 13.7, graphs a–c) and set-up of clinical trials is getting better with the arrival of each new decade. This is made more difficult in the transition from test-tube to human system in terms of medical limitations and intrinsic flaws (in the array of tests) given demographic or physiological inconsistency and variability. Clever construction of clinical trials and patient cross-sectional profiles can eradicate much inconsistency and difficulty in arriving at a reasonable conclusion. Additionally, more predictable [52] dosage form (chemistry and polymorphism), size range and

structural form (mushroom or brush surface conformation of stealth polymer coatings) [9, 14, 61] allow better therapeutic dosing coupled with a more convenient manner of fabrication and production.

13.10 Conclusions

The wealth of current academic and technological research provides a route to the expansion and continuation of useful drug product design. Given the predominance in research of a range of composite and “intelligent or responsive” environmentally sensitive therapeutic nanoparticles, it is likely that development of novel or better forms of existing therapeutic dosage forms is guaranteed to expand greatly over the next decade. At present obvious growth areas showing huge potential as highly adaptable platform technologies include PEG or poloxamer polymeric micelles [5, 65], multiple constituent particles (PLA, PCL, PLG nanogels), reduction-sensitive polymers, antibody bioconjugate nanoparticles [67] and further adaptation of colloidal (nanoscale) dispersed drug delivery systems [9], such as SLNs and LNCs [52].

Adjuvant therapy, where a drug is used to supplement invasive surgery, does work well and is likely to be the mainstay of the treatment of resilient diseases such as a range of cancers (ovarian, melanoma, lymphoma, lung, breast, colorectal, etc.). Currently, many forms of therapeutic DDS work by hitting the whole body with a drug that causes devastating cellular impact as a consequence of toxicity across the body. In more insightful technologies, specific tissues (due to antibody targeting), enhanced permeability (due to reduced size) and persistent long-term circulation or accumulation in tissue (due to size or antibody focusing of the drug) mean lower dosages can be used with greater success and less generic tissue damage. This helps to remove the rather clumsy approach where a multitude of therapeutics (e.g., polymer-coated platinates) can work *in situ* in the body. An approach where the recovery of normal cells is marginally better (more rapid cell turnover) than cancer cells and represents the sole basis for therapies that involve programmed cell death due to drug cytotoxicity. This toxic effect can be reduced in part by using a “hurdle approach” where cumulative toxicity is maximal for the cancer but by using a mixture, or synergistic cocktail, of drugs that is less devastating to normal cells but is considerably more lethal to the tumor.

Looking closely at 2009 as a particularly good year for the release of new therapeutics that were awaiting final approval or were approved by the FDA, new drug cancer products accounted for about 50% of all new drug products. Such products included, Folutyn (small molecule for lymphoma), Istodax (small molecule for lymphoma), Arzerra (antibody conjugate for leukemia), Votrient (small molecule for kidney carcinoma) and Cervarix vaccine (bivalent papilloma virus for cervical cancer). Other products submitted for FDA approval also accounted for roughly half of all new therapeutics and included Onorigin (small molecule for leukemia), Provenge (immunostimulant small molecule drug for prostate cancer) or XM02 (small molecule for the

neutropenia resulting from cancer chemotherapy). This prevalence of cancer therapeutics arises partly because of the difficulty and relatively poor success of conventional therapeutics or regimen, but also because the toxicity of these drug molecules or species mean encapsulation is required in a form that currently reduces drug product efficacy. In this way, the toxicity of a therapy provides the limitation because of large dosages due to use of non-nanoforms of many drugs. Toxicity induced by poor targeting can arise shortly after administration (immediate: nausea or early: diarrhea, alopecia, thrombocytopenia, leucopenia) and culminate in delayed toxicity (e.g., anemia and hepatitis) or long-term toxicity (e.g., gonadal atrophy and second malignancies).

Consequently, for most new products (or redesigns) the inherent failings seem to be negated in part when using biocompatible block copolymers and lipids. If composite structures can be small enough and targeted very specifically to certain cells or tissues then the efficacy of the DDS is effectively maximized. Any new developed drug must pass the rigors of regulatory approval and preclinical, and more specifically late-stage, clinical trials. Additionally, the product at the end of the development and approval process must be accessible. This is illustrated by two very effective drugs, Nexavar (small molecule sorafenib) and Avastin (antibody conjugate bevacizumab) used for cancer treatment, where the product is effective but the therapeutic cost of routine use is rather inhibitory because of the costs due to drug development itself. However, with a wide array of potential technological platforms (SLNs, polymer micelles, niosomes, antibody conjugation) in the new product pipeline the future for drug development actually looks very promising indeed.

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Progress of Nanobiomaterials for Theranostic Systems

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Abstract

Since its conception, nanotechnology has gained substantial momentum and transformed nearly every field of science and technology. In the field of nanomedicine, a variety of nanobiomaterials have been developed as unique platforms to detect and deliver therapeutic agents in the treatment of life-threatening diseases. A brief review of recent publications reveals that these therapeutic platforms can be nanoparticles, micelles, nanogels, liposomes, metallic nanoparticles and semiconductor derived quantum dots. When compared to traditional therapeutic systems, theranostic nanomedicine is an emerging field that employs a multifunctional approach to specifically target, diagnose and deliver therapeutic agents using a single platform to treat diseases such as cancers. This chapter will focus on the recent progress of nanobiomaterials toward theranostic nanomedicine for tackling unmet clinical problems.

Keywords: Nanomedicine, nanomaterials, drug delivery, imaging, theranostic system, fluorescent polymer, quantum dots, gold nanoparticles, iron oxide nanoparticles

14.1 Introduction

14.1.1 Nanomaterials and Nanomedicine

In 1959, physicist Richard P. Feynman laid out a vision of what would one day be called nanotechnology in his talk titled “There’s Plenty of Room at the Bottom” at a meeting of the American Physics Society [1]. Although as revolutionary and groundbreaking as his ideas were at the time, it was doubtful that his idea would be translated into the multidisciplinary field of applied science and technology of today. The term nanotechnology was first used at a conference in 1974 by Japanese scientist, Norio Taniguchi [2]. Since that time there have been numerous attempts to define nanotechnology, but Mihail Roco, who was the current head of the National Nanotechnology Initiative

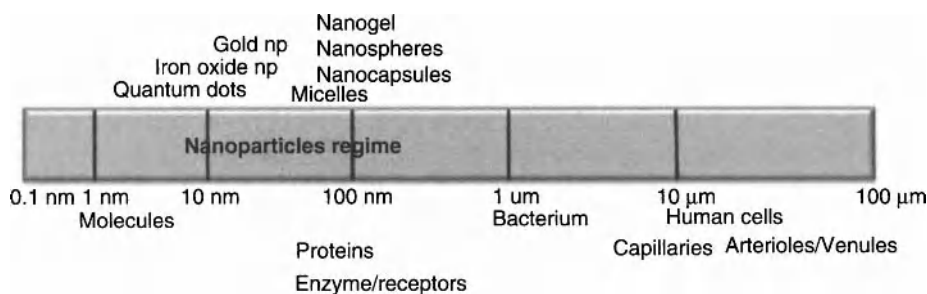


Figure 14.1 The comparison of size of nanostructures with biological components.

(NNI), proposed the most straightforward definition. “Nanotechnology is the creation of functional materials, devices, and systems through control of matter on the nanometer length scale, exploiting novel phenomena and properties (physical, chemical, biological) present only at that length scale” [1]. Since then, nanotechnology has garnered significant attention in diverse fields such as cosmetics, textile, energy, electronics, biomedicine, pharmaceuticals, imaging and diagnostic industries. As estimated in 2008, the global annual investment in nanotechnology research and development, from private and public sectors collectively, exceeded \$15 billion [3].

A nanometer (nm) is one billionth of a meter, which is equivalent to the diameter of a molecule of glucose. For comparison, the spacing between atoms in a molecule are in the range of one tenth of a nanometer [4]. The genetic instructions for development (a deoxyribonucleic acid double-helix) has a diameter of 2 nm [5]. The smallest cellular lifeforms (*Mycoplasma genitalium*) have a length of 200 nm [6]. Although, particles within the range of 10–1000 nm in diameter are considered nanoparticles [7], for biomedical applications it is preferred that particles are within the range of 10–200 nm, particularly in drug delivery, due to their longer circulation time and improved tissue penetrability. In the human body, most biological functions occur at the molecular level. Thus, nanostructures can be designed to interface and interact with cells and tissues at the molecular level (Figure 14.1).

Today scholars from diverse scientific communities are actively studying a broad range of materials in the nanoscale-size range and have observed new material phenomena that are absent in the microscale. Gold nanoparticles (GNPs), for example, when synthesized in various shapes, sizes and dimensions, demonstrate a wide range of optical properties that are not observed in the bulk material [8]. The advances in nanotechnology and other related fields are based upon this set of “new properties” in the nanoscale, which are not observed in the bulk material. Nanomaterials can be produced from a wide range of bulk materials such as natural polymers, synthetic polymers, semiconductors, metals and ceramics. In general, there are two approaches available to fabricate nanomaterials: top-down [9] and bottom-up [10]. The top-down approach, which is simply cutting, grinding and etching of a bulk piece of the material into the nanostructure, creates imperfections in the surface structure

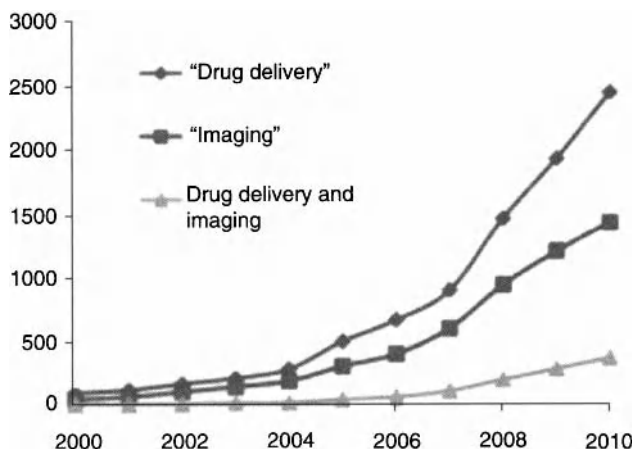


Figure 14.2 The total number of publications of nanomaterials for drug delivery, imaging, and dual purpose (Key word- Drug Delivery Nanoparticles, Imaging Nanoparticles, and Drug Delivery and Imaging Nanoparticles) (Source: Science Citation Index Expanded [SCI-EXPANDED]).

that significantly affects the overall physical properties and surface chemistry of the material's nanostructure. The bottom-up approach, which includes assembly of atomic or molecular species via weak noncovalent molecular interactions, creates nanostructures with homogeneous chemical composition and minimal defects. Most of the nanomaterials used in biomedical applications discussed in this chapter utilize the latter approach.

Nanomedicine is an emerging field which has produced new approaches to improve the diagnosis of and treatment of medical problems. The ability of nanomaterials to incorporate biologically important molecules within a matrix, selectively deliver these factors to tissues or cells, and demonstrate coveted optical properties that are easily identifiable with current imaging technology, has garnered interest and interdisciplinary research from proximal fields. One indication of this interest is the rapid increase in related publications since 2000 (Figure 14.2). Investigations and publications have doubled every year for the last decade. The clinical translation of this large body of research into commercial products has already begun. According to the April 2006 issue of *Nature Materials*, it was estimated that 130 nanotech-based drugs and delivery systems were in development worldwide [11].

14.1.2 Drug Delivery, Imaging, and Targeting

Nanoparticles are structures that are assembled either by physical or chemical crosslinking of several molecules in the range of 1–1000 nm. Small molecules such as drugs, imaging agents, and biomolecules can be adsorbed, dissolved, or dispersed throughout the matrix (as in nanosphere) or confined to an aqueous or oily core surrounded by a shell-like wall (as in nanocapsules). These entrapped molecules can be transported securely to the site of infection without

diffusing across the body's blood-brain barrier or being sequestered by various organs. In the clinical setting, the nanoparticles show therapeutic effect upon releasing the drugs from the matrix by diffusion, swelling, erosion or degradation. When nanoparticles are conjugated to targeting molecules, the delivery efficiency can increase by two orders of magnitude [12]. Researchers are currently studying incorporation of multiple drugs and/or other small molecules within a single nanoparticle [13].

As with any field, research in nanomedicine is sometimes thwarted by limitations such as the resolution limits of diagnosis and detection of biomolecules or microorganisms, localization to an infection site, and determining the fate of drug delivery vehicles. A thorough understanding of both biology and medicine at the molecular level is required to overcome the present challenges and limitations within the field [14]. Nanomaterials that possess unique optical, electronic and magnetic properties, which are amenable to detection by existing imaging modalities, are a promising solution to address these issues [15]. Nanoparticle-based imaging systems are believed to one day revolutionize how medicine is practiced. This is due to their ability to substantially improve the specificity and sensitivity of noninvasive diagnostic agents and detection of tissue specific biomolecules *in vitro* as well as *in vivo*. Further, pharmacokinetics can also be controlled through variations of size, shape and surface properties.

Cell surfaces are decorated with numerous biomolecules and are distinct for different types of tissue. In pathophysiological states some markers are overexpressed, or even in some cases, different sets of biomarkers are expressed when compared to healthy tissue [16]. A targeting agent that can recognize these diseased biomarkers located on the cell surface can be conjugated on the nanoparticles to selectively guide therapeutics and/or contrast agent to accumulate at the target molecules in the lesion. Targeting techniques are powerful tools to formulate individual effective dose treatment. However, this simple idea has practical difficulties in complex multi-compartmental organisms. For example, antibodies that specifically bind to target molecules can only recognize its counterpart only within a distance less than 100 nanometers, but nanoparticles decorated with these antibodies are thousands of centimeters away from the target site when in the blood stream [12]. Another difficulty often recognized in targeting techniques is that diseased sites that are located deep within an organ are segregated from the bloodstream with multiple stages of physical and functional barriers [17]. So, special consideration has to be made while formulating a targeted therapeutic or imaging system. Nonetheless, numerous monoclonal antibodies are already in clinical application and others are under investigation in clinical trial.

14.1.3 Theranostic Nanomedicine

Substantial progress of nanomaterials for delivering drugs or providing contrast agents within the vicinity of a diseased site has already been

translated into clinical uses. However, diseases which are heterogeneous or adaptive, such as cancer, require a therapeutic protocol which is tailored to the individual patient [18]. Recently, the therapeutic benefits of nanoparticle drug delivery and imaging functions were combined to yield particles that can diagnose, deliver targeted therapy and monitor the process and response of the treatment before, during and after treatment regimen to significantly improve prognoses. The marriage of diagnosis and therapeutics within a nanosystem is defined as a theranostic nanosystem and has been extensively investigated and reviewed within the past few years [19–22].

Traditional pharmacological agents (mostly small drug molecules) distribute not only into the diseased site but also into almost all organs. Recent advancement of nanomedicine includes the use of drug carrying nanovehicles with the hope of saturating the sight of interest with significant amount of drugs with minimal systemic distribution. These nanovehicles can further be decorated with targeting molecules to enhance targeting efficiency. However, diseases like cancer, which is described as uncontrolled growth of cells, is not only malignant, but also is highly adaptive and heterogenic. For instance, most chemotherapeutic drugs work by impairing cell division in fast-dividing cells. However, in succeeding generations of tumor cells (typically at the center of the solid tumors), cell division is typically lost, making them less sensitive to most chemotherapeutic agents. Further, in addition to cancerous cells, other fast-dividing cells, such as bone marrow cells, intestinal epithelium, and hair follicles, show rapid cell division in normal physiological conditions making them also highly affected by chemotherapy. So, it is extremely important to provide real-time validation and monitoring of the therapy in addition to targeting therapeutics to the tumor site. The numerous advantages of designing concurrent imaging systems in combination with therapeutic interventions are listed below.

1. Acquire complementary information such as systemic distribution, toxicity and pharmacodynamic of the pharmacological agents.
2. Track drug delivery vehicles systemically and understand the targeting capability.
3. Validate the treatment strategy.
4. Real-time monitoring of disease response to the therapy.
5. Provide early feedback of the therapeutic efficacy.
6. Track the expression pattern of surface markers in response to therapeutics.
7. Help to understand the adaptive nature of disease and alternate the treatment strategy.
8. Understand mechanism underlying regression of the disease and therapeutic effect.
9. Indication of the complete eradication of the disease.

14.2 Design Concerns for Theranostic Nanosystems

The efficiency of therapeutic or diagnostic particles is governed by parameters such as biodistribution (BioD), pharmacokinetics (PK), and pharmacodynamics (PD) [23]. For example, the extended blood circulation time is the primary determinant of attainable particle-plasma concentration. Elevated plasma levels result in an enhanced permeation and retention (EPR) effect for ligand-mediated targeting particles as the elevated localized drug concentrations shift binding equilibrium towards a bound configuration. On the other hand, for imaging agent-containing particles, it is important to excrete the excess nanoparticles from circulation after significant accumulation at the target to reduce signal-to-noise ratio prior to obtaining a diagnostic image. To design a functional theranostic system, careful optimization of the chemical synthesis methods and fabrication techniques to address many important parameters are discussed below.

14.2.1 Size and Stability

Nanoparticles with dimensions in the range of 10–1000 nm can cross the blood-tissue barriers, which is a significant barrier for other drug delivery and imaging approaches. Particles smaller than 150 nm diameters are also able to avoid macrophage engulfment [12]. The blood-brain barrier can be infiltrated by nanoparticles smaller than 100 nm [24]. Nanoparticles smaller than 150 nm can cross cell membranes in the caveolae, and particles 20–60 nm in diameter can specifically cross tissue layers by transcytosis [12]. Nanoparticles larger than 15 nm are not excreted renally, whereas nanoparticles smaller than 5.5 nm are rapidly cleared by urinary excretion [25]. Thus, the size of the nanoparticles should be carefully selected to avoid unwanted barriers, sequestration or excretion and to selectively target the intended site. Later in this chapter we will discuss various synthesis techniques for each different types of materials to fabricate nanoparticles with different sizes.

Another prime concern about nanoparticles utility is the stability of nanoparticles over the period of time (kinetic stability), and stability against dilution and other environmental changes (pH, charge, and temperature) [26]. First, nanoparticles have high tendency of adhesion and aggregation as compared to the submicron sized particles. Second, in clinical settings, nanoparticles encounter mechanical stress due to bloodstream turbulences. Nanoparticles with low thixotropic characteristics may be disrupted during their navigation. Third, nanoparticles will be attacked by many enzymes (amidohydrolases, esterases, and other proteolytic enzymes), which cause a premature breakdown of the nanoparticles prior to intended application. Thus, it is quite important to develop techniques to provide stability to the nanoparticles prior to formulating them as functional materials and products. One such technique can be surface modification of particles with polymers to generate an effective repulsive force between nanoparticles [27]. Another technique can be the use of stabilizer such as PEG, PVA, and polysaccharides such as dextran [28].

14.2.2 Surface Area and Chemistry

The large surface-area-to-volume ratio intrinsic to nanoparticles allows for a high density of functional moieties at the surface. The large surface area facilitates the development of “smart” nanoparticles within the system by conjugating multiple, complimentary targeting molecules to the surface. Throughout the journey, from injection to target cells, a nanoparticle will encounter multiple cell types and physiological conditions prior to reaching the destination. The presence of a high density of multiple binding epitopes specific to a particular cell type increase the odds of binding to the target cell type while concurrently minimizing off-target binding. The surface-area-to-volume ratio also modulates the elution rates of drugs. As the size of the particles decrease, the surface-area-to-volume ratio significantly increases, which results in a faster diffusion of encapsulated drugs from the nanoparticle matrix. Finally, the surface chemistry of the particles can produce a wide range of optical properties [29] (as in the case of quantum dots [QDs] and GNPs), which is critical for bioimaging applications. By simply changing the size of the nanoparticle, a volumetrically proportional change in the surface area is created to modify these important qualities. Numerous surface modification techniques are reported to create a wide range of surface chemistry. In general, surface modification can be done as post synthesis or controlled *in situ* modification. The post-synthesis technique has a benefit of easy processability, whereas the controlled *in situ* modification technique has a benefit of creating nonaggregated nanoparticles [27].

14.2.3 Drug Loading and Release

A successful drug carrier has high drug carrying capacity, feasibility of incorporation of both hydrophilic and hydrophobic drugs, and enables the controlled (sustained) drug release from the matrix. Basically, drugs can be incorporated within nanoparticles by one of three methods: one by conjugating drugs to the material of the nanoparticles prior to nanoparticle formulation, second by encapsulating during the course of production, and third adsorbing the particles after the nanoparticles formation [30]. In these strategies, drug molecules may be encapsulated, dispersed, absorbed, or chemically bound within the polymeric matrix. In any nanoparticle system, the chemical structure of drug molecules, the nanomaterials, and the conditions of drug loading determine the efficiency of drug incorporation [31]. It is critically important to understand the loading/binding rate to create the best possible formulation. In general, linear sorption isotherms can be employed to determine loading rate in case of drug encapsulation and Langmuir S-type Isotherm for surface adsorption [32].

Drug-loaded particles when delivered to targeted site by targeting strategies have the ultimate goal of demonstrating sustained release. Drug release can be achieved by desorption of surface bound drugs, diffusion through the nanoparticles matrix, degradation of the matrix, and combination of either of these. So it is critically important to understand the release mechanism and

factors affecting the drug release from the particular nanoparticle system. For example, smaller particles have higher burst release than larger particles [32], and nanoparticles composed of hydrophilic polymers show release rates largely controlled by diffusion [33]. If the drugs are conjugated to the nanoparticles system, then the drug release predominantly occurs due to the degradation of the matrix. When drugs are entrapped within low molecular weight polymers, the initial burst release is increased [34]. So, careful selection of both materials to formulate nanoparticles for specific drug types and drug loading strategies may be critical to design an effective therapeutic drug delivery system.

14.2.4 Imaging

Contrast agents that are tagged within the nanoparticle system should provide adequate signal that can be detected by currently existing imaging modalities such as fluorescent microscopy, magnetic resonance imaging (MRI), positron emission tomography, computer tomography, X-ray, radiography, and ultrasound. For example, as in the case of fluorescent technology, contrast agent demonstrate excitation/emission as a result of incident photons of a specific wavelength. As electrons decay from this excited state, they emit light with higher wavelength (fluorescent light) [22]. Fluorescent light that is emitted can be captured to quantify the illumination of the sample. The wavelength of the emitted light ranges from visible to the near-infrared (NIR) range, thus providing good spatial resolution. However, autofluorescence and light absorption from biological molecules such as proteins (257–280 nm), heme groups (max absorbance at 560 nm), and water (above 900 nm) [35] can produce significant background noise. These sources of noise must be considered when deciding which wavelength is selected for excitation and emission. Therefore NIR fluorescence imaging within the wavelength range of 700–1000 nm are central to research in order to avoid autofluorescence. Another known problem with fluorescent dyes such as fluorescein and sulforhodamine is that the fluorescence is considerably quenched after conjugation with biological molecules, particularly to proteins [36]. This quenching effect can be troublesome in the case of traditional imaging use but can be very beneficial while developing a “smart” theranostic system. Similarly in the case of MRI, contrast agent such as gadolinium diethylenetriaminopentaacetic acid (Gd-DTPA) is widely accepted in clinical use due to strong T1 shortening effect but suffers with low contrast effects and a very short retention time *in vivo* [37]. So as an alternative, magnetic iron oxide (IO) nanoparticle, whose magnetic properties can be manipulated by controlling the sizes of core and coating surface has emerged as an exciting target-specific MRI T2 contrast agent [38].

14.2.5 Targeting

Designing a targeting theranostic system has multiple advantages, such as maximum therapeutic efficiencies, minimized side effects by reducing toxicity

and immunogenicity, avoiding non-target specific treatment, and being rapidly cleared from the system upon target failure. However, except for some superficial lesion and haematological malignancies, as in the case with most other tumor tissue, nanoparticles have to navigate through a multistage path prior to reaching the final target molecules [12]. In order to invade all the barriers and reach the destination, natural nanoparticles (the wild-type human immunodeficiency virus HIV-1 is a natural nanoparticle) of diameter 126 nm bears 8–10 trimers targeting spikes. These spikes are 8 nm long and 16 nm apart oriented correctly around the 5000 nm² surface area. To engineer an ideal targeted particle, it is critically important to understand the surface chemistry of the nanoparticle and its conjugation with targeting chemistry [39].

Biomolecules such as antibodies, aptamers, ligands, enzymes and nucleotides are capable of binding with cell surface molecules with high stereospecific affinity. Nanoparticles with appropriate chemistry such as amine, carboxyl, thiol, carbonyl and many more can be easily conjugated with these biomolecules to create targeting ability of the nanoparticles. On the other hand, unwanted uptake of nanoparticles into elements of reticulo-endothelial system and attachment to non-target site should be avoided. Surface modification of nanoparticles with appropriate electric charges and hydrophilic group, such as PEG molecules, may confer stealth properties within the nanoparticles [40]. Another important concern while designing nanoparticles is to minimize immunogenicity. The most widely accepted technique in this regard is to construct the nanoparticle system with humanized glycosylated components [41].

14.3 Designing a Smart and Functional Theranostic System

Despite much progress, drug delivery systems still face numerous challenges, particularly nonspecific targeting and drug release. From administration to pharmacological activity, nanocarriers navigate through very different biological environments. To address this issue researchers are actively investigating various environmental responsive therapeutic nanocarriers. In the following section, we review studies in which environmentally responsive nanoparticles that change size, shape, degradation, surface chemistry and targeting molecules are discussed.

14.3.1 Tailoring Size and Shape of the Particles

Particle size is the prime determinant of a particle's physical properties. A small change in the diameter of the particles from 15 nm to 25 nm translates into an increase in the surface area equivalent to more than three hundred human bodies in length [12]. Thus, changing the size of a particle during the course of therapy by various stimuli such as pH, temperature, light and hydrophobic-hydrophilic switching of the surface can create a "smart" theranostic system [42]. In most cases, these parameters alter the particle swelling, resulting in a

change of the particles size. Particles that are composed of PEG and polycarbonate block copolymers (polymersomes) show significant swelling behavior (expanding from 120 nm to over 1000 nm within 24 hours) when suspended in a buffer of pH 4.0 [43]. Changes in particle size, in response to varied pH of the surrounding environment, also improves cytosolic delivery of membrane-impermeable proteins by disrupting the endosomal membrane, as in the case of pH-sensitive PDEAEMA-core/PAEMA-shell nanoparticles [44]. Similarly, particles composed of poly(NIPAAm) based [45], Pluronic [46] and pluronic/poly(ethylenimine) [47] also show changes in size in response to temperature changes. In the case of pluronic/poly(ethylenimine), fast and reversible swelling induced by a short cold shock treatment (20°C) in the endosome increase the size of particles from 100 nm to 300 nm resulting in rupture of the endosomal membrane [47]. Another example of swelling induced size change occurs in virus mimicking particles composed of a pH-sensitive hydrophobic core (poly (His-co-Phe)) and two layers of hydrophilic shells (PEG and BSA). The particle hydrophobic core at pH 7.4 becomes hydrophilic at pH 6.4, and exhibits a reversible size change from 55 nm to 365 nm due to instantaneous swelling [48]. Another interesting size change behavior has been observed in poly(4-cinnamic acid)-co-poly(3,4-cinnamic acid) (PCA) particles in response to radiation wavelength. These particles decrease in size when exposed to UV irradiation above 280 nm and reversibly increase when exposed to 254 nm wavelengths [49, 50].

Similar to size change, particles that can undergo shape deformation in response to environmental stimuli have also been reported. In an *in vitro* study led by Yoo and Mitragotro, macrophages were unable to phagocytose particles when in the elliptical disk-like form. However, when particles were switched from elliptical disk-like to sphere shape macrophages, the ability to phagocytose them was regained. These particles, composed of PLGA, demonstrate switching behavior in response to high temperature, low pH or chemicals [51]. Another shape change in the nanoparticles system was observed in amphiphilic DNA-brush copolymers. In this system, amphiphilic polymer chain assemble into 25 nm spherical micelles that transform into cylindrical shape in the presence of DNAzyme and return to their original spherical shape when a specific DNA sequence is added to the micelle [52].

14.3.2 Degradation and Drug Release Kinetics

The ultimate goal of therapeutic nanoparticles is to carry maximal drug payloads to the targeted disease site and demonstrate a controlled, “smart” drug release profile. Controlled release can be achieved by degradation/dissolving of the nanocarriers or through diffusion. Tailoring these two parameters through environmental stimuli can optimize smart drug release kinetics. For example, poly (β -amino ester) exhibits different solubility at different pH level (completely soluble below pH value of 6.5 and insoluble at pH 7.4) [53, 54]. This property can be utilized to design a smart delivery system with a rapid payload release when it encounters an acidic microenvironment. The

drug otherwise remains safely encapsulated within the mechanically stable, insoluble nanoparticles. This system was further illustrated in poly(B-amino ester) based mPEG-PbAE block copolymer where stable micelles (at pH 7.4) were demicellized selectively at tumoral acidic pH. In two separate studies, this micelle system was used to deliver anticancer drug (Doxorubicin) to the tumor site [55] and MRI contrast agents (Fe_3O_4) to cerebral ischemic area [56]. As mention earlier, other factor such as pH, temperature, hydrophobic-hydrophilic switching, and light induced swelling also effect on the over drug release kinetics.

14.3.3 Surface Properties and Placement of Targeting Molecules

While travelling to targeted tissues, nanoparticles interact with numerous living components and tissue barriers. The surface chemistry determines the fate of each particle. For example, hydrophilic surfaces (PEG and dextran coated particles) show poor protein adsorption and delayed circulation clearance [57]. Particles with positively charged surfaces exhibit improved cellular attachment and internalization. Hydrophilic surface of the particles can prolong circulation but also may inhibit particles-cell interaction. Surface modification of particles with moieties such as antibodies, aptamers, proteins, and peptides sequences results in selective localization to a target tissue [58–61].

Surface chemistry can also be altered to create smart delivery systems. Smart nanoparticles with PEGylated surfaces can be designed in such a way that de-PEGylation occurs only within the vicinity of diseased tissues. Sun and coworkers formulated a drug carrying micelle system by conjugating PCL chains to PEG molecules through a disulfide bond. These disulfide bonds can be reduced to achieve rapid drug release at target cells [62]. In two separate studies, matrix metalloproteinase-2 (MMP-2) (an enzyme released by tumor cells) liable PEG was coated in hepatic tumor-targeted liposomes [63] and cell penetrating peptide (CPP) conjugated QDs [64] to enhance the cellular uptake in presence of MMP-2 molecules. In addition to this, surface charge switching particles are also studied to avoid off-site nonspecific binding of particles. For example, polymeric micelles composed of a hydrophobic core, poly-L-lactide, and two hydrophilic shells, PEG and poly-L-lysine (PLys)-dimethylmaleic anhydride (DMA) show surface charge switching according to the change in pH of the environment. In this system, negatively charged, DMA molecules at the outmost surface molecule can be cleaved (at pH 6.5–6.0), exposing the underlying positively charged, PLys molecules to enhance cellular uptake [65].

Alternatively, targeting molecules can be hidden by PEGylation to avoid off-site recognition. Lee and coworkers have developed a polymeric micelle system to deliver doxorubicin to tumor cells based upon masking and unmasking of functional ligands. These micelles are composed of two block copolymers, polyHis-b-PEG and pLLA-b-PEG-b-PolyHis-biotin. Above pH 7.0 (physiological pH), the hydrophobic polyHis segment sequestered biotin and exposed hydrophilic PEG as the outer shell. However, below pH 7.0 (tumoral pH), ionized polyHis was liberated from the hydrophobic core, extending the biotin

beyond the PEG shell to interact with tumor cells. Furthermore, below pH 6.5 (endosomal pHs), the micelles dissociate and disrupt the endosomal membrane to release doxorubicin into the cytosol [66].

14.4 Materials for Theranostic System

14.4.1 Polymeric Systems

Research on biodegradable nanoparticles (NPs) has intensified over the last 10 years [14, 67]. These NPs are formulated with biodegradable and biocompatible polymers that can be further surface modified or functionalized with targeting molecules, drugs and imaging agents [68]. Factors such as particle surface charge, functionalities and hydrophilicity/hydrophobicity ratios are easily manipulated in polymeric nanoparticles, which are critical determinants of drug release pharmacokinetics [69]. Polymeric nanoparticles are on the front line of development for diverse therapeutic applications, ranging from cancer therapeutics to antimicrobial actions, vaccine delivery to gene delivery or site-specific targeting. The following section will be subdivided according to polymer hydrophobicity or hydrophilicity in order to understand their role in drug delivery.

14.4.1.1 *Hydrophobic Polymers*

Polymers that are miscible in organic solvent, but not aqueous solvents, are called hydrophobic polymers. Polymers such as poly(lactic acid) (PLA) [70–74], poly(lactide-co-glycolide) (PLGA) [75–78], poly(ϵ -caprolactone) (PCL) [79–83], and poly(alkyl cyanoacrylate) (PACA) [84–88] are the most extensively characterized hydrophobic polymers that can be used to fabricate nanoparticles. The surface hydrophobicity of nanoparticles is an important factor in drug delivery applications. Nanoparticles made up of hydrophobic polymers have limited water absorbing capacities (5–10%) [33] prolong drug release by preventing rapid diffusion, and have demonstrated higher cellular uptake [69]. However, these polymers also elicit unintended protein absorption [89] and rapid clearance by macrophages [90]. Due to their ability to dissolve in organic solvent but not in aqueous medium, these polymers are easily fabricated into nanoparticles with desired size, morphology, polydispersity, stability, and drug-loading ability by dispersion of preformed polymers.

Solvent evaporation [91–93], salting-out [72, 94], nanoprecipitation [95], and dialysis [96–98] are commonly used techniques that utilize dispersion of preformed polymers. The general principle shared by these techniques is that the polymer is dissolved in organic solvent and subsequently subjected to a nonsolvent system (typically water) with a surfactant (optional). Once the solvent leaves the system (by displacement or evaporation), the polymer is precipitated into a nanostructure (nanospheres or nanocapsules). The organic solvents used in these techniques are typically toxic and recent research has highlighted the need for organic, solvent-free methods. This, in turn, led to the

use of supercritical fluid technology for the production of nanoparticles [99, 100]. Techniques such as rapid expansion of supercritical solution (RESS) [101] and rapid expansion of supercritical solution into liquid solvent (RESOLV) [102] are based upon the ability of supercritical solvent to expand and decrease the solvent density dramatically from liquid-like to gas-like density. When the nanoparticle precursor (polymer) is dissolved in these solvents, the polymer precipitates into a nanostructure upon expansion. Nanoparticles precipitate into ambient air via the RESS technique while RESOLV fabricated nanoparticles precipitate into a liquid medium [103].

14.4.1.2 *Hydrophilic Polymers*

Polymers that have high affinity for aqueous solvent systems are called hydrophilic polymers. Polymers based upon poly(ethylene glycol) (PEG) [104–109], poly(vinyl alcohol) (PVA) [110–112], poly(ethyleneimine) [113], poly(vinyl pyrrolidone) [114–117], and poly-N-isopropylacrylamide [118–122] are the most common synthetic hydrophilic polymers. Polymers based on chitosan [123–127] and alginate [128–130] are examples of natural hydrophilic polymers that are frequently investigated in nanoparticle fabrication studies (recently referred to as nanogels) for drug delivery system. NPs made up of hydrophilic polymers do not require toxic organic solvents and surfactants, and demonstrate extended life spans in circulation [131, 132]. However, their hygroscopic nature may result in rapid dissolution of the drugs from the system through diffusion [33]. Despite an affinity for water hydrophilic polymer chains they do not rapidly dissolve in an aqueous environment. Dissolution is prevented by the critical crosslinks present in these nanostructure. Nanoparticle crosslinking is created by either chemical bonds between the polymer chains or physical entanglements of the polymer chains.

Several strategies are used to crosslink polymer chains by covalent bonding such as radical (induced either by photosensitive or chemical initiators) initiated polymerization, high-energy irradiation, enzymes, complementary group chemical reaction (aldehydes, Michael addition, and click reactions) [33]. The general principle behind covalent crosslinking assumes that the polymer chains are dispersed in aqueous solvent at low concentration, under continuous stirring. When a crosslinking initiator is introduced these polymeric chains crosslink, randomly, into nanoparticles. These crosslinking strategies, which rely on specific functional group reactions, preserve other functionalities present in the polymer networks. Thus, crosslinked nanoparticles retain the "smart" functionality for drug delivery or conjugation to other biomolecules of interest [133]. Chemical-based crosslinking carries with it toxicity issues associated with the crosslinking initiators. In contrast to this, physical gelation strategies eliminate the need for external crosslinking agents. During gelation, polymeric chains uniformly disperse in the aqueous system, and physically entangle in response to an environmental change (pH, temperature, and ionic strength within the polymeric chain) [133–135] of the aqueous system to create nanostructures. Since the nanostructures are assembled from

water-soluble polymeric chains in response to an environmental change, this polymeric assembly is highly reversible [33].

14.4.1.3 *Amphiphilic Polymers*

Polymers composed of both hydrophobic and hydrophilic segments in the same polymeric chain are known as amphiphilic polymers. Polymers such as poly(ethylene glycol)-poly(amino acid) (PEG-PAA) [136, 137], poly(ethylene oxide)-b-poly(propylene oxide) [138–140], and poly(ethylene oxide)-b-poly(ester)s [141–144] are examples of synthetic amphiphilic polymers. Liposomes represent natural amphiphilic lipid core polymers [145]. Micelles are formed when the hydrophobic core of amphiphilic chains assemble together to minimize hydration, thus creating a nonpolar core, which can act as a reservoir for hydrophobic drugs. The hydrophilic segment of the block polymer preserves the thermodynamic stability of the system in aqueous solvents [146, 147]. Micelle preparation from amphiphilic polymers is a simple process. When hydrated in an aqueous solvent amphiphilic polymeric chains self-assemble, due to the desolvation, collapse, and the intermolecular association of the hydrophobic portions and electrostatic interaction between charged block copolymers of monomers [148]. The following features of micellar delivery systems distinguish them from other drug delivery systems: 1) they are of small size (< 100 nm) with a narrow size distribution [149]; 2) they can be prepared in large quantities easily and reproducibly [150]; 3) they can physically entrap hydrophobic therapeutics at concentrations above their intrinsic water solubility [150]; and 4) the hydrophilic corona, usually composed of PEG molecules, increases the thermodynamic stability and also increases circulation time [151].

14.4.1.4 *Review on Application of Polymeric Nanomaterials in Drug Delivery*

Polymers have been utilized as a therapeutic agent since 1940. Products such as Povidone (PVP-iodine), Stimaler and Adagen (polymer-protein conjugates) are early works that have found approval for human clinical use [152]. However, polymeric nanomaterials as drug carriers have only garnered significant interest over the last two decades. These nanoparticles can act as a drug reservoir and can also be functionalized with targeting molecules for controlled and targeted drug delivery, thus making them a promising approach within the medical field. Molecules of therapeutic agents are either dispersed within the polymeric matrix or conjugated to the polymeric backbone of the nanoparticles. In the former case, the encapsulated drugs can diffuse out of polymer matrix by fluid uptake or in response to environmental changes. In the latter case, degradation of the polymer matrix or cleavage of polymer-drug conjugating bond determines drug release kinetics. In recent years, various techniques have been developed to adjust the release rate and to avoid premature drug release prior to reaching the targeted tissue. As a result, numerous polymeric

particles are currently in the early stages of clinical evaluation to characterize their therapeutic relevance. Polymeric nanoparticles can be formulated into nanospheres, nanocapsules, dendrimers, micelles, nanogels, vesicles or liposomes according to the nature of the polymeric chain (hydrophilicity and hydrophobicity). In all cases, however, these particles have proven to be effective drug carriers by increasing the efficacy of the drug and safely delivering highly toxic, poorly soluble, or relatively unstable drugs [153]. Polymeric nanoparticles are the leading nanocarriers for wide range of drug molecules as tabulated in Table 14.1.

Two clinical advances are particularly noteworthy given their low therapeutic toxicity and ability to extend the product life cycle. Liposomal drugs and polymer-drug conjugates account for greater than 80% of the nanoscale-based therapeutics market [154]. The first liposome-based drug delivery system approved for human use was Doxil (doxorubicin liposomes) for the treatment of acquired immunodeficiency syndrome (AIDS)-associated Kaposi's sarcoma. Since that time the FDA has cleared other liposome-based drug delivery systems. In addition to this, several other modified liposome-based drug delivery systems are also under investigation, such as surface functionalized targeted liposome, polymer combined liposome, photosensitive liposomes for stimuli responsive drug release and cationic liposomes for nucleic acid-based therapeutics delivery [153]. Similarly, Abraxine (paclitaxel bound albumin) is an example of an FDA approved drug conjugate for the treatment of breast cancer [155]. Abraxane is a nanosystem (130 nm) that significantly increases the therapeutic response rate at the tumor site, retards disease progression and ultimately increases the survival rate of breast cancer patients [156]. Paclitaxel albumin-bound systems have been clinically investigated for targeting metastatic breast cancer by conjugation with transtuzmab (antibody) [157, 158]. More recently Nanoxel, a nanoparticle-based delivery system composed of synthetic biodegradable amphiphilic block copolymer mPEG and PDLLA, has been approved for human use. Nanoxel increases the efficacy and minimizes the cytotoxicity of paclitaxel in breast cancer and ovarian cancer treatment [14].

14.4.2 Diagnostic and Imaging Materials

Nanotechnology has also become increasingly prevalent in the development of many innovative biological applications, such as biosensors, labeling of cells or cellular component, detecting biomarkers and diagnosing diseased site. Materials such as semiconductor nanocrystals (QDs), metallic nanoparticles (gold), polymers (Biodegradable photoluminicient polymer (BPLP), Poly(amido-ester) (PAMAP)), and small molecules (fluorescent dyes) have demonstrated optical properties that can be detected by fluorescent technology.

Alternatively, iron oxide is a powerful imaging agent in magnetic resonance imaging (MRI) technology. This technology relies upon the precession of water hydrogen nuclei by pulses of radiofrequency under a magnetic field. In this process the difference in energy will be released as a photon that can be detected as an electromagnetic signal [22]. Although this technology

demonstrates excellent spatial resolution, it suffers from low sensitivity. Other imaging modalities such as positron emission tomography, computer tomography, X-ray, radiography, and ultra-sound are used in a clinical setting but lack specificity, sensitivity, or may suffer from radioactivity-associated health risks [202]. In this chapter we have limited our discussion on fluorescence- and magnetic resonance-based contrast agents.

14.4.2.1 QDs

Quantum dots (QDs) are nanocrystals composed of semiconductor matter that exhibit optical and electrical properties according to the size and shape of the individual crystal [29]. Binary alloys such as cadmium selenide, cadmium sulfide, indium arsenide, and indium phosphide are semiconductor material that can be grown into nanocrystals ranging from 2–10 nanometers in diameter (about the width of 50 atoms), collectively known as QDs [29]. In semiconductors, electrons can have energies only within certain bands. When an electron is excited and moves from the valence band into the conduction band, due to photon impingement of energy higher than the band-gap energy, it generates a short-lived electron–hole pair (exciton). When the crystal returns to its resting state, the electrons and holes recombine quickly and a fluorescent emission of photons are detected with a lower energy than that of the excitation energy [203]. As the size of the crystal grows bigger, the band gap gets smaller; the energy between the highest valence band and the lowest conduction band significantly decreases. As a consequence a lower energy level is required for excitation and lower energy fluorescent light (such as red light) is emitted.

14.4.2.1.1 *Synthesis of QDs*

Techniques such as photolithography, chemical synthesis and self-assembly are some of the common routes used to synthesize QDs. Numerous improvements in the synthesis process have resulted in low size dispersity and higher fluorescence efficiencies since the initial report by Efros and Ekimov in 1982 [204, 205]. For example, preparation of QDs in aqueous solution mixed with stabilizing agents (e.g., thioglycerol or polyphosphate) was a common practice prior to 1993. The resulting QDs had large size variations and poor fluorescence efficiencies [29]. In 1993, the size variability was significantly reduced by the use of a high-temperature organometallic procedure, though products still suffered from low quantum yields [206]. Later techniques, such as layer deposition of surface-capping agents (ZnS or CdS), significantly improved the quantum yield of QD particles [207]. In addition to this, the quality of particle was improved by varying the precursor materials (such as CdO) [208]. As discussed earlier, variations in the size of QDs has a significant impact on tuning the emission wavelength from the blue to the near infrared. It is also critical to understand the techniques that control the size of the particles [209].

Though QDs are attractive vehicles because of their superior fluorescent properties, such as tunable emission, wavelength, quantum yield, molar absorption coefficients and low photo bleaching, they are frequently criticized

for toxicity [210]. Toxicity issues commonly associated with QDs include desorption of free Cd (QD core degradation) [211], free radical formation, and interaction of QDs with intracellular components [212]. The extent of cytotoxicity depends upon factors such as the choice of precursor Cd materials, capping materials, surface chemistry, processing parameters, size, color, and dose of QDs [213–215]. Despite persistent effort the toxicity associated with QDs remains; a technique which yields highly monodisperse, luminescent and non-toxic QDs remains to be discovered [210].

14.4.2.1.2 Review of QDs for Diagnosis and Imaging

Since its first discovery almost 30 years ago, QDs have been employed in diverse biomedical applications such as *in vitro* cell labeling, *in situ* tissue diagnostics, targeted *in vivo* imaging, monitoring intracellular drug/gene trafficking, and more recently as a theranostic tool [209, 216–218]. It was not until 1998 that Bruchez and coworkers reported for the first time the use of QDs in immunofluorescence to detect actin filaments in mouse fibroblasts [219]. Subsequently, Wu and coworkers reported the use of QDs in static immunostaining of specific cellular targets. In this work, it was demonstrated that the QDs conjugated to immunoglobulin G (IgG) and streptavidin can label breast cancer marker (Her2) on the surface of fixed and live cancer cells, stain actin and microtubule fibers in the cytoplasm, and detect nuclear antigens inside the nucleus. They concluded that QD-based probes could offer substantial advantages over organic dyes in cellular imaging [220]. During the same year, Dahan and coworkers reported the use of QDs to track individual glycine receptors (GlyRs) and characterized multiple diffusion domains in relation to the synaptic, perisynaptic, or extrasynaptic GlyR localization [221]. It was concluded that this real-time visualization of single-molecule movement in single living cells was possible only with QDs which, unlike organic dyes, are not susceptible to photobleaching and can be effectively applied in time-lapse studies [218]. Since that time, QDs have been frequently reported in *in vitro* cell labeling, receptor diffusion dynamics, ligand–receptor interactions, biomolecular transport and enzyme activity studies.

There has been considerable interest in the use of QDs for mapping lymph node and vascular structure, tracking cells *in vivo* and tumor imaging. This niche within the field continues to expand at a greater rate than *in vitro* diagnosis. QDs demonstrate strong fluorescence, good photochemical stability and, most importantly, a narrow and tunable emission spectrum (from near-ultraviolet to near-infrared region) that reduces background noise from tissue autofluorescence [222]. The first study, reported by Akerman, to use peptide conjugated to QDs targeted tumor vasculature for imaging. Although *in vitro* histological results revealed that detectable amount of QDs localized to tumor vessels, the level of QDs were below the range of detection *in vivo* [223]. Since that time many successful approaches have been reported with improved QDs systems. QDs encapsulated in phospholipid micelles were injected into frog oocyte cells for real-time tracking of embryonic development [224].

QD encapsulated triblock polymer conjugated with tumor-targeting ligands have successfully imaged human prostate cancer grown in nude mice [225]. QDs conjugated to arginine-glycine-aspartic acid (RGD) peptide were used to image the tumor vasculature in living mice [226]. Similarly, numerous lymph node mapping studies were successful with the assistance of QDs [227–229] and were observable for longer than 4 months [230]. Recently, a QD-based *in vivo* imaging system was reported in coordination with bioluminescent protein. In this system, external illuminating sources were completely abandoned and QDs were illuminated by resonance energy transfer (BRET) system. This system aims to eliminate autofluorescent background noise and avoid superficial location of excitation light, which is a major disadvantage of external light-source-dependent systems [231].

QDs have also been evaluated for pharmacokinetic and pharmacodynamic modulation of drug and intracellular drug/gene trafficking mechanisms. Manabe and coworkers developed a QD-captrophil (antihypertensive drug) conjugated system to characterize the half-life and plasma concentration of captrophil in blood. However, nonspecific uptake by macrophages and endothelia cells of QD-captrophil significantly reduced the efficacy of drug performance [232]. Significant efforts have also been made to understand intracellular drug/gene trafficking by utilizing QD-mediated Forster Resonance Energy Transfer (FRET). Ho *et al.* investigated the structural composition and dynamic behavior of polymeric-DNA nanocomplexes intracellularly based on a QD-FRET system. In this system, plasmid DNA (pDNA) was conjugated with 605QD (donor) and a cationic polymer (chitosan) conjugated with Cy5 dye (acceptor). When the pDNA-chitosan nanocomplexes formed, fluorescence from both 605QD and Cy5 were simultaneously obtained, but when disrupted, only fluorescence was observed from the donor indicating the release of drugs (pDNA) from the system [233]. In addition to this, Chen and coworkers developed a two-step QD-FRET system by pairing QD and nuclear dye on the pDNA to study both polyplex dissociation and DNA degradation in a simultaneous and noninvasive manner [234]. These approaches are invaluable for studying mechanisms involved in polyplex unpacking and trafficking within live cells.

14.4.2.2 Gold Nanoparticles (GNPs)

Like QDs, gold nanoparticles (GNPs) also exhibit optical properties that are not observed in the bulk metal or in molecular compounds. These optical properties are dependent upon factors such as the shape and size, interparticle distance, and nature of the protecting organic shell [8]. Nanoparticles composed of metals such as gold, silver, and copper have the ability to produce an intense absorption (530 nm in the 5–20 nm diameter range of GNPs) due to the characteristic collective oscillation of electrons on the surface (plasmon resonance), when subjected to an electromagnetic field [235]. Absorption wavelength of these gold particles can be tuned (redshift) according to the size and shape of the particles. For example, spherical particles with 48.3 and 99.4 nm in diameter exhibit the maximum absorption of Au at 533 nm and 575 nm, respectively [236].

In addition to this, if the shape of the particles is changed into rod-like particles, absorption can be pushed to the NIR region (650–900 nm) [237]. Another interesting property of GNPs is the ability to convert light energy into heat energy (energy transducers) upon laser irradiation, which makes them desirable candidate materials in photothermal therapy [235].

Apart from these, GNPs are gaining interest in surface-enhanced Raman scattering (SERS) technology. In general, every molecule when subjected to laser light shows signals within the 400–1800 cm^{-1} range that is unique to each molecule, due to their characteristic molecular vibrations [238]. This technique shows clear differences in the Raman spectra of the cell nucleus and the cell cytoplasm [239], however, identification of other low concentration biomolecules or drug molecules within the cell suffer from extremely weak signal for detection. In order to solve this problem, GNPs can be attached to a probe molecule to increase the local optical fields of these structures by 10- to 12-fold in magnitude (SERS) [240, 241]. This property of gold particles can help identify the fate of a therapeutic agent within the infected cells or tissues.

14.4.2.2.1 *Synthesis of Colloidal Gold Particles*

Discovery of “soluble” gold has been dated as early as the 5th century B.C. [242]. However, the first formal report of colloidal gold formulation (deep red solutions) by reduction of an aqueous solution of chloroaurate (AuCl_4^-) was purposed by Michael Faraday in 1857 [243]. Consequently, various methods for the preparation of gold colloids have been reported and reviewed [242, 244–246]. For example, the Turkevich method [247], Brust-Schiffrin method [248], Perrault Method [249] and Martin Method [250] are a few of the pioneering works which led to increasingly stable, monodispersed and nanosized gold particles. Although each method has furthered the goal “high quality” gold particles, the principle of synthesis still follows Faraday’s (reduction of AuCl_4^-) concept, though with novel reducing agents and stabilizing agents.

14.4.2.2.2 *Review of Colloidal Gold Particles for Diagnosis and Imaging*

Colloidal gold particles are another promising nanomaterial used in various aspects of biomedical application such as *in vitro* assays, *ex vivo* and *in vivo* imaging, cancer therapy (photothermal), radio/photo sensitizers and drug delivery. In early attempts by Mirkin and coworkers, it was reported that gold particles (13 nm diameter) coated with 5’-(alkanethiol)-capped oligonucleotide and coupled with a complementary target oligonucleotide sequence absorbed at longer wavelengths when aggregated. This technique was translated into a colorimetric DNA-hybridization assay that can distinguish sequences containing one base-end mismatch, a deletion or an insertion from a fully complementary target [251]. GNPs are now routinely used in many other *in vitro* assays such as immunoassays, protein assay, time-of-flight secondary ion mass spectrometry, capillary electrophoresis and detection of cancer cells. Gold particles have found success in the handheld immunodiagnostics and histopathology fields because they do not alter the biological activity of bound proteins.

Over the last three decades endoscope-compatible microscopies, such as optical coherence tomography and reflectance confocal microscopy, have been

used to obtain three-dimensional pictures of tissue microanatomy with excellent spatial resolution (1–10 μm). However, to identify molecular biomarkers associated with diseases such as cancer, specific molecular contrast agents are required. Contrast agents based on GNPs have been used in techniques such as reflectance microscopy, optical coherence tomography, photoacoustic tomography and SERS to acquire *ex vivo* and *in vivo* imaging of disease state. Sokolov and coworkers realized this limitation and developed a GNPs-based probe molecule that can image precancerous stage via reflectance laser-scanning confocal microscope [252]. Similarly, numerous studies were reported in tissue phantom to study GNPs as a contrast agent in OCT until Kirillin and coworkers demonstrated real-time *in vivo* skin images comparable to histology (gold standard) by Monte Carlo calculations [253].

In another study, Zharov and coworkers developed a photoacoustic flow cytometry model for real-time detection of circulating cancer cells labeled with gold nanorods in the vasculature of mouse ears. This technique is expected to expand to study pharmacokinetics and the immune response to apoptotic and necrotic cells because the estimated threshold sensitivity was one cancer cell in the background of 10^7 normal blood cells [254]. Another promising technique for *in vivo* imaging is based on Raman spectroscopy and SERS nanoparticles. In clinical settings, optical imaging using Raman spectroscopy is only relevant for superficial tissues such as breast or tissues and those accessible by endoscopy such as the esophagus and colon. However, Keren and coworkers demonstrated a deep tissue whole-body Raman imaging by using SERS nanoparticles composed of a gold core, a Raman-active molecular layer, and a silica coating [255].

14.4.2.3 Iron Oxide Nanoparticles

Similar to QDs and GNPs, iron oxide nanoparticles (IONPs) are other interesting metallic nanoparticles that have demonstrated significant importance in biomedical applications. In the past few decades, many IONPs (bare nanoparticles or polymer, liposome or micelles coupled) have been reported, some of which are in clinical trials today. A few of these candidates have also entered the market as pharmaceutical products [256]. It is the superparamagnetic property of IONPs (particles less than 20 nm), which are not observed in bulk material, that makes them an attractive contrast probe for magnetic resonance imaging (MRI) [235]. Individual atomic magnetic moments present in these particles are typically oriented randomly, resulting in a net zero magnetic moment in the particles absent of external magnetic fields. In the case of nanoparticles, a small change in thermal energy can cause the magnetic moment to fluctuate, resulting in a superparamagnetic state. The high magnetic moments of each particle can further reduce the T2 relaxation time leading to signal attenuation on a T2 or T2*-weighted mapping system. Materials such as magnetite or maghemite are the most commonly used metals for the formulation of IONPs due to their superior magnetic properties, biocompatibility, and low cost compared to other magnetic materials (oxides and pure metals).

14.4.2.3.1 *Synthesis of Iron Oxide Nanoparticles*

Since 1970, extensive research on the synthesis and characterizations of magnetic particles has been reported. Many of these techniques rely on the ability of ferrous, ferric ions, or salt to precipitate into nanocrystals in potassium nitrate and potassium hydroxide (in Sugimoto's method), oxygen-free-base (in Massart's method), or water in oil phase (microemulsion method). Recently, advanced techniques have reported sonochemical routes, electrochemical deposition under oxidized conditions (EDOC), hydrothermal method and synthesis by magnetotactic bacteria. Apart from the synthesis, another important process is surface coating. Polymer surface coating can occur either during the process of synthesis or post-synthesis. Polymer coatings modulate the solubility, stability, biocompatibility, and circulation time of ferrous particles *in vivo*.

14.4.2.3.2 *Review of Iron Oxide Nanoparticles for Diagnostic and Imaging*

Over the past two decades numerous IONPs systems have been translated into medical practice and many more are under investigation in clinical trials. These particles are typically employed as contrast agents in MRI for imaging bowel, liver, spleen, lymph node, bone marrow, MR angiography, tissue perfusion, and many other tissues. IONPs, when administered intravenously, show rapid accumulation in the reticuloendothelial system (RES) and cells of the liver and spleen. Owing to this property, IONP-based contrast agents such as Feridex IV® and Endorem® have already been approved for hepatic imaging [257]. These contrast agents are capable of distinguishing lesions as small as 2–3 mm of liver tumor and metastases [258]. *Seneterre and coworkers* demonstrated through their study that ferumoxides-enhanced T2-weighted MR imaging was comparable to that of CT during arterial photography in identifying liver metastases [259]. Another contrast agent, Combidex®, is in late stage clinical trials for use in the detection of lymph node metastase [260] and has effectively identified lymph node metastases in prostate cancer with a diameter of 5–10 mm under MRI. Similarly, Lumiren® and Gastromark® are examples of other IOPN available for medical doctors as bowel contrasts [261]. More recently, IOPNs have also been proposed as contrast agents (MRI) to diagnose cardiovascular diseases such as myocardial injury, atherosclerosis and other vascular disease. Kelly and coworkers have identified the adhesion molecule (VCAM-1) of endothelial and macrophage cells as a target of IONPs, to study its role in atherosclerosis and more than 30 families of new peptides which bind to atherosclerotic lesions [262]. In two separate studies, it was concluded that MR images obtained by the use of IOPNs could be valuable tools to evaluate the risk of acute ischemic events [263, 264].

14.4.2.4 *Fluorescent Dyes*

Fluorescent dyes are small molecules that cannot be fabricated into nanoparticles, but can be incorporated into a nanoparticle system either by encapsulation or conjugation. In general, fluorescent dyes can be categorized into two groups according to the underlying process of emission. First, resonant dyes exhibit

emission that originates from the process of an optical transition delocalized over the whole chromophore. Fluoresceins, rhodamines, most 4,4'-difluoro-4-bora-3a,4a-diaza-s-indacenes (BODIPY dyes) and most cyanines are some examples of resonant dyes. Alternatively, in intramolecular charge transfer (CT) dyes the emission originates from an intramolecular charge transfer transitions. Coumarin and coumarin-based dyes are examples of CT dyes. Resonant dyes show poorly separated but slightly structured absorption and emission bands, and higher molar absorption and fluorescent quantum yield, whereas CT dyes show well-separated, broader and structureless absorption and emission bands [210].

14.4.2.4.1 *Synthesis of Fluorescent Dyes*

Synthesis of *De Novo* fluorescent dyes with the required properties can be complicated by an incomplete understanding of how the molecular structure determines the fluorescent properties, despite similarity to known molecules. Fortunately, existing molecules can be derivitized to yield molecules with desired fluorescent properties [265]. The monomethine and trimethine cyanines (Cy3), with absorption bands in the visible region, can be shifted by about 100 nm just by extending the chromophore by one vinylene moiety ($\text{CH}=\text{CH}$) [266]. As a result, pentamethine (Cy5) derivatives and heptamethine cyanines (Cy7) have absorption at a near-infrared region (>700 nm) and beyond 1000 nm, respectively. One of the advantages of organic fluorescent dyes is ease of functionalization with other nanomaterials [267]. The commercial availability of functionalized dyes in conjunction with established labeling, purification, characterization protocols, and information on the site-specificity of the labeling procedure has facilitated the conjugation of fluorescent molecules to nanoparticles of interest.

14.4.2.4.2 *Review of Fluorescent Dyes for Detection and Imaging*

In most *in vivo* applications it is desirable to use near infrared (NIR) fluorescent dye to avoid background interference from tissues, to improve tissue penetration depth, imaging sensitivity and noninvasiveness. Fluorescent dyes can be conjugated with targeting ligands, encapsulated within or conjugated with nanoparticles, and used in coordination with other contrast agents to achieve quenching/non-quenching effects. For example, Cy5, Cy5.5 and Cy7 have been conjugated with various chemical ligands, peptides, proteins and antibodies to target different types of cancers. For example, Garanger and coworkers reported a Cy5.5-based formulation that was conjugated with cRGD cyclopeptide and drug molecules. This system was employed in a mouse model and demonstrated significant reduction of tumor even at low doses [268]. Derycke and coworkers encapsulated aluminum phthalocyanine tetrasulfonate (AlPcS4), a photosensitized agent that shows absorption at 672, to visualize bladder cancers and to provide photodynamic therapy [269]. Conditionally activated fluorescent dyes lack fluorescence without the presence of an enzymes associated protease, predominantly expressed in inflammatory condition and in tumor cells, which stimulates excitation. These types of dyes are particularly suited

for disease severity characterization because the fluorescence intensity can be directly related to the quantity of enzyme present within the inflamed zone [267]. Similarly, many other fluorescent dyes are reportedly sensitive towards small fluctuation in the pH value of the environment [270]. Recently, Lee and coworkers illustrated the ability of pH-sensitive cyanine dye conjugated to a cyclic arginine-glycine-aspartic acid (cRGD) to detect primary and metastatic breast tumors with high sensitivity and specificity [271].

14.4.2.5 *Fluorescent Polymers*

A large number of light emitting polymers have been discovered over the last two decades [272, 273]. However, none of them have been translated in biomedical applications. These polymers typically contain complex conjugated systems of multiple benzene rings with nondegradable bonds [274] and unknown toxicity to living cells. Researchers have only recently realized the potential of fluorescent polymers for drug delivery purposes. Polymers such as poly(amino ester)s (PAMAM) [275] and biodegradable photoluminescent polymer (BPLP) [276] are some of the more interesting biodegradable polymers that show inherent fluorescent properties. As discussed earlier, polymeric materials can be fabricated into numerous types of nanostructures and are superior in formulating drug delivery vehicles. Thus, the use of fluorescent polymers to fabricate these nanostructures can achieve additional visualization capability within the same nanostructures. Traditionally, drug delivery vehicles possessing therapeutic and diagnostic functions (theranostic system) are fabricated by conjugation or encapsulation of a drug molecule and fluorescent molecules [235]. However, this methodology suffers for numerous limitations, such as tedious multiple fabrication steps, nonhomogeneous distribution of drug and fluorescent molecules to each particle, low loading efficiency for each molecule, low dye-to-particle ratio and available surface functionalities for further conjugation with targeting units.

Polymers such as BPLPs [276], which have demonstrated high quantum yields, intrinsic bright and tunable fluorescence (up to near infrared) without adding any organic dyes and QDs, photobleaching resistance, cytocompatibility and pendent functionalities can be synthesized as hydrophobic, hydrophilic and amphiphilic polymers by simple and cost-effective one-pot-polycondensation reactions. BPLP polymers can be synthesized by reacting citric acid with any α -amino acid in presence of any aliphatic diol to create a fluorescent, low-molecular-chain polymer. These fluorescent chains can be further crosslinked into thin film or fabricated into nanoparticles. The primary advantage of the BPLP system is that it is strictly composed of metabolites and aliphatic diols and can be biodegraded back to the respective monomers (citric acid and amino acids). The major concern for existing contrast agents is the creation of toxic degradation compounds, which are incompatible with human use. The BPLP system may ameliorate these toxicity concerns. In our laboratory, we are investigating the utility of versatile BPLPs to formulate next generation theranostic nanoparticles.

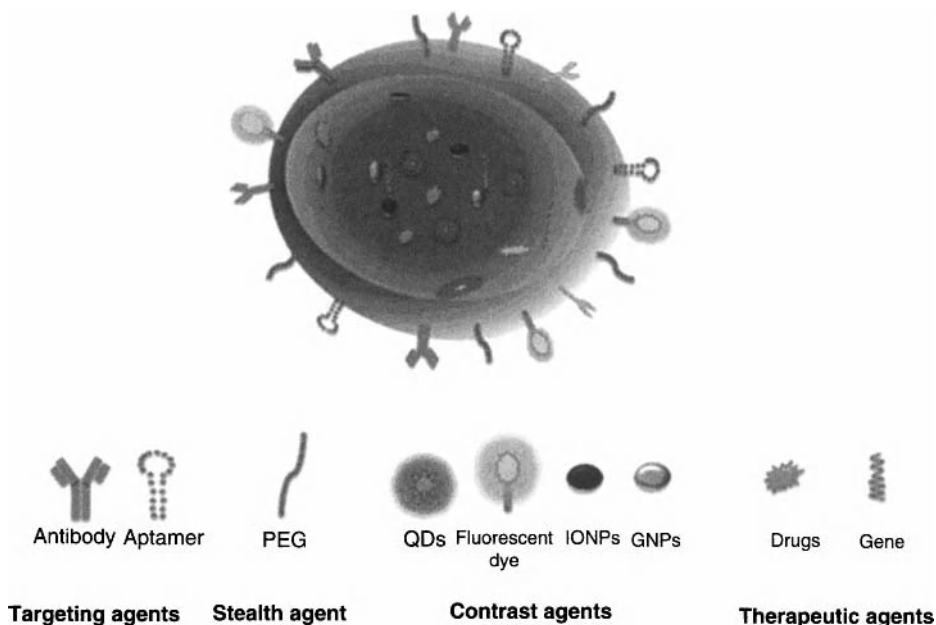


Figure 14.3. Theranostic nanoparticle. The theranostic nanoparticle has the capability to simultaneously carry therapeutic agents (drugs, gene or other pharmaceutical agents), targeting agents (antibodies, aptamer or other recognition agents), contrast agents (QDs, fluorescent dyes, IONPs, GNPs or other imaging agents) and stealth agents (PEG or other hydrophilic polymers).

14.5 Theranostic Systems and Applications

As the potential for nanoparticles in biomedical applications, such as drug delivery and imaging, continues to expand, researchers are intensely investigating the development of an imaging system with therapeutic intervention via a targeting delivering system (Figure 14.3). One theranostic design approach is to integrate existing nanomaterials or imaging agents for simultaneous drug delivery and imaging [235]. A recent approach employs intrinsically fluorescent polymers to create visually identifiable drug carrying vehicles.

14.5.1 Polymeric Nanoparticle-based Theranostic System

Polymers are the first line materials for multifunctional nanoparticle and theranostic systems. The versatile functions of polymers allow for coating, encapsulation and conjugation to any contrast agent and therapeutic agent, in combination with targeting moieties within the same system. Researchers are also developing fluorescent polymers as theranostic system. For example, Choi and coworkers reported that folate-targeted PAMAM dendrimers can release DOX molecules in the presence of light [277]. In another study, Feng and coworkers characterized a self-assembly fluorescent cationic polyfluorene polymer with DOX conjugated anionic poly(L-glutamic acid) polymer. In a

nanoassembly system (50 nm), the fluorescence is in a quenched state due to an electron transfer mechanism. Upon hydrolyses of the anionic polymer the system disintegrates, releasing DOX molecules and exciting PFO to monitor the DOX release [278].

In our laboratory, we are currently designing a theranostic system based upon biodegradable photoluminescent polymers (BPLPs). The BPLP system can also be incorporated into any other polymeric system to provide an innate fluorescence property and can be completely degraded into its starting monomers (citric acid and amino acid). This ability of BPLP to degrade into cyto-compatible monomers may solve the issue of toxicity accompanied with most of the imaging agents. In a recent study, we formulated an amphiphilic copolymer (ABPLP) based on BPLP (hydrophobic block) and PEG (hydrophilic block). ABPLPs demonstrated unique intrinsic fluorescence that can be visible *in vivo* and able to self-aggregate into nanostructures (average diameter of 50–120 nm) encapsulating anticancer drug (paclitaxel). Upon incubation of micelles with cells, they were rapidly internalized with minimal toxicity (below 5%), but induced cell death (80%) upon paclitaxel delivery over a 5 day period. This micelle system can potentially serve as an *in vivo* safe theranostic nanodevice for cancer management. In a separate study, we have also synthesized hydrophilic BPLP containing radical liable bonds. These BPLPs can be photo-crosslinked into fluorescent nanogels (80–200 nm) and further loaded with anticancer drug (5-fluorouracil). These nanogels also possess rich surface functional groups (carboxylic groups) that can be further functionalized with targeting molecules. *In vitro* studies suggested that these nanogels demonstrated effective delivery of drugs to cancer cells inducing significant cytotoxicity (92%) over 48 h.

Apart from these intrinsically luminescent polymeric systems, any other polymeric system can be conjugated to, or encapsulated with, existing fluorescent dyes to acquire imaging ability within a polymeric nanoparticle system. In fact, dyes such as FITC, Cy7, Cy5, and ICG are actively used in monitoring drug release from nanoparticles and detecting various tumor locations [267]. In addition, polymeric systems are also used for coating or surface modifying other contrast agents such as QDs and IONPs to construct theranostic systems that will be discussed in a later section.

14.5.2 QD-based Theranostic System

Another application of QDs is the integration of therapeutic interventions, also known as theranostic QDs [279]. The innate toxicity of QDs has inspired researchers to coat the particles with polymeric materials, thereby reducing toxicity, prolonging the circulation time, and diversifying the surface chemistry for further modifications. In addition to this, the coated polymeric shell can also be loaded with drug molecules to induce therapeutic effects within these contrast agents as tabulated in Table 14.2. QD drug delivery systems were first reported by Lai and coworkers, where they used CdS particles to cap pores of mesoporous silica particles after encapsulating drug molecules

Table 14.1 Examples of formulation methods used for fabricating polymeric nanoparticles for drug delivery.

Polymer	Formulation Method	Drug Type	Reference
PLGA	Solvent evaporation Nano precipitation Emulsion diffusion Solvent displacement	Paclitaxel, Doxorubicin, 5-fluorouracil, 9-nitrocamptothecin, Cisplatin, triptorelin, dexamethasone, xanthone, Estradiol, Docetaxel, 2-Aminochromone, Thymopentin, Etanidazole, Insulin, Haloperidol, Tetanus drug	[159–167]
PLA	Solvent evaporation Emulsion diffusion Double emulsion Salting out Spray drying	Dexamethasone, Hemoglobin, Ellagic acid, Haloperidol, Savoxepine, Tyrphostins, Progesterone, Vinblastin, Insulin, Oridonin, BSA, Neurotoxin-1, Protein-C	[34, 168–175]
PCL	Solvent evaporation Nano precipitation Emulsion diffusion Solvent displacement Micelles	Tamoxifen, Taxol, Insulin, Clonazepam Amphotericin B, Saquinavir, Docetaxel, Vinblastine	[31, 176–181]
PACA	Anionic polymerization	Ampicillin, Indomethacin, Ftorafu, Doxorubicin, 5-Fluorouracil	[182–186]
Chitosan	Ionic gelation, Desolvation, Emulsion-droplet coalescence, Reverse micellar, Self-assembly	Glycyrrhizin, Insulin, Cyclosporin, BSA	[187–190]
Alginate	Solvent evaporation, Emulsion diffusion, Double emulsion, Salting out, Spray drying	5-fluorouracil, doxorubicin, antisense oligonucleotide, Insulin, isoniazid, rifampicin, pyrazinamide, ethambutol, econazole,	[128, 191–195]

PNIPA-based	Chemical crosslinking, Free radical initiating, Seeding and feed- ing precipitation polymerization, Atom transfer radical polymerization	Dextran, clonazepam, naltrexone	[178, 181, 196–199]
PVA-based	Chemical crosslink- ing agents, electron beam, γ -irradiation, Water-in-oil emul- sion/cyclic freezing–thawing	human serumalbumin, teta- nous toxoid and cytochrom C, ibuprofen, nandolo, propranolol, and tacrine	[30, 200, 201]

and neurotransmitters. In response to stimuli, these caps open and release the drugs. A decrease in the fluorescence intensity of CdS particles within the silica particles confirms the release of the drug's molecules [280]. Nurunnabi and coworkers formulated a QD-based Herceptin conjugated micelle system for cancer therapy and imaging. This theranostic system achieved significant reduction of tumor size and good visualization of the tumor site, thereby allowing both treatment and monitoring of the tumor [281].

In another study, Wu and coworkers immobilized QDs in polysaccharide-based nanogels, which could perform optical pH sensing, tumor cell imaging, and anticancer drug (temozolomide) delivery [282]. Park and coworkers reported a PEGylated phospholipid micelle system that co-encapsulate QDs and IONPs, along with DOX, and further conjugated a tumor homing peptide for simultaneous magnetofluorescent imaging and drug delivery [283]. Recently, Xu and coworkers used Mn-doped ZnS QDs (core) and glycopolypeptides (shell) to deliver an anti-inflammatory drug (ibuprofen). This study, however, did not utilize the optical properties of QDs [284]. Bagalkot and coworkers reported an interesting variant of the theranostic system composed of QDs conjugated to an aptamer (Apt), loaded with DOX molecules as intercalating agents. In this nanosystem, the fluorescent properties of both QDs and DOX, intercalated within the aptamer sequence, were both in a quenched state. However, commensurate with the gradual release of DOX molecules at the targeted tumor cells, QDs recovered fluorescence, signaling the delivery of the anticancer drug [285]. A similar photoluminescent quenching effect was also reported by Yuan and coworkers when they conjugated mitoxantrone (MTX) onto QD surfaces. The photoluminescent property of QDs can be restored when this nanosystem is exposed to a species with higher affinity (DNA) for MTX [286].

Table 14.2 Examples of QD-based theranostic system.

Drug loading dock	Imaging Agents	Therapeutic	Targeting Agent	Targeted Disease	Ref
Chitosan	QDs	siRNA	HER2 antibody	breast cancer cells	[287]
Poly(ethylene glycol)-phospholipid	QDs/iron oxide nanoparticles	Doxorubicin	Tumor-homing peptides (F3)	MDA-MB-435 human cancer cells	[283]
Poly(ethylene glycol)	quantum dot	siRNA	Tumor-homing peptides (F3)	HeLa cells	[288]
Amphipol	QDs	siRNA	Her-2/neu antibody	SK-BR-3 cells	[289]
Aextran-conjugated poly(l-alanine)	QDs	Ibuprofen	Passive	HEK293 cell lines	[284]
A10 RNA aptamer	QDs/doxorubicin	Doxorubicin	A10 RNA aptamer	Prostate cancer cells	[285]
Methoxy poly(ethylene glycol) pentacosyonic acid	QDs	Herceptin	Herceptin	MDA-MB-231 tumors	[281]
Hydroxy-propylcellulose-poly(acrylic acid)	QDs	Temozolomide	Passive	Melanoma B16F10 cells	[282]

14.5.3 Colloidal Gold-particle-based Theranostic System

Due to their intrinsic diagnostic ability, gold nanoparticles can also be incorporated into theranostic systems. In recent years, researchers have reported the therapeutic ability of GNPs such as anticancer drug delivery, gene delivery and photothermal therapy (Table 14.3). Therapeutic GNPs are an attractive approach due to their unique characteristics such as strong surface Plasmon absorption, stability, biosafety, and ease of modification [235]. In fact, a PEG-modified gold-nanoparticle-based drug delivery system, developed under the trade name of Aurimune (CYT-6091) to deliver recombinant human tumor necrosis factor alpha (TNF) (tumor-killing agent), is already showing

promising results in Phase I clinical trials. Paciotti and coworkers first reported the formulation of this system and demonstrated rapid accumulation of these particles in colon carcinoma tumors without significant accumulation in the liver, spleen, and other healthy organs of the animals [290]. Further, this conjugate system shows maximal antitumor responses with lower drug doses, proving its efficacy over native TNF vector. Subsequently, Phase I data demonstrate that delivery of TNF in humans could achieve concentrations far beyond what was attainable in previous studies along with manageable side effects. The particles accumulated in and around tumor sites, avoided uptake by healthy tissues and immune system detection [291].

Other studies have investigated anticancer drug delivery to the tumor sites. Modified PTX conjugated with GNPs [292], Methotrexate conjugated GNPs [293], and DOX conjugated GNPs [294] are some of the chemotherapeutics developed in recent years to target various cancers. Park and coworkers reported another interesting use of GNPs in tumor therapy. Park demonstrated the efficacy of a paired-nanoparticles system that can work together to detect a diseased site and more effectively deliver chemotherapeutics to the site than individual nanoparticle treatments. In this system, the first nanoparticles composed of Cetyltrimethylammonium bromide (CTAB)-coated gold nanorods coated with a mixed monolayer of poly(ethylene glycol) (PEG) and SERS-active reporter molecules were directed to the tumor site. Once these particles entered the tumor zone they were subjected to photothermal heating, selectively at the tumor site, by excitation with an 810 nm laser. Next, a thermally sensitive liposome and micelles carrying DOX molecules were delivered to the tumor site where selective drug accumulation was observed without systemic toxicity [295].

Numerous other photothermal therapies using GNPs take advantage of the unique surface plasmon resonance (SPR). It was found that: 1) Elevated temperature at the tumor region is capable of inducing irreversible tissue damage that cannot be achieved by GNR-free NIR light exposures [296]; 2) the absorbance wavelength (in the visible range) of small gold nanospheres is not optimal for *in vivo* applications, so particles that attain SPR peaks in the NIR region either by clustering small diameter particles [297], large (100–300 nm) nanoparticles or different shapes of particles (nanorod, nanocage, and nanoshell) [235] should be used; 3) as many as 5000 gold nanoshells per prostate cancer cell are needed to achieve cell death [298], and; 4) monocytes can be recruited into hypoxic regions within tumors induced by gold nanoshells [299]. Although photothermal technique sounds promising in cancer therapy, future investigation and optimization are required.

14.5.4 Iron-oxide-based Theranostic Systems

IONPSs coated with other polymeric materials are easily coupled with drug molecules to provide therapeutic intervention while retaining their diagnostic capability. Many types of chemotherapeutic agents, proteins, peptides, DNA and siRNA can be stored in the IONPS particles to achieve synergic effects

Table 14.3 Examples of GNP-based theranostic system.

Drug docking zone	Imaging agent	Therapeutic	Targeting molecules	Cellular target	Reference
Poly(ethylene glycol)	GNPs	PTX	Tumor necrosis factor	Tumor	[300]
Poly ethylenimine)	GNPs	Plasmid DNA	Passive	monkey kidney (COS-7) cells	[301]
Methoxy Poly(ethylene glycol)	Fluorescein/ GNPs	Photo-thermal	Tumor homing ligand (NDP-MSH)	MC1R-positive B16/F10 tumors	[302]
Gold particles	GNPs	Photo-thermal	Anti-epidermal growth factor receptor antibody	A431 tumor	[303]
Poly(l-aspartate)/ poly(ethylene glycol)	GNPs/ doxorubicin	Doxo-rubicin	Folic acid	4T1 mouse mammary carcinoma cells	[294]
Cationic lipid bilayer	GNPs	Plasmid DNA	Passive	human embryonic kidney cells (HEK 293)	[304]
Thiol-derivatized PEG	GNPs	Tumor necrosis factor	Passive	MC-38 colon carcinoma tumors	[305]
Gold particles	GNPs	Methotrexate (MTX)	Passive	Lewis lung carcinoma (LL2)	[306]

(Table 14.4). Anticancer drugs such as doxorubicin, paclitaxel, methotrexate, cisplatin, and gemcitabine are encapsulated or linked to IONPSs for potential diagnosis and treatment of various tumors. Kohler and coworkers formulated biostable methotrexate-immobilized iron oxide nanoparticle drug carrier coated with PEG for the real-time monitoring of drug delivery through MRI. The controlled release of MTX from the conjugated IONPS system in response to the pH changes and in the presence of lysozymes was also evaluated [307, 308]. In another study, Jian and coworkers evaluated efficiency of doxorubicin and paclitaxel (alone or in combination) loaded IONPS coated with oleic acid and pluronic. They also evaluated the MRI signal intensity of this system in the carotid arteries of mice, and reported superior sensitivity in terms of T_1 and T_2 relaxivity [309]. Piao and coworkers developed porous IONPS nanostructures and demonstrated successful loading of DOX molecules [310]. Chen and coworkers modified this technique to create porous IONPs loaded with cisplatin and coupled with Herceptin to confer targeting specificity. As a result, this

Table 14.4 Examples of IONPs-based theranostic system.

Drug docking zone	Imaging agent	Therapeutic	Targeting molecules	Targeted tissue	Reference
IONPs	IONPs	Doxorubicin	Passive	Lewis lung carcinoma	[314]
IONPs	IONPs	Cisplatin/ Herceptin	Herceptin	ErbB2/ Neu-positive breast cancer cells	[315]
IONPs	IONPs	Epirubicin	Magnetic field	Various sarcomas	[316]
Poly (ethylenimine)	IONPs	LacZ gene	Magnetic field	Ileum lamina propria	[317]
IONPs	IONPs	Antisense oligodeoxy-nucleotides	Magnetic field	Cremaster vessels	[318]
Liposomes	IONPs	Silencing RNA	Magnetic field	Gastric tumours	[319]
Cationic Liposomes	IONPs	Hyperthermia	Direct injection	Solid glioma tissue	[312]

IONPS system demonstrated selective affinity towards ErbB2/Neu-positive breast cancer cells and provided sustained cytotoxicity [311].

Another interesting therapy that can be aided with IONPS is hyperthermia. Yanase and coworkers demonstrated that the Fab fragment from an anti-human MN antigen-specific antibody, anchored phospholipid-coated IONPs administered systemically, and significantly improved tumor uptake. When exposed to an AMF, the temperature of the tumor was elevated above 43°C within 15 min, resulting in tumor regression but no effect on the control group (no use of IONPs) [312]. Recently, this hyperthermia effect was further enhanced by Primo and coworkers with Zn-Pc (a photodynamic therapeutic [PDT] agent) loaded IONPSs. A synergistic toxic effect was observed in J774-A1 macrophage cells from both PDT and magnetohyperthermia [313].

14.6 Future Outlook

In the late 19th century, Nobel laureate, Paul Ehrlich, coined the phrase “magic bullet,” to describe a drug that selectively attacked diseased cells without harming healthy cells. Over the past several decades, scientists have investigated and developed numerous drug types and delivery systems, but have failed to achieve a magic bullet. However, within the past two decades, scientists have revolutionized therapy systems by taking a “plenty of room at the bottom” approach. One example of this breakthrough is the use of nanomaterials that can selectively deliver therapeutic and diagnostic agents specifically to the diseased site. As this technology has matured, the need for simultaneous, noninvasive quantification of the diseased site and an individualized pharmacotherapy system has become apparent. This, in turn, has driven the development of theranostic systems.

Theranostic nanoparticle system development is still in its infancy; consequently, there remains much to investigate. From the perspective of the biomaterial scientist, it is important to characterize the safety of the biomaterial before designing a theranostic nanosystem to carry therapeutics. Future studies must focus on characterizing biomaterials with novel chemistry to impart smart functions such as stimuli sensitivity and tissue/cell-specific targeting to theranostic nanoparticle systems. Theranostic nanomedicine is at the intersections of cancer biology, diagnostic imaging, and nanobiomaterials. Integrated with the evolving understanding of cancer biology, the explosive development on nanobiomaterials and diagnostic imaging/instrumentation will be predominant in theranostic nanomedicine for the treatment of cancers and other fatal diseases.

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Intelligent Drug Delivery Systems for Cancer Therapy

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Abstract

Most cancer chemotherapies cause severe side effects due mainly to the toxicity of the drugs to normal healthy cells. Conventional drugs lack satisfactory specificity towards cancer cells, and have difficulties overcoming biological barriers; this highlights the importance of developing safe and effective delivery systems. Opportunities for the design of intelligent drug delivery vehicles are provided by the unique physiological properties of tumor tissues and the overexpression of specific receptors in tumor cells and neovasculature. These delivery vehicles include peptides, polymers and lipids. Targeting ligands, such as cell targeting peptides, have been used to decorate delivery vehicles in order to guide the therapeutic or diagnostic agents to tumor sites and enhance their efficacy. Recently, attention has been drawn to bacteria-mediated cancer therapy. This approach targets the tumor environment by taking advantage of the wide flexibility of bacterial genetics. This chapter focuses on recent progress in drug delivery approaches in cancer therapy.

Keywords: Cancer, drug delivery, targeting, peptide, lipid, polymer, bacteria

15.1 Introduction

With almost 13 million new cases and 7.6 million deaths each year, cancer is a leading cause of death worldwide. The slow progress in cancer treatment has caused a growing demand for novel approaches to the effective and targeted delivery of therapeutics. Several drug delivery systems have been developed to overcome the delivery obstacles, but only a few have proven sufficiently promising that they have been developed into clinical applications. Recent advances in engineering, material science, physics and chemistry have

produced novel materials and devices in nanoscale (1–100nm) proportions. The unique physical and chemical properties of nanomaterials allows them to be used in ways much different than bulk materials. Nanomaterials that are found naturally as components in biological systems are nucleic acids, lipids, proteins, carbohydrates and surface receptors and other nanosized materials, which can easily interact with similar biological components in the system. Thus nanotechnology platforms based on various nanomaterials are being developed for medical applications. Many nanobiomaterials have been developed as carriers for therapeutic agents. The most common examples of nanotechnology platforms for cancer therapy include polymeric nanoparticles, liposomes, dendrimers, nanoshells, carbon nanotubes, and superparamagnetic nanoparticles [1].

The design of effective delivery vehicles plays a crucial role in the development of intelligent drug delivery systems for clinical use. Some factors that are determinant in obtaining an ideal delivery system are biocompatibility, biodegradability, surface charge, size, circulation time, loading capacity, and targeting. The development of drug delivery systems for cancer treatment has focused on the following three strategies: (i) targeted delivery, where surface ligands are introduced to enhance nanoparticle accumulation in cancerous tissue, (ii) triggered release: nanoparticles sensitive to external stimuli such as light or ultrasound, or nanoparticles sensitive to changes in the microenvironment in cancerous tissue such as enzymes or pH, and (iii) passive drug release from the nanoplatform on accumulation in the diseased tissue due to the enhanced permeability and retention (EPR) effect [2].

In addition to carrier-mediated drug delivery platforms, biologically-based delivery systems are also being developed. Bacteria-mediated tumor therapy is a quickly growing research area that offers an effective approach for treatment of solid tumors. Taking advantage of the unique physiological conditions of solid tumors, genetically engineered bacteria can be designed to carry, deliver, release, and activate prodrugs to targeted tumors.

In this chapter, we will discuss the major advantages, challenges and shortcomings of different carrier-mediated and bacteria-mediated drug/gene delivery systems for cancer therapy.

15.2 Peptides for Nucleic Acid and Drug Delivery in Cancer Therapy

15.2.1 Self-assembling Peptides as Carriers for Anticancer Drugs

Because of their biocompatibility, small size and relative stability, self-assembling peptides are emerging as potential carriers for anticancer drug delivery. Peptide sequence can be manipulated to yield a variety of stable nanostructures such as fibers, tubes, rods, and nanovesicles which can be used as models of molecular self-organization [3]. Another important feature of peptide systems that render them intelligent biomaterials is their specific response to local environmental

stimuli (e.g., temperature, pH, ionic strength, and light), caused by their unique physicochemical properties [4].

Over the past decade, Chen *et al.* have studied and characterized peptide-peptide and peptide-drug interactions. Peptide-peptide assembly can be achieved through noncovalent interactions such as ionic bond formation, hydrophobic interactions, hydrogen bonding and π -stacking [4]. A special class of self-assembling peptides, the ionic-complementary peptides, has shown great promise for drug delivery. Fung *et al.* investigated the capability of the self-assembling peptide EAK16-II to deliver a hydrophobic anticancer drug. Their results confirmed the ability of the EAK16-II peptide to stabilize the hydrophobic anticancer drug Ellipticine in an aqueous solution. They also showed that the molecular state of Ellipticine (protonated or crystalline) in solution is dependent on the concentration of EAK16-II. The anticancer activity of peptide-ellipticine complexes was determined *in vitro* on two cancer cell lines, A549 and MCF-7 [5]. More recently, they have designed and investigated the physicochemical properties and anticancer drug delivery potential of an amino acid pairing peptide (AAP8) as a novel self-assembling peptide [6]. Their study holds promise for future development of peptide-mediated delivery of chemotherapeutics [5, 7].

15.2.2 Different Classes of Peptides Used in Gene Delivery

Over the past two decades, peptides have been employed to deliver nucleic acids (NAs), by mechanisms that still remain controversial [8–11]. These peptides include cationic peptides [12], protein-derived cell penetrating peptides (CPPs) [9], designed amphiphilic peptides [13], cell targeting peptides (CTP) [14], endosomolytic peptides [15], fusogenic peptides [16], and nuclear localization peptides [17]. Because of their diversity and versatility in design, which takes advantage of the physicochemical properties of different amino acids, peptides might be the only biomaterials which can actually play all the roles required to safely and efficiently deliver genes to their target sites. The designed peptides must be able to (i) condense NAs into compact nanoparticles; (ii) target the complex to cell or tissue-specific receptors; (iii) penetrate through the cell membrane; (iv) induce endosomal escape; and (v) release the NA cargo at the target site. Cationic peptides rich in basic amino acids can electrostatically interact with small NAs or condense DNA into small stable particles. CPPs can facilitate the translocation of the complex through the cell membrane. Endosomolytic and fusogenic peptides can enhance the endosomal escape and cytoplasmic release of the gene complex. Decorating the gene delivery system with CTPs mediates cell and/or tissue-specific targeting. Finally, attachment of a nuclear localization sequence (NLS) improves nuclear localization of the condensed DNA. Lee *et al.* reported a polypeptide-based targeted gene delivery system consisting of (i) a cationic peptide for DNA condensation, (ii) a ligand peptide for epidermal growth factor receptor (EGFR) recognition, and (iii) an endosome-releasing peptide for endosomolysis [18]. This gene delivery platform successfully delivered the p53 gene into the Huh-7 cells, causing

significant inhibition of growth. As another example, a multifunctional peptide-poly(ethylene glycol) (PEG) intercalating conjugate was developed as a novel gene delivery system to target the blood-brain barrier. Four peptides with different functionalities were employed in this system: TAT, as DNA condensing and cell penetrating peptide; NLS, as nuclear localization sequence; and brain-homing (BH), and apolipoprotein E receptor-specific peptides for targeting the brain tissue [19].

Most peptides used in gene delivery systems are net positively charged. The main obstacle of using peptide-based carriers *in vivo* is their cationic nature which causes aggregation with negatively charged biomolecules in the blood stream. Several modifications, e.g., PEGylation have been made to achieve safer and more efficient delivery carriers *in vivo*. Harris *et al.* developed poly(glutamic acid)-based peptide coatings to change the exterior composition of a gene delivery nanoparticle. This modification not only reduced the toxicity of nanoparticle but also mediated specific gene delivery to the liver or to the spleen and bone marrow, depending on the coating surface density [20].

Table 15.1 shows some examples of peptides employed in NA delivery purposes. For in-depth reviews, see Refs. [15, 21].

Table 15.1 Some peptide sequences used in gene therapy.

Peptide	Sequence	Ref.
<i>Protein-derived CPPs</i>		
Penetratin (43-58)	RQIKIWFQNRRMKWKK	[22]
Tat (48-60)	GRKKRRQRRPPQ	[23]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	[24]
R9	RRRRRRRRR	[25]
E5WYG	GLFEAIAEFIEGGWEGWEGWYG	[26]
HA	GLFEAIAAGFIENGWEGMIDG	[27]
bPrPp	MVKSKIGSWILVLFVAMWSDVGLCKKRPKP	[28]
gp41 fusion	GALFLGWLGAAGSTMGA	[29]
TP10	AGYLLGKINLKALAALAKKIL	[30]
pVEC	LLIILRRRIRKQAHAAHSK	[31]
<i>Designed amphiphilic CPPs</i>		
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	[32]
MPG ^{ΔNLS}	GALFLGFLGAAGSTMGAWSQPKSKRKV	[33]
MPG8	AFLGWLGAWGTMGWSPKKKRK	[34]

CADY	GLWRALWRLRLSLWRLLWRA	[35]
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	[36]
SP	MGLGLHLLVLAAALQGAWSQPKKKRKV	[37]
SP-NLS	MGLGLHLLLAALQGAKKKRKV	[38]
MAP	KLALKLALKALKAALKLA	[13]
hCT-K7	KFHTFPQTAIGVGAPKKKKKKK	[39]
<i>Cell targeting peptides</i>		
RGD	ACDCRGDCFCG	[40]
NGR	CNGRCVSGCAGRC	[41]
Leukemia cancer targeting	CAYHRLRRC	[42]
VEGF targeting	ATWLRRR, RRKRRR	[43, 44]
Ovarian cancer targeting	GTRIIYDRKFLMECRNSPVT	[45]
Breast-homing	CPGPEGAGC, CGNKRTRGC	[46]
Prostate targeting	CVSYCLFEFCYVC, CVFTSDYAFC	[47]
Lung targeting	CGFECVRQCPERC	[48]
<i>Nuclear localization peptides</i>		
SV-40 T antigen	PKKKRKV	[49]
SV-40 Vp3	KKKRK	[50]
Xenopus nucleoplasmin	KRPAATKKAGQAKKKK	[51]
Human c-myc	RQRRNELKRSP, PAAKRVKLD	[19]

15.2.3 Protein-derived and Designed CPPs

Tat and Penetratin are the most widely studied peptides among all protein-derived CPPs. Discovered by Frankel and Pabo in 1988, Trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) has been shown to be efficiently taken up by several cell types in culture [52]. It has been successfully used to deliver oligonucleotides [53], plasmid [54], and proteins [55].

Penetratin is a 16-mer peptide, derived from the third helix of Antennapedia (a membrane transduction protein [56]). It has been widely applied in gene

delivery [57, 58]. At endosomal pH, Penetratin adopts an α -helical secondary structure that can facilitate the peptide interaction with the endosomal membrane and eventually cause membrane disruption and pore formation [15].

To take advantage of the amphiphilic nature of the cell membrane, some peptides have been designed to include both hydrophilic and hydrophobic moieties. The hydrophilic domain can interact with hydrophilic drugs, genes and the hydrophilic heads of the lipid bilayer; while the hydrophobic domain is anchored in the hydrophobic core of the bilayer, mediating the translocation of peptide-cargo complex to the cytosol.

The MPG peptide was one of the first designed amphiphilic peptides successfully used for nucleic acid delivery. MPG includes a hydrophilic part (an NLS of Simian virus 40 large T-antigen), a four amino acid linker, and a hydrophobic domain, derived from an HIV glycoprotein 41 fusion sequence [32]. The positively charged NLS domain mediates the electrostatic interaction of the peptide with NAs, while the peptide-peptide assembly through hydrophobic domains provides a shield around NAs which protects NAs against degradation and improves the complex stability. A single lysine to serine residue mutation in NLS sequence significantly reduced the nuclear localization of NAs, highlighting the importance of the NLS in targeting the nucleus. The resulting mutated peptide, MPG^{NLS} (MPG with mutated NLS), has shown high efficiency in siRNA delivery, for which the target site is the cytoplasm. MPG-family mediated delivery of siRNA has been shown to cause significant gene knock-down both *in vitro* [33, 34, 59, 60] and *in vivo* [9]. More recently, The MPG-8 and CADY peptides were reported to have high siRNA delivery efficiency in different cell lines [35, 61].

15.2.4 Cell Targeting Peptides

Because of their biocompatibility and small size, cell-targeting peptides (CTPs) are emerging as alternatives to monoclonal antibodies for targeting purposes. These peptides can localize the decorated complex at the target site by directly binding to cell surface receptors or the endothelial cell surface of tumor vasculatures. Among all CTPs, a tripeptide RGD (Arginine, Glycine, Aspartate), is the most studied peptide. RGD is a short segment of Fibronectin protein which has shown high affinity to most integrins, the cell surface glycoproteins overexpressed in neovasculature of tumor sites [62]. The conformation of the RGD sequence plays an important role in its selectivity for a specific integrin. The cyclization of RGD, as the main way to constrain this conformation and gain more rigid structure, has been shown to significantly improve the affinity of peptide for integrins, compared to the linear RGD [63].

Several cell or tissue specific peptide ligands have been reported in the literature. Wood *et al.* identified and employed a peptide ligand (WIFPWQL) which was capable of selectively targeting glucose-regulated protein-78 kDa (GRP-78), a functional tumor antigen identified in human cancer patients. They synthesized and used a hybrid polymer system, including this peptide ligand, in tumor-targeted gene delivery [64]. A peptide ligand (ASSLNIA) that targets a

muscle-specific receptor was used for targeted antisense oligonucleotide delivery. When conjugated to oligonucleotides, the T9 peptide (SKTFNTHPQSTP) displayed increased specificity for the heart and quadriceps muscles as compared with the kidney, liver, and diaphragm [65]. Liposomes decorated with The RGD and GGP (GGPNLTGRW) peptides, to target neutrophils and monocytes, showed high drug/gene delivery efficacy to the brain [66, 67]. Examples of other CTPs have been listed in Table 15.1.

15.2.5 Nuclear Localization Peptides

The nuclear pore complex (NPC) is believed to serve as the sole gateway to the nucleus. However, the inner diameter of the NPC is too small for passive diffusion of condensed DNA [68]. The nuclear localization peptides have been developed to efficiently translocate therapeutic DNA from the cytoplasm into the nucleus. These peptides either directly bind to the DNA or decorate the gene delivery complex so that the resulting complex can be recognized as a nuclear import substrate by intracellular receptor proteins [69]. Due to their net positive charge, the NLS peptides can also electrostatically interact with DNA without another condensing agent such as CPPs. The most used NLS in gene delivery is the one derived from Simian virus 40 large T-antigen with a sequence of PKKKRKV. More examples of NLSs used in NA delivery are shown in Table 15.1.

15.3 Lipid Carriers

A range of lipid-based carriers have been explored. Liposomes, solid lipid NP's (SLN) and nanostructured lipids (NSL) have been investigated extensively, and many lipid-based formulations are in clinical use. The use of lipid carriers for drug delivery has been extensively reviewed [70]. Modifications to lipid carriers have given rise to various types of designer lipid molecules. Cationic lipids are used to produce lipoplexes—positively charged lipids associated with negatively charged DNA—a successful tool for gene transfection. Drug-loaded liposomes can be targeted to specific sites by linking tumor-targeting agents (e.g., antibody fragments or small molecules) to the lipid. The tumor-targeting liposome nanoimmunocomplex, prepared using GMC (an anticancer small-molecule quinazolinone analogue), encapsulated within a cationic liposome and surface-decorated with an anti-transferrin receptor single chain antibody fragment (TfRscFv) showed improved cytotoxicity of GMC due to specific targeting [71].

15.3.1 Liposomes

Liposomes are phospholipid-containing vesicles that can encapsulate therapeutics for efficient delivery. A number of liposomal formulations are in clinical applications and the list is growing, with many in phase III clinical trials.

The first clinically approved liposomal drug formulation, DOXIL, is a treatment for breast cancer, advanced ovarian cancer, multiple myeloma and AIDS-related Kaposi's sarcoma [72]. Others now in use are Marqib-Vincristine sulphate liposome injection, for which the liposome consists of a sphingomyelin bilayer [73]; DuanoXome, a duanorubicin citrate liposome injection [74]; and DepoCyt, a liposomal cytarabine formulation [75]. These are used in the treatment of acute lymphoblastic leukemia, advanced HIV-associated Kaposi's sarcoma and lymphomatous meningitis respectively. The inclusion of PEGylated lipids in the liposomes increases accumulation of the drug in the tumor by avoiding the reticulo-endothelial system.

15.3.2 Modified Liposomes

Modification of phospholipids can result in engineered liposomes with desirable properties. Attaching functional ligands, such as maleimide, to the carrier lipid, allows conjugation of antibodies (for targeting) or polymerizable molecules (for stabilizing liposomes). Modifications of the lipids to modulate *in vivo* circulation have also been attempted. Modified lipids for production of thermosensitive liposomes, photoactivated liposomes and pH-sensitive liposomes have been reported.

Thermosensitive liposomes are stable at body temperature but are destabilized (through a phase transition) above their liquid crystal transition temperature T_c and thus release their cargo. Dipalmitoylphosphatidylcholine (DPPC) has a T_c of 41°C and is the matrix lipid used in construction of thermosensitive liposomes [76]. Smith *et al.* have successfully delivered doxorubicin using thermosensitive liposomes [77]. Chemical alterations to photosensitive segments of modified lipids that would enable release of the payload on light exposure (by several mechanisms) have been reviewed by Yavlovich [78]. Wang *et al.* have reviewed the different phototriggering mechanisms for controlled release from liposomes, with special reference to azobenzene-based drug delivery systems [79].

Liposomes consisting of a mixture of cationic and anionic lipids destabilize when the pH of the surrounding environment changes. Shi *et al.* demonstrated that liposomes composed of egg phosphatidylcholine, dimethyldioctadecylammonium bromide (DDAB), cholesteryl hemisuccinate (CHEMS), and Tween-80 (25:25:49:1, mol/mol), which were stable at pH 7.4, underwent rapid content release and irreversible aggregation under acidic pH [80]. A novel system using sugar-containing Gemini surfactants, which exist in the lamellar phase at neutral pH, can be triggered to convert to a micellar phase at a mildly acid pH, thus releasing their cargo [81]. In a similar study, pH-triggered delivery of fluorescent probes into cells by Diphosphatidylethanolamine (DOPE) liposomes containing a bacterial rhamnolipid has been demonstrated, and may be a candidate for drug delivery [82]. Andresen has reported the use of enzymes for drug release from liposomes. Liposomes containing modified enzyme-sensitive lipids were prepared; overexpression of these enzymes caused the liposomes to release their payload [2].

Nanostructured aqueous dispersions of liquid crystalline particles have attractive applications as carriers for drug delivery [83]. These dispersions with lipids and polymers form cubosomes and hexosomes. When compared to liposomes, these structures are capable of solubilizing higher amounts of amphiphilic, hydrophobic, and hydrophilic drugs in their highly ordered self-assembled interiors. Liquid crystal nanoparticles based on phospholipids as carriers of docetaxel have shown better penetration/absorption compared to Taxotere, a commercially available micellar formulation of docetaxel [84].

Liposome constituents, especially PEG-lipopolymers (which seem to absorb ultrasonic energy) and molecules that compromise the close packing of lipids in the bilayer, have a large effect on increasing ultrasonic responsivity and may be used for ultrasound-induced drug release from liposomes [85].

15.3.3 Targeted Lipid Carriers

Lipid carriers can be modified for drug targeting, for instance, by coating liposomes with antibodies (forming immunoliposomes) or linking the carrier to vitamins, peptides, aptamers or affibodies.

15.3.3.1 Immunoliposomes

Immunoliposomes contain antibodies linked to the liposome that directs it to the target site. Many immunoliposomes make use of monoclonal antibodies directed to specific receptors on target cells as targeting agents [86]. The linking of the antibody to the lipid may be performed in one of two ways. In the first method, the antibody is coupled to a dual-functionalized PEG molecule which is incorporated into the drug-loaded liposome to yield the drug-carrying immunoliposome. This method was used to prepare immuno-Doxil by reacting doxil containing liposomes with pNP-PEG-PE-modified anticancer 2C5 monoclonal antibodies [pNP-PEG-PE = (poly(ethylene glycol)-phosphatidyl ethanolamine conjugate (PEG-PE)] [87].

The second approach involves linking the antibody to a functionalized lipid on the surface of the drug-loaded liposome. Unlike the random incorporation of drug-containing liposomes in the first method, this method is controlled. Kwong *et al.* used a modification of this strategy to couple anti-CD 40 antibody and CpG oligonucleotide to the surface of functionalized PEGylated liposomes [88]. They found that by anchoring these immunostimulatory molecules to liposomes, a more confined biodistribution and enhanced local retention was observed following intratumoral injection, compared to systemic exposure to the same agonists. Another variation, in which pegylated immunolipopolyplexes (PILP) were constructed for gene delivery, is reported by Hu *et al.* [89]. They used DNA/polyethylenimine polyplexes and anionic liposomes to form a complex. This complex was stabilized with PEG bound to biotin, and employed streptavidin-monoclonal antibody conjugating through the biotin group located at the distal end of the PEG spacer as the targeting antibody (see

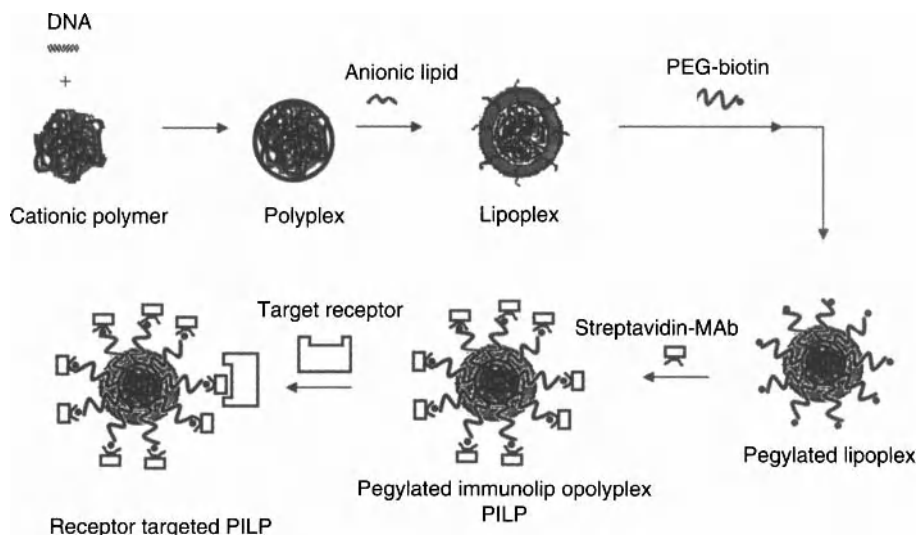


Figure 15.1 Assembly of the pegylated immuno-lipopolyplexes (PILP) composed of PEI/DNA complex, streptavidin-monoclonal antibody (SA/MAB), anionic lipids composed of liposomes.

Fig. 15.1). These complexes showed efficient transfection of liver cancer cells *in vitro* and *in vivo*, as well as decreased cytotoxicity.

15.3.3.2 Affisomes

Affisomes are antibody mimics and consist of small proteins (6–8 kDa) that can have better binding affinities than the corresponding antibodies. Puri *et al.* and Smith *et al.* have used an HER 2 specific affibody as the targeting agent to direct thermosensitive liposomes to their target site [77, 90]. Their studies show that the affibody-conjugated drug-loaded liposomes are more efficient at killing cells than the unconjugated Dox-loaded liposomes (Doxil). The function of these thermosensitive liposomes is sensitive to the nature of the phospholipid composition; lecithin-containing liposomes are not able to release a high percentage of drugs.

15.3.3.3 Receptor Targeting Ligands

Small-molecule receptors that are overexpressed in tumors can be used as targets for drug delivery to cancer cells. One example is the folate receptor (FR), which is upregulated in ovarian, lung, breast, kidney, colon and breast cancers. The vitamin folic acid is required for cell growth; increased expression of FR in tumor cells enhances cell proliferation by internalizing the folic acid. The use of folic acid as a targeting ligand for drug delivery is well documented, and has yielded promising results. Folic acid-linked drug-loaded liposomes have much higher cytotoxicity than untargeted liposomes [91]. Folate conjugates of cytotoxic drugs, such as paclitaxel, maytansinoids, vinca alkaloids and mytomyacin C, have been used for chemotherapy [92]. A potential folate siRNA

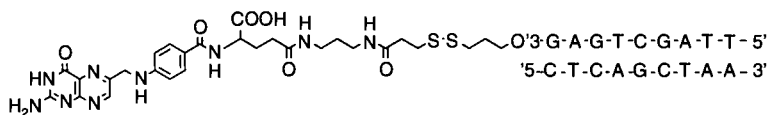


Figure 15.2 siRNA-folate conjugate.

Table 15.2 Use of folic acid as a targeting agent.

Application	Folate conjugate	Folate linker	Ref.
Chemotherapy	Folate-drug	Camptothecan	[93]
Chemotherapy	Folate-drug	Doxorubicin	[94]
Enzyme prodrug cancer therapy	Folate-enzyme	Penicillin-V Amidase	[95]
Immunotherapy	Folate-antibody	Hapten	[96]
Imaging	Folate-Radionucleide	^{111}In -DTPA*	[97, 98]
Gene Therapy	Folate-viral vector	Baculovirus	[99]
Gene Therapy	Folate-non viral vector	Polyplex, lipoplex	[100, 101]
Gene Therapy	Folate-siRNA	siRNA	[102]

* DTPA = diethylenetriamine pentaacetic acid

conjugate for use in gene therapy is shown in Figure 15.2. Other uses of folic acid as a targeting agent are listed in Table 15.2.

15.3.4 Bolaamphiphiles

Bolaamphiphiles are molecules having two hydrophilic head groups—one at each end of the hydrophobic domain [103]. Bolaamphiphiles form monolayer membranes (as opposed to the bilayers formed by phospholipids). Compared with bilayers, these monolayers are less permeable and more durable. Moreover, they can aggregate into nanosized particles; shortchain bolaamphiphiles form micelles, whereas long chain bolaamphiphiles form spherical vesicles [104]. Although the physicochemical properties of these bolaamphiphiles have been studied [105], their use in drug delivery has not yet been investigated. The use of similar amphiphiles for gene delivery [106, 107] and peptide delivery to the brain [106] have yielded successful results. Unsymmetric bolaamphiphiles bearing neutral sugar (gluconic acid) and dicationic ornithine head groups have been designed to bind DNA (Figure 15.3). The transfection efficiency of these bolaplexes (*in vitro*) was significant, when either DOPE or chloroquine were used as helping agents, suggesting that the key barrier for their internalization is the endosomal escape [108].

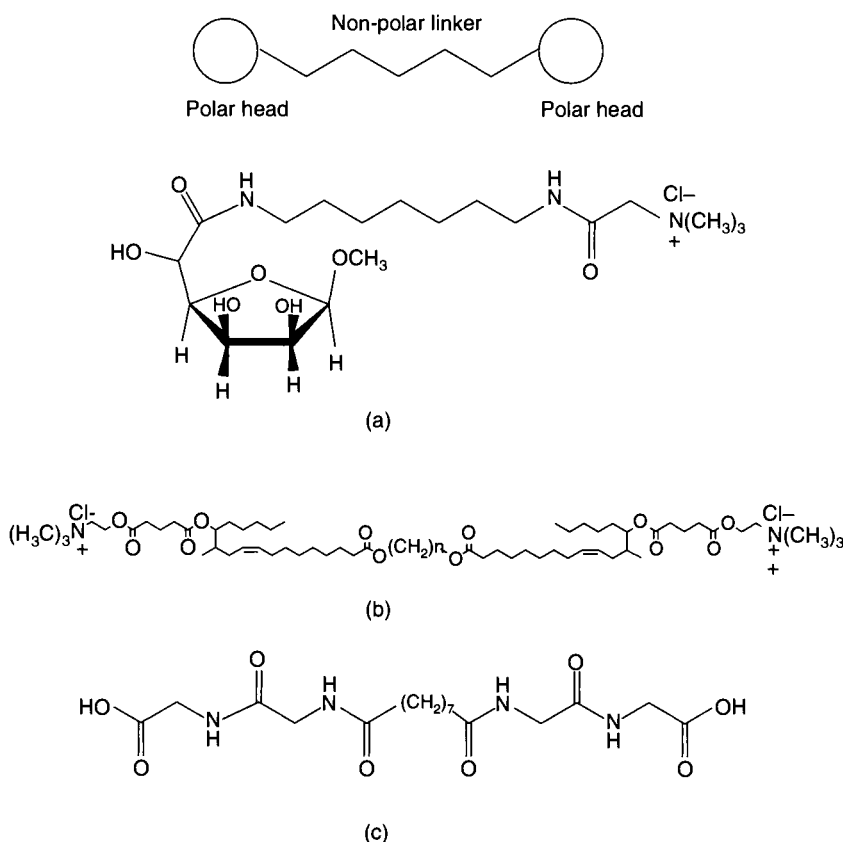


Figure 15.3 Bolaamphiphiles: A) nonsymmetrical bolaamphiphile with a sugar at one end, B) lipid based bolaamphiphile, C) peptide bolaamphiphile.

Bola lipids extracted from archaebacteria [109] (referred to as archaeosomes since they contain ether linkages to the glycerol backbone) and synthetic analogues of these bolaamphiphiles may yield intelligent devices for targeted drug delivery.

15.3.5 Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs)

SLNs and NLCs are lipid-based nanoparticles that have a hydrophobic core, enabling entrapment of hydrophobic drugs. The SLN structure is a solid lipid core made of triglycerides, mixtures of glycerides, or wax, which remains solid at room temperature and body temperature. SLNs have perfect crystal lipid matrices to load the drug, but suffer the disadvantage of burst release. In contrast, NLCs consist of an imperfect crystal or amorphous lipid matrix. They are constructed using a blend of solid lipids and liquid lipids (oils) in a proportion that ensures that their solid phase is maintained at temperatures up to 40°C.

Drugs are loaded into lattice imperfections, enabling enhanced drug loading and improved release kinetics. The lipid is more stable when triglycerides constitute the major lipid component [110]. PEGylation is used to stabilize many liposomal drug formulations. SLNs and NLCs are commonly stabilized by PEGylation or polymer coating with PEG 2000, PVA, or poloxamer. Lecithin-based surfactants have been shown to stabilize SLNs and increase their circulation time [111].

The main disadvantages of SLNs and NLCs are their short circulation half-lives and limited ability to cross cell membranes. Because the formation of these lipid carriers requires high temperatures and stringent solvents, they are unsuitable for encapsulation of denaturable proteins and peptides [112]. However, high pressure homogenization, which can be performed both at elevated temperature (hot HPH technique) and below room temperature (cold HPH technique), enables the incorporation of heat labile drugs into SLNs [113].

The efficiency of therapeutic nucleic acids for silencing oncogenic and chemoresistance targets in chemotherapy is improved when an appropriate carrier is used as the delivery vehicle. The translational success of siRNA-based cancer medicine depends on effective devices to provide sustained, time-controlled siRNA supply without resorting to excessive dosing. Xue *et al.* have developed a novel lipid-based “nanostructured siRNA carrier” (NSC), which incorporates variable amount of oil phase into the solid-lipid matrix to modify its siRNA release behaviors. They obtained effective *in vitro* and *in vivo* chemosensitization of prostate cancer to docetaxel for over a week by tailoring the lipid constituents in the NSC [114]. The use of SLNs for the encapsulation and delivery of cytotoxic anticancer compounds, chemosensitizers and cytotoxic therapeutic molecules has been reviewed by Wong *et al.* [115].

As with targeting strategies used with other nanomaterial carriers, attaching a targeting molecule to the lipid carrier improves the delivery of the drug to the carrier and increases the cytotoxicity of the drug. Zhang *et al.* found that using folate-conjugated stearic acid as a lipid in the NLC formulation enhanced the transport of paclitaxel and doxorubicin into cancer cells, in comparison to unmodified NLCs; this could yield an approach to reversal of multidrug resistance in human cancer cells [116]. The asialoglycoprotein receptor is overexpressed in human hepatoma cell lines and can facilitate the uptake of galactosylated nanoparticles. Galactosylated dioleoylphosphatidylethanolamine-containing SLN loaded with docetaxel displayed significant antitumor efficacy, due to increased accumulation of drug in the tumor caused by increased uptake of the SLNs by the hepatoma cells [117].

15.3.6 Mixed Systems

Mixed nanosystems have recently been developed, in which nanomaterials are used in combination to improve stability, release, effectiveness and pharmacological properties. Combinations of two different nanobiomaterials in one

nanocarrier have been classified as chimeric advanced Drug Delivery nano Systems (chi-aDDnSs). This combination of two independent technologies has produced systems with unique properties. Mikhaylov *et al.* proposed the combination of iron oxide nanoparticles and liposomes to produce ferri-liposomes, which have the dual property of delivering a drug as well as monitoring the drug uptake as an MRI contrast agent [118].

Incorporation of PAMAM (Generation 3,5 Polyamidoamine dendrimer) into liposomes consisting of dioleoyl phosphatidyl choline (DOPC) and dipalmitoyl phosphatidyl glycerol (DPPG)-encapsulating doxorubicin yielded a new chi-aDDnS. This mixed system demonstrated improved encapsulation efficiency, drug release profile and pharmacological properties, when compared to the respective conventional liposomal system [119].

In a clever application exploiting the fact that high-density lipoprotein (HDL) particles are known to escape the reticuloendothelial system while their core constituents undergo receptor mediated uptake, reconstituted HDL (rHDL) nanoparticles were tested by Shahzad *et al.* for their ability to deliver siRNA. These rHDL nanoparticles display long circulation and facilitate highly efficient delivery of siRNA *in vivo*, through scavenger receptor class B type 1 (SR-B1), which is overexpressed in the liver and most malignant cells [120].

To overcome the difficulty of loading water-soluble drugs into NLCs, a mitoxantrone-dextran sulphate drug complex has been loaded into the lipid matrix to form a lipid-dextran sulphate hybrid carrier. Dextran sulphate is an anionic polymer and forms an electrostatic complex with cationic water-soluble drugs. This nanostructured lipid-dextran sulphate hybrid carrier exhibited high encapsulation efficiency, sustained release, and desirable pharmacokinetics and biodistribution; it is a potential candidate for treating multidrug-resistant tumors [121].

15.4 Polymeric Carriers

Polymeric nanoparticles—one of the most widely investigated nanotechnology platforms—have emerged as a versatile carrier system for targeted delivery of anticancer drugs and macromolecules such as genes and proteins. The therapeutic applications of these nanosized medicines, which are now routine clinically, address a variety of diseases and exploit a range of different administration routes. Successful applications have used both naturally occurring and synthetic polymers (biodegradable and nonbiodegradable). Nontoxic and biocompatible polymers (natural and synthetic) are especially good candidates for use as carriers. Synthetic polymers, including poly(lactic acid) (PLA) and poly(lactic co-glycolic acid), and natural polymers, such as chitosan and albumin, have been used to encapsulate drugs without chemical modification. Table 15.3 lists some carriers and the cells/organs used to test their efficiency of delivery.

A wide range of engineered polymer carriers are being used in medical/pharmaceutical applications, including polymer-drug and polymer-protein conjugates, block copolymers, polymeric micelles (to which a drug can be covalently bound) and polyplexes (nucleic acid containing complexes) (see Fig. 15.4). These complex multicomponent polymer-based drugs and delivery systems are defined by Peer [130] and Duncan [131] as “new chemical entities”

Table 15.3 Type of polymers used for carrying drugs and nucleic acids.

Polymer	Drug/NA	Cell type/organ	Ref.
Chitosan	P53 gene	Hela cells	[122]
Chitosan	tobramycin and ofloxacin	Eye	[123]
PLGA/PLL copolymer	curcumin	A2780CP cells	[124]
Polyalkylcyanoacrylate	doxorubicin	Mice	[125]
Albumin	Paclitaxel	abraxane-in clinic	[126, 127]
PEG/PLA copolymer	siRNA	HepG2 cells	[128]
PEI	DNA	Mousa brain	[129]

PLGA= Polylactic-co-glycolic acid, PLL= poly-L-lysine, PAMAM= Polyamidoamine, PLA= Polylactic acid, PEG= Polyethyleneglycol, PEI= Polyetheleneimine, A2780CP cells (a cisplatin resistant/metastatic ovarian cancer cell line).

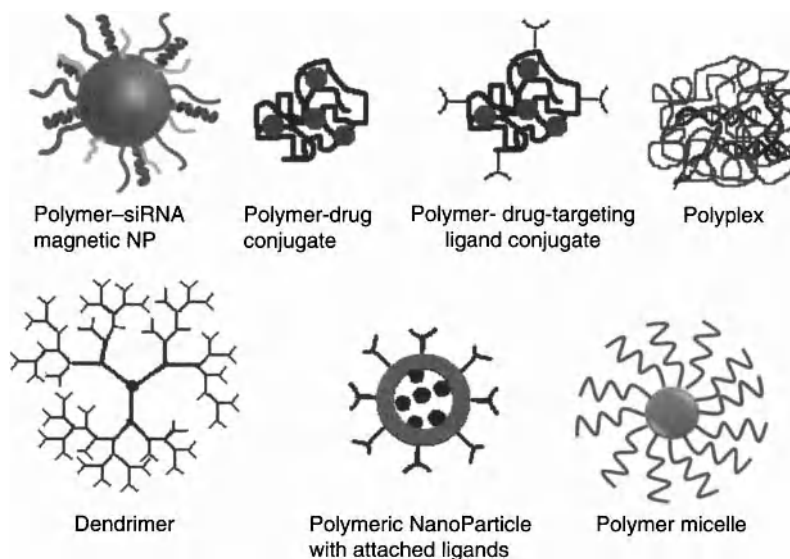


Figure 15.4 Different polymer-based drug delivery systems.

(NCEs) rather than conventional drug-delivery systems or formulations that simply entrap, solubilize or control drug release. The designer molecules currently available display diverse morphologies: polymeric nanoparticles, dendrimers, polymer-protein/aptamer complexes, polymeric micelles, polymer drug conjugates and cationic polymers with modifications for targeting, triggered release and dual/multi functions.

15.4.1 Polymeric Nanoparticles

Conventional polymer nanotechnology platforms were based on polymeric nanoparticles prepared from polymers such as poly(D, L lactide-co-glycolide) (PLG), poly(D, L lactic acid) (PLA) and polythyleneimine (PEI). Biodegradable polymers, such as polylactide (PLA), poly(lactide-co-glycolic acid) (PLGA), gelatin, and albumin, have been used as carriers for small molecules and biologically active peptides and proteins and are listed in Table 15.3.

Polyesters belonging to the poly(malic acid) family (e.g., poly(benzyl malate) (PMLABe) and poly(ethylene glycol)-b-poly(benzyl malate) (PEG(42)-b-PMLABe)) have been shown to effectively carry and deliver doxorubicin [132].

A hitherto underutilized polymer is hyaluronic acid (HA), a biocompatible, nontoxic and noninflammatory material. Ossipov *et al.* reviewed a variety of chemically-based approaches in which small molecule drugs, proteins and nucleic acids are chemically or physically bound to hyaluronic acid. These include hyaluronic acid-drug conjugates, hyaluronic acid nanogels, and hyaluronic acid-decorated nanoparticles, in which HA is coated on nanogels, liposomes, polyplexes, polyester nanoparticles, and inorganic NP's [133]. In addition to being biodegradable, polymeric nanoparticles have the advantages of reduced frequency of administration, sustained drug release, reduced dosage, and few systemic side effects.

15.4.2 Dendrimers

Dendrimers are hyper-branched, roughly spherical, large molecules. A range of targeting ligands, drugs, imaging agents, etc., can be linked to surface functional groups on the branches. Their monodispersity, three-dimensional structure, and multilinkage ability make them useful as targeted drug carriers, gene delivery systems or imaging agents. The dendrimer PAMAM has been shown to be a versatile nanobiomaterial for intelligent delivery, demonstrating an energy-dependent uptake process and targeting ability when linked to biotin [134]. Jang *et al.* constructed a PAMAM dendrimer with arginine-grafted branches. This cationic dendrimer formed a complex with luciferase gene as well as with the EGFR antisense gene. They demonstrated an absence of cytotoxicity and high transfection efficiency, recommending them as a potential therapeutic agent for the treatment of ovarian cancer [135].

A novel polymer conjugate of camptothecin (CPT), employing a core-functionalized, symmetrically PEGylated poly(L-lysine) (PLL) dendrimer,

has been reported by Fox *et al.* [136]. Poly(ethylene glycol) (PEG) and CPT were attached through a lysine dendrimer functionalized with aspartic acid to yield a final load of 4–6 wt% CPT. The PEGylated PLL-CPT dendrimer showed effective treatment in murine (C26) and human colon carcinoma (HT-29) tumor models.

Multifunctional dendrimers with drug, imaging agent, and targeting molecules conjugated to their branches have been reported as efficient therapeutic and diagnostic agents [137]. A detailed account of recent progress in the uses of dendrimers as delivery systems for anticancer drugs, as well as boron carriers for boron neutron capture therapy, and photosensitizers for photodynamic therapy, is provided by Gillies and Frechet [138].

15.4.3 Polymer-Protein/Aptamer Conjugates

Aptamers are short single-stranded DNA or RNA oligonucleotides or small peptides that have strong binding affinity to selected targets, such as intracellular proteins, transmembrane proteins, soluble proteins, carbohydrates, and small molecule drugs. Aptamers that bind specifically to receptors on cancer cells have shown promise as targeting agents for nanoparticle-aptamer conjugate therapy.

A cisplatin-encapsulated PLGA-PEG NP conjugated to the A10 2'-fluoropyrimidine RNA aptamer, which recognizes the extracellular domain of prostate-specific membrane antigen PSMA (A10 PSMA Apt), has been constructed [139]. This design enabled the selective delivery of cisplatin to prostate cancer cells. In another example, docetaxel (Dtxl)-encapsulated nanoparticles, whose surface was modified with an aptamer that targets the antigen on the surface of prostate cancer cells, were delivered with high selectivity and efficacy *in vivo* [140].

PEG is used as a coating for liposomes to produce “stealth” nanoparticles which escape endosomal uptake and remain longer in circulation [127]. In an adaptation of this design, polymer protein conjugates employing PEG attached covalently to protein were found to have elongated circulation half-lives [141]. Macugen®, which contains a nucleic acid encapsulated in PEG, is the only formulation that has been approved for treatment of a non-cancer-related disease: age-related macular degeneration. The PEG polymer is linked to an aptamer targeting vascular endothelial growth factor (VEGF) for efficient targeted delivery [142].

Other applications involve the linkage of therapeutic enzymes to polymers, as in acute lymphoblastic leukemia: Pegylation of asparaginase enzyme prolongs the half life, enabling administration of single doses of PEG-asp, compared to multiple injections of the unpegylated formulations [143].

Acharya *et al.* linked antibodies to a drug-encapsulating polymer for targeting purposes. Rifamycin-loaded PLGA nanoparticles were covalently linked to EGFR antibodies, resulting in specific molecular targeting, and efficient uptake in malignant MCF 7 cells, enhancing the cytotoxic effect of the encapsulated drug in the breast cancer cell line [144].

15.4.4 Polymer-Drug Conjugates

The use of polymer-drug conjugates as carriers for releasing the drug at a defined target can increase the local drug concentration and reduce toxicity. Conjugating an insoluble drug to a hydrophilic polymer can alter its solubility and improve its bioavailability. Polymers that have been tested clinically include N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, polyglutamic acid (PGA), polyethyleneglycol (PEG), polysaccharides (such as dextran), and block copolymers (that are used to assemble micelles). Several polymer-drug conjugates have entered Phase I and II clinical trials; these are reviewed in detail by Peer *et al.* [130]. The drugs that have been covalently linked to the polymers listed above are doxorubicin [145], camptothecin [146], paclitaxel [147] and platinite [148]. A list of polymer-drug conjugates, as well as a list of polymer-protein conjugates, is included in the review by Duncan [149].

A camptothecin analogue, 7-ethyl-10-aminopropoxy-camptothecin (T-2513) has been linked (through a Gly-Gly-Gly linker) to the polymer carboxymethyl-dextran; the resulting construct has demonstrated potential for antimetastatic therapy [150]. To improve the cellular uptake of drug-polymer conjugates, a cell-penetrating peptide (TAT), has been linked to a chitosan/doxorubicin (chitosan/DOX) conjugate to form a chitosan/DOX/TAT hybrid. This conjugate exhibited efficient cell entry, was highly toxic, and decreased tumor growth in tumor-bearing mice [151].

An interesting account of the evolution of drug-conjugated N-(2-Hydroxypropyl) methacrylamide (HPMA) copolymers and concerns regarding the use of this particular nanomaterial is presented by Duncan and Vicent [152]. The importance of rational design and modifications of nanobiomaterials is evident from the results observed in the progress of drug polymer conjugates, from which no marketable product has been developed over the past thirty years [153].

The linking of nucleoside analogs to polymers has given rise to polymer drug conjugates capable of efficient transport and sustained release of phosphorylated drugs [154]. These nucleoside analogs are linked to cholesterol-modified mucoadhesive polyvinyl alcohols or biodegradable dextrin through a tetrakisphosphate linker. The nucleoside analog floxuridine 5'-phosphate is released by enzymatic action or under hydrolytic conditions (pH 1.0–7.4). The researchers claim that polymeric conjugates of phosphorylated nucleoside analogs are novel therapeutic agents against drug-resistant tumors.

15.4.5 Noncovalent Drug Conjugates

Apostolovic *et al.* constructed a polymer conjugate with a noncovalently linked drug which is able to unload its cargo in the endosomal compartment [155]. The conjugate consists of two complementary peptide sequences forming a "coiled coil" heterodimeric peptide motif, where one peptide is linked covalently to the polymer, while the other is covalently attached to the drug. The release strategy is based on the unfolding of the otherwise stable heterodimeric

coil at the low pH found in the endosome; unfolding releases the drug-linked peptide. Studies indicate that the polymer conjugate is not toxic and is transported into lysosomes. Further studies are being conducted to measure the distribution and fate of the trafficked drugs [155].

15.4.6 Cationic Polymers

Chitosan [122], poly-L-lysine, and polyethylene amine (PEI) [156] are cationic polymers widely used for gene delivery. They have several advantages, such as efficient condensation with DNA and simple formation of a relatively small and stable DNA-polymer complex at low cost. However, their use as an *in vivo* gene delivery vector is limited by some undesirable characteristics, such as inherent cytotoxicity and relatively low transfection efficiency *in vivo* (in comparison to viral vectors). Polyplexes having low transfection ability or lacking specificity for a target could be made more efficient by modification of the carrier. Sun *et al.* have shown that rational design of cationic polymers such as PEI, Chitosan and Polyamidoamine (PAMAM) can improve transfection efficiency to some extent [157].

Poly β -amino esters are another class of cationic, degradable, polymers that can form polyplexes; they exhibit controlled release of short DNA fragments like ODNs and small interfering RNA (siRNA). 1,4-butanediol diacrylate, and 1,6-hexanediol diacrylate polymers have been used to form polyplexes that induced efficient siRNA-mediated gene silencing in primary rat hepatocytes and in hepatoma cells without causing significant cytotoxicity [158].

One of the most important concerns in the design of cationic polymers for gene delivery is the release of the gene/nucleic acid once inside the cell. Although some polyplexes may be effectively taken up by the cell, their transfection efficiency may be reduced if the nucleic acid is not released from the polyplex. (Release mechanisms will be discussed in the next section.) However, a polycation that can release its cargo without any trigger has been reported [159]. The polymer, a self-catalyzed degradable poly(2-dimethylaminoethyl acrylate) (PDMAEA) [160] is able to protect an oligo DNA for a sufficiently long period of time, transfect most cells (80% transfection after 4 h) and then, through autodegradation, release the DNA inside the cells after approximately 10 h.

15.4.7 Polymers for Triggered Drug Release

Drugs can be released from polymeric micelles composed of polymers that contain acid-cleavable covalent linkages. Acetal, orthoester, oxime, hydrazone, imine and cis-acotinyl linkages respond to mild acid and have been used in polymeric carriers to obtain pH-sensitive materials. A pH-sensitive doxorubicin-loaded micelle, formed from a PEO-dendritic polyester copolymer having acid-labile acetal groups on the outer ends of the dendrimer, releases its drug content

when acidic pH cleaves the acetal linkages. The release is caused by a change in the solubility of the polymer micelle that destabilizes the structure. It was found that the acetal groups undergo hydrolysis and that DOX is selectively released at acidic pHs (such as those encountered in tumor tissue) and in endocytic vesicles, including endosomes and lysosomes [161].

A novel amphiphilic dynamic diblock copolymer PS-*r*-PEG (polystyrenehydrazone-polyethylene-glycol) micelle containing the reversible acid labile acylhydrazone moiety exhibits disruption at low pH and reassembles into micelles at higher pH. Hydrolysis of acylhydrazone bonds results in the release of its encapsulated contents. This construct can thus be used as a novel sustained-release drug carrier [162].

In another example, oxime linkages have been used to design and prepare a pH-sensitive triblock copolymer (PEG-OPCL-PEG), consisting of hydrophilic poly(ethylene glycol) (PEG) and hydrophobic oxime-tethered polycaprolactone (OPCL) [163]. It was found that DOX-loaded PEG-OPCL-PEG micelles had potent anticancer efficacy (due to the acid labile oxime groups) in addition to being effectively taken up by HeLa cells.

The anticancer drug bortezomib (BTZ) contains borate groups, which can form borates with hydroxyl-containing molecules. This principle was used by Su *et al.* to conjugate BTZ to catechol-containing polymeric carriers (e.g., catechol-conjugated PEG), since catechol can bind and release borates in a pH-dependant manner [164]. They demonstrated that by including biotin as a targeting molecule, the cancer-targeting drug-polymer conjugates showed dramatically improved cellular uptake, proteasome inhibition, and cytotoxicity toward breast carcinoma cells, in comparison with nontargeting drug-polymer conjugates.

Doxorubicin encapsulated poly(D, L-lactide-co-glycolide)-methoxypoly(ethylene glycol) PLGA-mPEG nanodroplets that have been transformed to nanobubbles at a temperature of 37°C are able to release the drug under ultrasound at low pH (e.g., pH 6.5). The drug is not released in the absence of ultrasound and so this construction can be used as a triggered release carrier [165].

15.4.8 Polymerosomes

Polymerosomes are polymeric micelles or polymer vesicles. They are fabricated from di- or tri- block copolymers to generate polymer vesicles similar to liposomes. Depending on the application, rational copolymer design can impart flexibility for controlling the fluidity, membrane permeability and triggered release from the polymerosome [166]. The hydrophobic core of these polymer micelles is capable of dissolving hydrophobic drugs, while the hydrophilic chains of the outer layer make them water soluble. Several hydrophobic core-forming biocompatible and biodegradable polymer micelles such as poly(ethylene-co-propylene-co-ethylene oxide) (PEOb-PPO-b-PPO) or poly(ethylene-co-propylene oxide) (PEO-b-PPO), poly(lactic acid) (PLA), poly(D, L-lactide) (PDLLA), poly(lactic-co-glycolic acid) (PLGA),

poly(ϵ -caprolactone) (PCL), poly(hydroxybutyrate) (PHB), and poly(beta-benzyl L-aspartate) are being used in drug delivery applications [167].

Polymeric micelles are able to inhibit p-glycoprotein at drug-resistant tumors, in the gastrointestinal tract, and at the blood/brain barrier, and may overcome drug resistance in cancer [168]. Flexible polymerosomes constructed using poly(caprolactone)-poly(ethylene glycol)-poly(caprolactone) copolymer showed promise as transdermal drug carriers, and may be useful in topical applications, such as treatment of melanomas [169].

Extensive reviews of the different types of polymer micelles, such as tri-block copolymer, PEG, PVA or PLL coated polymerosomes, polymerosomes functionalized for targeted delivery, and pH-sensitive and thermosensitive polymer micelles have been provided [167, 170, 171]. The versatility of polymerosomes in applications such as drug delivery, imaging, triggered release, and targeted delivery is reviewed by Oerlemans *et al.* [170]. The design of polymer material for pH-sensitive and light-sensitive release, and the preclinical studies of multifunctional micelles, have also been discussed [170].

An example of intelligent design of nanobiomaterials for drug delivery is presented by Li *et al.* who had previously reported the development of micelles using amphiphilic PEGylated oligomers of cholic acids, synthesized via peptide chemistry [172]. Their present design attempts to minimize premature release of drugs during circulation in the blood stream by introducing nontoxic building blocks, such as hydrophilic spacers and amino acids, into the telodendrimers at the desired position, in a stepwise manner. Thereafter the introduction of cysteine into the telodendrimers generates a reversibly crosslinked micelle system. Intramolecular disulfide bonds will be cleaved under reductive conditions, such as in the intracellular environment (glutathione), and the drug payload will be released only after reaching the tumor sites [173].

Polymeric micellar drug delivery is a means for advanced nanoengineering of polymeric micellar carriers. The rational drug- and disease-based development of polymeric micellar carriers has resulted in seven formulations being advanced to clinical trials; these are reviewed by Xiong, *et al.* [174].

15.4.9 Other Applications

Polymers that encapsulate magnetic nanoparticles [175] have been used as dual drug delivery and imaging systems. Magnetic nanoparticles embedded in polylactide-co-glycolide matrices (PLGA-MNPs) are capable of encapsulating both hydrophilic and hydrophobic drugs. The attachment of Herceptin as a ligand results in enhanced cellular uptake in an *in vitro* system (when compared with native drugs). These PLGA-MNPs displayed better contrast effects than commercial contrast agents, verifying the efficacy of this multifunctional system [176]. Other surface-functionalized polymer-associated magnetic nanoparticles for imaging and siRNA delivery are described by Boyer *et al.* [177].

The diversity of engineered polymers is limited only by the imagination; in recent years a nearly unlimited ingenuity has been demonstrated. As more and more intelligent applications emerge, the future of designer nanobiomaterials looks brighter than ever.

15.5 Bacteria-Mediated Cancer Therapy

Bacteria-mediated cancer therapy has a long history. Bacteria were first associated with cancer almost 200 years ago. In 1813, Vautier reported that patients with cancer were cured after suffering from gas gangrene caused by *Clostridium* infections. In 1868 W. Busch inadvertently used *erysipelas* bacterial infection to treat a case of sarcoma cancer. The patient was accidentally placed onto the bed of another patient with an *erysipelas* infection. The patient's tumor shrunk by half in a week, but she died from the infection after nine days [178]. About 30 years later, William B. Coley cured a cancer patient by introducing a severe *erysipelas* infection. He reported that at New York hospital a patient with a malignant sarcoma of the neck was cured after being infected with *erysipelas* twice. Surprisingly, the tumor disappeared completely after the second attack. Dr. Coley found the patient still alive seven years later, after which he repeatedly applied the bacterial infection to treat cancers [179].

Despite this anecdotal clinical evidence, research into bacteria-mediated cancer therapy did not begin until the mid-1900s. A range of bacterial species, such as *Salmonella*, *Bifidobacteria* and *Clostridia*, have been explored. Most research on this area is focused on *Salmonella* and *Clostridium*.

15.5.1 The Tumor Microenvironment

Every cell in our body follows a highly regulated cell division cycle. If a single cell loses its control over the cycle, it could proliferate quickly, which may result in the formation of a tumor. As the tumor grows, the surrounding blood vessels become inadequate to supply nutrients for the abnormal cells. This triggers the secretion of tumor angiogenic factors (TAFs). TAFs stimulate differentiation, division and migration of endothelial cells of the blood vessels to the tumor site. The imbalance between different TAFs typically causes abnormality in the blood vessels of the tumor. As the tumor grows, the distance between microvessels and some tumor cells increases, causing reduced oxygen levels in the inner part of the tumor. Consequently, the interior of the tumor may become highly hypoxic; some parts may form necrotic areas that are oxygen-free [180, 181].

Figure 15.5 shows the structure of an idealized solid tumor. In the proliferative part, the abnormal cells are close to blood vessels and receive sufficient nutrition and oxygen for growth. In the quiescent or hypoxic part, the concentration of oxygen is less than 0.33% (2.5 mmHg). The abnormal cells in this region no longer proliferate. The necrotic core of the tumor consists of dead cells; the oxygen level in this core is almost zero. (For comparison, the

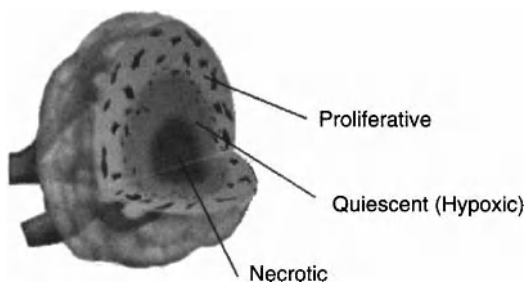


Figure 15.5 Structure of solid tumors [182]. National Academy of Sciences, USA, copyright (2001).

oxygen concentration in normal tissue ranges from 3.1–8.7% (24–66 mmHg) [183, 184]).

The lymphatic vessels in the hypoxic and necrotic parts are abnormal and cannot discharge waste water from within the tumor. Consequently, the interstitial pressure increases in these regions. The high interstitial pressure and low oxygen concentration make hypoxia a barrier against a range of cancer therapy methods, including chemotherapies and radiotherapy [185].

Although hypoxia poses a barrier against traditional therapies, it can be used as a marker to distinguish solid tumors from normal tissues. The necrotic part of a tumor is oxygen-free and rich in nutrients from dead cells. It is, therefore, an ideal environment for anaerobic (and facultative anaerobic) bacteria to germinate.

15.5.2 *Salmonella*-mediated Cancer Therapy

Salmonella is a gram-negative facultative anaerobic bacterium that causes intestinal infections. Early studies of *Salmonella* focused on reducing its pathogenicity (septic shock). In 1952, Graham and Coleman showed that *Salmonella montevideo* colonizes the interior of carcinoma tumors [186]. In order to use *Salmonella* as an antitumor agent, it had to become nonpathogenic, or at least attenuated, to reduce its harm to the body.

Early works on *Salmonella* were focused on making vaccines; these works were also helpful for providing a bacterial strain that could be used as an anti-cancer agent. In 1951, Bacon *et al.* showed that *Salmonella* can be attenuated by an auxotrophic mutation, such as those mutations that affect the biosynthesis of purines [187]. In 1981, Hoiseth and Stocker attenuated *Salmonella typhlimurium* by mutations that affect the biosynthesis of aromatic amino acids [188]. They also showed that the attenuated *Salmonella* can be used as a live vaccine.

It was not until 1997 that researchers reported that attenuation increases the colonizing capability of *Salmonella typhlimurium* in tumor and that this strain can be used as a gene delivery vector. Pawelek *et al.* hypothesized that the necrotic part of a tumor provides essential nutrients for auxotrophs [189]. They used melanoma-bearing mice as animal models with different implanted human tumors such as human lung carcinoma A549, human colon carcinoma

HCT 116, human breast carcinoma BT20, human renal carcinoma CRL 1611, and human hepatoma HTB 52. They showed that attenuated *Salmonella* germinate in tumors to levels 250–9000 fold higher than in normal tissue, such as liver. Therefore attenuation of *Salmonella* not only makes them less pathogenic but also increases their tumor-targeting abilities. They also engineered *Salmonella* to express genes, such as the thymidine kinase from herpes simplex virus; tumor growth regression was observed when the engineered *Salmonella typhimurium* was injected into the tumor-bearing mice.

Low *et al.* showed *Salmonella* retains its tumor suppression properties when two genes from its chromosome are deleted. The deletion of the *msbB* gene reduces induction of TNF α (Tumor necrosis factor α), which in turn reduces the risk of septic shock. The deletion of the *purI* gene makes the bacteria dependent on an external source of adenine. They showed that these mutated *Salmonella* continue to replicate with tumors and cause tumor suppression [190].

In 2005, Ming Zhao *et al.* reported development of a genetically modified strain of *S. typhimurium*. This strain, which is also known as *S. typhimurium A1*, selectively grows in prostate tumors implanted in mice and causes tumor regression [191]. Normal tissue was cleared from *S. typhimurium A1* bacteria even in immunodeficient mice; no side effects were observed. *S. typhimurium A1* is auxotrophic (*leucine-arginine* dependent) and apparently receives sufficient nutritional support only from tumor tissue. When the bacteria were injected intravenously, they germinated inside PC-3 prostate tumors and caused tumor regression.

In 2006, Ming Zhao *et al.* modified *S. typhimurium A1* further to increase its tumor-targeting ability [192]. The strain was reinsulated after infection of a human colon tumor growing in mice. They inject the modified strain into the breast tumors in mice models. This strain, known as *S. typhimurium A1-R*, exhibits increased tumor targeting *in vitro* and *in vivo*, compared to *S. typhimurium A1*.

Current research on *Salmonella* has focused on delivery and expression of therapeutic agents such as cytokines, prodrug-converting enzymes, and agents toxic to tumors. Even though attenuated *Salmonella* shows good tumor colonization and regression in animal models, the clinical results on human are disappointing. The colonization of *Salmonella* in patients is insufficient. Moreover the colonization of attenuated *Salmonella* in normal tissue, even transiently, causes significant side effects and reduces the specificity of this gene transfer system [193].

15.5.3 Clostridium-mediated Cancer Therapy

Clostridia are gram-positive obligate anaerobic bacteria. Even though some strains of *Clostridia*, such as *C. botulinum* or *C. tetani*, are well known as pathogens, most strains are nonpathogenic. In 1935, Connell used *C. histolyticum* to treat advanced cancers [194]. He concluded that the production of proteolytic enzymes in *C. histolyticum* causes tumor regression. Parker *et al.* were the first to infect tumor-bearing mice with *C. histolyticum* to study tumor regression

by bacteria [195]. They observed considerable regression of sarcoma tumors. These studies suggested that *Clostridium* spores are good candidates for anti-tumor agents.

In 1955, Malmgren and Flanigan intravenously administered *C. tetani* spores into tumor-bearing and normal mice [196]. All tumor-bearing mice died within 48 hours because of the production of tetanus toxin in the tumor, but the non tumor-bearing mice survived without any tetanus symptoms. Microscopic examination of tumor and normal tissue sections revealed that the spores germinated exclusively within the tumor, where they released tetanus toxins.

In 1964, Möse and Möse intravenously injected *C. butyricum* M55 (later named *C. oncolyticum*, and now classified as *C. sporogenes* ATCC 13732) into mice with solid Ehrlich carcinomas [197]. Bacteria colonized the tumors and the necrotic part of the tumor was discharged as brownish liquid. In the few mice that survived this deadly stage, tumor regrowth was observed from the remaining outer rim, which consists of viable cancer cells. These results were confirmed by further studies with different nonpathogenic spores of *Clostridium* and different types of tumor models [198, 199]. In 1967, Möse and Möse demonstrated that *C. sporogenes* is nonpathogenic by injecting this strain into themselves.

Corey *et al.* reported treatment of five patients with neoplastic diseases by injection of 10^{10} spores of *C. sporogenes*. The patients developed only a mild fever during treatment [200]. However, surgery was required before completion of oncolysis to prevent death. Because of tumor regrowth from the outer rim, the clinical trial was discontinued. These studies show that *C. sporogenes* is a safe tumor targeting agent.

Combined treatments were examined in an effort to destroy the viable outer rim of the tumor. For example, *Clostridium* administration was accompanied by chemotherapeutic agents such as 5-Fluorodeoxyuridine and cyclophosphamide [199, 201]. Other combined treatments such as *Clostridium* spores, local irradiation, and high frequency hyperthermia showed significant result in mice bearing melanomas [202]. In 1979, Möse administered *Clostridium* spores to tumor-bearing rats while the oxygen level in the respiratory air of the animals was decreased to 11–12% [203]. Despite these efforts, regrowth continued to occur from the viable outer rim; complete tumor regression could not be achieved.

Dong *et al.* screened different anaerobic bacterial species, such as *Bifidobacteria*, *Lactobacilli* and pathogenic *Clostridia*, for their tumor targeting capability. He reported that *C. novyi* has the best colonizing behavior [204]. They removed a lethal toxin expressed by this strain, resulting in a nontoxic strain named *C. novyi*-NT. Intravenous administration of these bacteria into mice bearing Ehrlich ascites tumors resulted in tumor colonization and extensive oncolysis. They also showed that *C. novyi*-NT can efficiently infiltrate and spread extensively through necrotic tumor regions. Similar to *C. butyricum* M55, germination of the spores led to enlargement of the necrotic regions, with a consequent inhibition of tumor growth. These observations were tumor-type dependent; some combinations led to severe toxicity as a consequence of “tumor lysis” syndrome.

Clostridia have also been used as tumor-specific gene delivery systems. As these gene systems were only applicable to saccharolytic strains, the initial experiments were undertaken with *C. acetobutylicum* and *C. beijerinckii* [205]. Unfortunately, these strains have been shown to have limited tumor colonization activity. Indeed, upon systemic administration of spores, colonization levels of the saccharolytic *C. acetobutylicum* and *C. beijerinckii* are 1000-fold lower than proteolytic *C. sporogenes* strains [206]. Despite their weak tumor colonization properties, the use of saccharolytic strains may be beneficial if the introduction of the desired therapeutic gene is required, as degradation of extracellular therapeutic protein might be enhanced, as compared with proteolytic strains [178].

There have been efforts to genetically manipulate strains with desirable colonization properties, such as *C. sporogenes*. In 2002, Liu *et al.* described an electroporation protocol for gene transformation of *C. sporogenes* [207]. They injected engineered *C. sporogenes* accompanied by an administration of a 5-fluorocytosine (5-FC) prodrug. *C. sporogenes* was genetically engineered to express the *E. coli* cytosine deaminase enzyme, which converts prodrug 5-FC to fluorouracil, an anticancer drug (Fig. 15.6). Unfortunately, their results were not repeatable. In 2006, Theys *et al.* developed a gene transfer protocol, based on conjugation, which allows the construction of recombinant *C. sporogenes* strains [208]. They genetically engineered *C. sporogenes* to produce Nitrogen reductase (NTR), which converts prodrug CB1954 (5-aziridinyl-2, 4-dinitrobenzamide) to its 10000-fold more toxic 4-hydroxylamine (4HX) derivative, which can act as an apoptosis agent (Fig. 15.6). Consequently, it is now possible to use the strain with the highest tumor colonization rate (*C. sporogenes*) and thus the highest therapeutic gene expression levels. Not surprisingly,

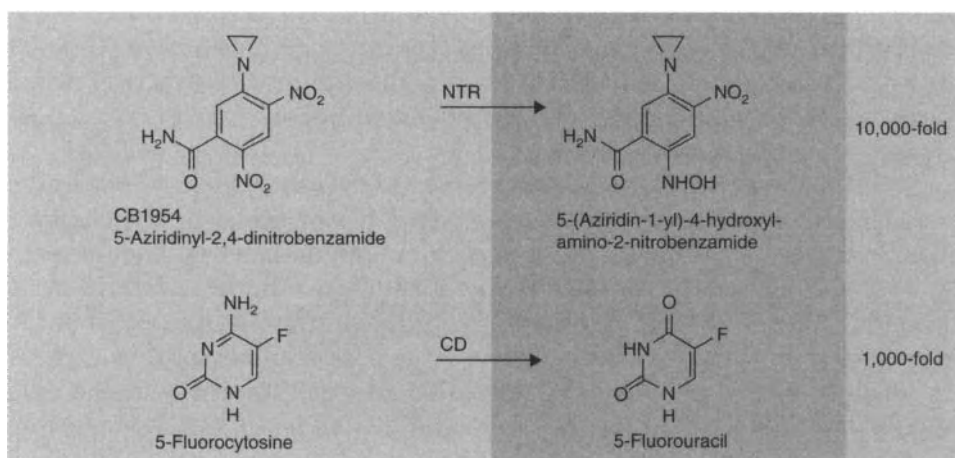


Figure 15.6 CD and NTR convert prodrug to drugs which are highly toxic. Reprinted by permission from Macmillan Publishers Ltd: (*Clostridia in Cancer Therapy*) [209], copyright (2003).

preclinical experiments with recombinant *C. sporogenes* have shown increased antitumor efficacy in comparison with *C. acetobutylicum* or *C. beijerinckii* [209].

Besides *Clostridium*, other anaerobic bacterial species, such as *Bifidobacterium*, can be used to deliver genes to tumors [210]. However, the rather low colonization efficiency and the tendency to clump rather than distribute within necrotic areas, appears to make *Bifidobacteria* inferior to the optimal strain of *Clostridia*.

15.6 Conclusion

Developments in the area of nanotechnology to enhance the delivery of anticancer drugs to tumor tissue have been targeted at minimization of drug distribution and toxicity in healthy tissue. Various nanotechnology platforms have evolved and numerous reports are available for polymeric nanoparticles, liposomes, dendrimers, nanoshells, carbon nanotubes, superparamagnetic nanoparticles, and peptide-based nanocarriers for the delivery of nucleic acids and specific anticancer drugs.

The attempts to use peptides for delivery of drugs and nucleic acids for cancer therapy is promising and more work is required before such applications reach clinical trials. Polymeric nanoparticles have been shown to be versatile carriers of both drugs and nucleic acids. Some of the polymers have been designed to perform specific functions such as targeting specific receptors in cancer cells. Several of these carriers have successfully gone through clinical trials and obtained approval for use. Lipid carriers of various forms such as liposomes, emulsions and solid lipid nanoparticles are used in many anticancer drug formulations as well as for delivering nucleic acids for cancer therapy. In all these systems, the physicochemical characteristics such as composition, particle size, surface charge, surface functionalization with hydrophilic polymers, and inclusion of tissue recognition ligands play an important function in their biodistribution and pharmacokinetics. A promising, but poorly researched, carrier system could be found in bacteria which can mediate cancer therapy. By engineering bacteria to survive within tumors and attack from the inside, tumor necrosis has been observed. However, the results obtained so far have not been very promising. This chapter is an overview of current nanotechnology platforms and their intelligent modifications for the purpose of cancer therapy. The variety and versatility of the various carrier systems designed by intelligent manipulation indicates the possibilities hitherto available in the field of cancer therapy.

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PART IV

TISSUE ENGINEERING AND ORGAN REGENERATION

The Evolution of Abdominal Wall Reconstruction and the Role of Nanobiotechnology in the Development of Intelligent Abdominal Wall Mesh

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Abstract

Advancements in surgical techniques include the introduction of abdominal wall resection of metastatic tumor implants and intraoperative use of heated intraperitoneal chemotherapy. These advancements along with a consistent increase in the average age and comorbidities of the surgical patient, delineate the challenge of post-surgical abdominal wall reconstruction. Parallel to this increasing need, a technical and industrial evolution has occurred in an attempt to create the ideal approach for abdominal wall reconstruction. The issue of mesh versus primary repair without mesh for ventral incisional hernia was carefully studied to demonstrate that the long-term recurrence outcome is improved by the use of mesh-reinforced repair. Following that, an era of increased use of synthetic mesh started, and consequently, its limitations became more apparent. Nonabsorbable mesh carries a risk of infection that requires its excision leaving a bigger abdominal wall defect, it is not integrated in the host tissue and is surrounded by a seroma collection, and finally it cannot be used in a potentially contaminated environment. Absorbable synthetic mesh, typically consisting of a polyglycolic acid material, is associated with a high frequency of enterocutaneous fistulas, largely thought to be related to the desiccation of bowel loops.

Later on, biomaterial, known as biological meshes, entered the arena of clinical care in the late 1990s to early 2000s. Included among these were materials derived from human skin, porcine skin, porcine intestine and materials derived from bovine pericardium.

In considering the characteristics of an ideal prosthetic for contaminated incisional hernia repair, such a material would not incite an inflammatory response

or foreign body reaction. Similarly, it should not promote seroma formation, intestinal adhesion, bowel obstruction, or erosion of the prosthesis into an adjacent viscus. Lastly, the prosthetic material should not contract over time, thus not giving rise to the now present terms in the hernia clinical lexicon such as mesh-odyna and inquin-odyna. None of the previously mentioned material fulfills the criteria of the ideal prosthesis.

Recently, nanotechnology was introduced to the medical field and was integrated into the manufacturing of different intelligent smart biodevices and prothesis. For instant, biodegradable poly(lactic-co-glycolic) acid (PLGA) nanoparticles (NPs) have been manufactured using a double emulsification-solvent evaporation method for use as a vehicle for delivering various bioactive molecules such as nucleotides and drugs-for therapeutic purposes. This integration of biomaterial also induced a homogenous support of cell adhesion, proliferation, and cell growth and improved mesh integration and remodeling. Similarly, a biconanocomposite mesh was developed by cross-linking a decellularized biomaterial mesh tissue with a functionalized nanomaterial. Also, nanotechnology was used for the first time to design a composite mesh with titanium fibers which is known for its inertness and immunity to corrosion by all body fluids and tissue and with the comfort and flexibility of polypropylene.

This chapter will briefly review the evolution of abdominal wall reconstruction and highlight the need for an ideal material for this purpose. The development of nanobiotechnology and current attempts to create an ideal abdominal wall reconstruction material using this new technology will also be discussed. Finally, a review of all nanobiotechnology materials available for abdominal wall reconstruction will be discussed as well as necessary future directions.

Keywords: Abdominal wall reconstruction, hernia, mesh, nanotechnology, biomaterial

16.1 The Complex Structure of the Abdominal Wall

Before discussing the evolution of abdominal wall reconstruction, it is important to understand the complex function of that part of the body. For instance, a poor understanding of its function as only limited to prevent herniation of the intra-abdominal viscera was responsible for an era of treating defects with a plugging concept in order to fulfill this mechanical function, and yet overlooking the importance of its dynamic characteristics. In fact, the abdominal wall complex fulfills multiple functions (Figure 16.1). The most interior layer which is lined by the peritoneum prevents the adhesion of intra-abdominal viscera. This is followed by a strong fibromuscular layer that provides mechanical support of the abdominal viscera and allows for the dynamic function of trunk mobility, and for expansion to accommodate abdominal distention with an elastic component allowing for the return to its original dimension when indicated. The outermost layer is the skin and skin appendages that have the same role of the skin covering the whole body. However, its particular importance in the field of abdominal wall reconstruction is based on the fact that some material used for reconstruction is dependant on skin coverage to prevent their premature degradation, while other need this coverage to minimize their infection.

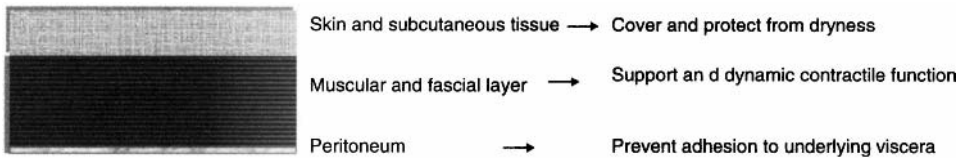


Figure 16.1 The complex structure and function of the abdominal wall.

16.2 Need for Abdominal Wall Reconstruction

Incisional hernia continues to be a common occurrence subsequent to major abdominal operations. The increasing use of laparoscopic techniques for an expanding array of abdominal operations may decrease the incidence of incisional hernias in the future; however at present, incisional ventral hernia repairs constitute a significant portion of the general surgical caseload. Moreover, the advance in surgical techniques including the introduction of abdominal wall resection of metastatic tumor implants and intraoperative use of heated intraperitoneal chemotherapy, add another major demand for abdominal wall reconstruction [1].

Traditional approaches to the repair of abdominal wall defects has centered primarily on the use of native host tissues (primary repair) or synthetic mesh material. For the vast majority of patients these approaches are sufficient to achieve the goal of repair. However, in some yet undefined subset(s) of patients, as has been demonstrated by clinical trials, primary repair without mesh leads to a high rate of recurrence, and incisional hernia repairs employing synthetic mesh have a potential infection rate in the range of 10–30% in selected cohorts [2].

16.3 Failure of Primary Repair

Previous randomized prospective trials comparing the mesh versus primary repair for ventral incisional hernia revealed superiority of the mesh repair [3]. For patients in this study, the use of an underlay synthetic mesh material led to a recurrence rate of 23% as compared to 46% for primary nonabsorbable mesh repair ($p < 0.05$). Interestingly, for relatively small hernias less than 3x3 cm, the use of mesh material resulted only in a 6% recurrence rate as compared to a 44% recurrence for primary repair. This particular study can be seen as the benchmark study demonstrating that for incisional hernia repairs the long-term recurrence outcome will be improved by the use of a mesh reinforced repair in the underlay position.

It is important to understand the terminology utilized within incisional hernia literature. There are three specific terms: *inlay* denotes that the mesh is sewn directly to the fascial edge and directly into the defect; *overlay* that the mesh is sewn directly on anterior fascial aponeurosis of both edges along the fascial defect, and; *underlay* that the mesh is sewn below the fascial edges of the hernia defect onto the posterior aponeurosis with a recommended >3cm overlap of fascia by the mesh (Figure 16.2). Respectively speaking, the documented

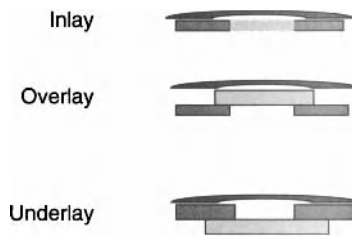


Figure 16.2 Terminology used to describe the type of repair according to the mesh position to the native abdominal wall.

recurrence rate for hernia repair using these variable mesh positions are as follows: inlay, 20–30%, overlay, 20–40% and lastly, underlay, <4%.

Taken together, it is clear that mesh-reinforced repairs result in a decreased abdominal incisional hernia recurrence rate and that when mesh repairs are utilized, placement of the mesh in an underlay position is optimal in terms of recurrence.

16.4 Limitations of the Synthetic Meshes

Optimal incisional hernia repair, particularly for the subset of patients in which there is a contaminated operative field, an infected synthetic mesh, or a mesh that has been extracted from an infected field was well described by T.C. Fabian [4]. In this landmark work, Dr. Fabian and his group discuss the need for an alternative method of closure for the 5–10% of open ventral hernias that will ultimately become infected. Contained within that article is the observation of a University of Washington study which observed a 50–60% failure and reinfection rate for synthetic mesh materials when placed in the setting of a previous infection. It was for this population of patients that the so-called Fabian approach became popularized.

The Fabian approach requires that in a grossly infected or contaminated field that has not had synthetic mesh put in, or in a similar field from which the synthetic mesh has/is being extracted, that the wound be closed with an absorbable mesh, typically consisting of a polyglycolic acid material intended to promote granulation and prevent evisceration. The skin edges, in keeping with standard surgical practice, are not closed and the patient is allowed to granulate the wound. As denoted in his article and mirrored in abundant literature over the years, the use of a synthetic absorbable mesh is associated with a high frequency of enterocutaneous fistulas, largely thought to be related desiccation of bowel loops. These enterocutaneous fistulas have typically been demonstrated to heal spontaneously and, subsequent to the formation of appropriate bed of granulation tissue, a split thickness graft will be placed on the wound. Approximately 2 years are required for separation of the split thickness skin graft and the underlying bowel which will enable the removal of the split thickness skin graft and a reattempt of closure with synthetic mesh.

It is important to recognize that synthetic mesh is all that was available in that particular era. The reader can quickly recognize that if a surgical field was repaired using the Fabian approach, that this was, in fact, a contaminated field and that the risk of subsequent mesh infection would be prohibitively high. It is specifically in this type of clinical scenario that the use of biomaterials began in earnest in the late 1990s.

16.5 Introduction of Biomaterials To Overcome Synthetic Mesh Limitations

Biomaterial or biological meshes, as they are often called, entered the arena of clinical care in the late 1990s and early 2000s. Included among these, which now number approximately 14 which are FDA approved, are: materials derived from human skin, materials derived from porcine skin, materials derived from porcine intestine and materials derived from bovine pericardium (Table 16.1). From a nomenclature perspective, biomaterials can be divided into two large categories, dermal origin and non-dermal origin. Additionally, these materials can be further subdivided into allografts (human derived) or xenografts (non-human origins). For the basis of this report we will concentrate largely on three separate materials which general consensus would agree are the three predominant materials utilized clinically in North America for the repair of contaminated incisional hernia and/or abdominal wall reconstruction.

Alloderm®, manufactured by Lifecell Company, is comprised of dermatome harvested human dermis. Because it is derived from human dermis it has a high elastin relative to collagen content making “stretch” an inherent property of this material. This stretch or relaxation of human acellular dermis, as it is also referred to, makes the material strong, pliable and elastic. Biochemically, the material has been demonstrated to initiate integration, vascular ingrowth and angiogenesis and is reported to be infection resistant [5–7].

Next is **Strattice®**, also manufactured by Lifecell Company. This is derived from porcine dermis with a reported 30–40% reduced elastin content as compared to acellular human dermis. However it is elastin containing nonetheless. This particular material was introduced into the clinical market

Table 16.1 Ideal material for abdominal wall reconstruction.

Ideal material for abdominal wall reconstruction
1 Strong 2 Does not produce inflammatory reaction (seroma-adhesions to underlying viscera) 3 Innate to the immune system 4 Does not contract 5 High biocompatibility and remodeling

in 2008, and as of the writing of this report limited literature exists to support its use and define its clinical characteristics.

Within the same category as Stratattice is the product **Permacol**[®]. This is an acellular porcine dermis. The significant difference between this product, which is manufactured by Tissue Science Laboratories and the Stratattice mesh is the chemical crosslinking of the collagen which is designed to promote increased resistance to collagenase degradation [8]. Based on the IFU (instructions for use) literature this material does not absorb or shrink over time, however there are only limited published clinical series in ventral hernia repairs for the use of this material. The material has largely sat dormant since its introduction in the early 2000s until the acquisition of the Tissue Life Sciences company by Covidien that is at present marketing it for incisional hernia repair.

Last, under the discussion of the selected biomaterials is the material **Surgisis**[®] manufactured by Cook Medical. Surgisis is porcine derived from small intestine submucosa. This particular material has a high collagen-to-elastin ratio so that it demonstrates less stretch and elastic activity [9–13].

Taken as a whole, these four mentioned materials constitute the most widely used biomaterials in North America. Categorizing them as either human or porcine in origin is not particularly clinically useful. Rather, these materials can be broadly classified into materials that are usable in tension repair situations and materials that require non-tension repair. In large part, due to the high elastin content, dermal derived materials would theoretically perform superiorly in tension requiring fields and/or repairs for which relaxation, elasticity and contouring over time are required. The non-dermal materials such as Surgisis which are predominantly collagen in composition theoretically are expected to result in strong, nonelastic, non-contouring repairs over time [14].

16.6 Ideal Material for Abdominal Wall Reconstruction

In considering the characteristics of an ideal prosthetic for contaminated incisional hernia repair, such a material would not incite an inflammatory response or foreign body reaction. Similarly, we would want this material to not promote seroma formation, intestinal adhesion, bowel obstruction, or erosion of the prosthesis into an adjacent viscus. Lastly, and most clinically apparent in the present day, we would want this prosthetic material not to contract over time. Unfortunately, this ideal synthetic prosthetic is not yet available (Table 16.2). Surprisingly, even in the year 2009, our choice of synthetic materials for incisional hernia repair is largely limited to materials that have more than a 50-year history: for example, nylon 1944, Dacron 1956, polyethelene 1958, ethelene 1958, with the most recent addition to the synthetic armamentarium being PTFE in 1970. Subsequent novel synthetic materials after the 1990s are largely a combination of these standard synthetic materials with a coating or combination of materials to prevent bowel adhesions in a short-term period prior to peritonealization. It is from this need of an optimal reconstructive prosthetic for which biomaterials have been created.

Table 16.2 Most common biomaterial used for abdominal wall reconstruction.

	Origin	Autogenous	Example	Limitation
Dermal	Human skin	Yes	Alloderm	Stretchable
	Porcine skin	No	Stratice Permacol	Stretchable Limited reports
Non Dermal	Porcine small intestinal submucosa	No	Surgisis	Seroma, tension free repair
	Bovine pericardium	No	Tutomesh	Tension free repair

16.7 Role of Bionanotechnology in Providing the Ideal Material

Over the last decades, technological advancement have allowed for the development of structures in which scientists can dictate their characteristics at a nanolevel. The new nano-controlled material opened a new horizon in multiple industrial fields. Similarly in the medical field there is the potential for this newly created material to accomodate the needs of the ideal implant. Most of the newly developed material was used for cartilage and bone regeneration/replacement with very few reports exploring the field of abdominal wall reconstruction. For abdominal wall reconstruction, it should provide the necessary strength while promoting cellular attachment, tissue ingrowth and integration, and an improvement in overall biocompatibility. The current role of nanotechnology in the feld of abdominal wall reconstruction can be divided into either improving the characteristics of currently present products (biomaterials or synthetic mesh) by special characteristics provided by the integrated NPs, or; creating a denovo nanomesh material with controlled characteristics (Figure 16.3).

A good example of the application of nanotechnology to improve current biomaterials is the incorporation of poly(lactic-co-glycolic) acid (PLGA) nanoparticles (NP) into porcine small inestineal sumucosa biomaterials [15]. The aim of this attempt was to overcome the heterogeneity limitation of this biomaterial, since mechanical properties and regenerative capability of SIS depend upon species of animals, ages of animals, and locations of small intestine being used for SIS preparation. In this model, PLGA NPs ranging between 162 nm and 306 nm in diameter were added onto the mucosal side of SIS. The result was a NP modified SIS with less permeability without compromising the SIS mechanical properties. Moreover, the NP-modified SIS showed significantly higher cell adhesions and growth. The mechanism that is responsible for the enhanced cell proliferation in PLGA NP-modified SIS is not clear. The elevated cell proliferation might result from a uniform porous structure

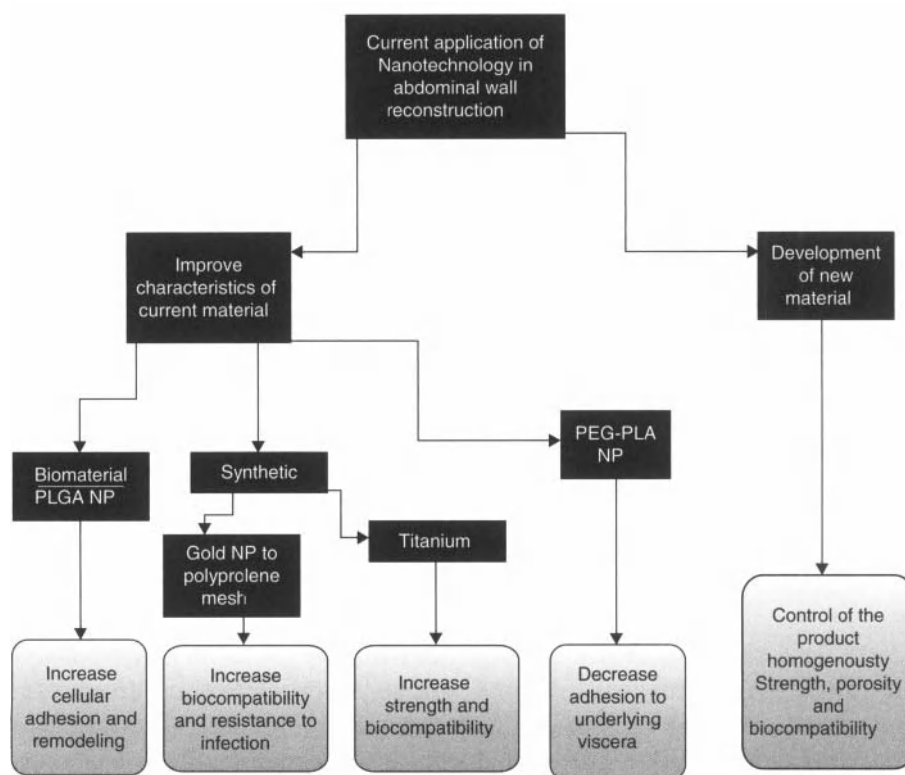


Figure 16.3 Current application of Nanotechnology in abdominal wall reconstruction.

provided by NP-modified SIS for easier cell migration and distribution, or the synergistic effects of PLGA and SIS to support cell growth [16–18].

Another example is provided by Grant *et al.* [19]. In this example, researchers integrated gold NP in a polypropylene mesh in order to improve cellular response. Differential scanning calorimetry and mechanical testing confirmed that the polypropylene did not undergo any significantly detrimental changes in physicochemical properties. On the other hand, A WST-1 cell culture study showed an increase in cellularity on the gold nanoparticle-polypropylene mesh as compared to pristine mesh. This study showed that biocompatibility of polypropylene mesh may be improved via the conjugation of gold nanoparticles.

Another example to improve mesh biocompatibility is to use nanotechnology to integrate titanium nanofilament in a synthetic mesh. The favorable characteristics of titanium that makes it desirable for implantation are its corrosion-resistance and biocompatibility. TiMESH is light, strong, totally biocompatible and FDA approved. Because of its inertness and immunity to corrosion by all body fluids and tissue, titanium is one of the few materials that naturally match the requirements for implantation in the human body. TiMESH's unique design combines the proven benefits of titanium with the comfort and flexibility of polypropylene. Cutting-edge nanotechnology is used to develop TiMESH with its ideal surgical mesh properties, including biocompatibility, resistance to infection and the ability to be recognized by the body as a solid

titanium implant. Results of the studies reporting the potential beneficial effect of titanium are controversial. Regarding biocompatibility, titanium layering of a polypropylene mesh implant to improve its biocompatibility is not well supported. Studies checking this hypothesis failed to prove it, and found no significant improvement of biocompatibility when analyzing the effect of titanium coating compared to the pure polypropylene mesh structure [20]. Regarding the strength and the lightweight of the titanium-coated mesh, a randomized clinical trial of patients who underwent inguinal hernia repair found that patients with the lightweight mesh returned to work after 4 days, compared with 6.5 days for the standard mesh ($P = 0.040$). The lightweight group returned to normal activity after 7 days, versus 10 days for the standard group ($P = 0.005$). There was no difference in postoperative pain or recurrence at the 1-year follow-up [21].

A different track, is to create a completely new nanomaterial. A good example of such a track is the study of Soliman *et al.* [22]. In this model, a novel (scalable) electrospinning process was developed to fabricate bioinspired multiscale three-dimensional scaffolds endowed with a controlled multimodal distribution of fiber diameters and geared towards soft tissue engineering. The resulting materials finely mingle nano- and microscale fibers together, rather than simply juxtaposing them, as is commonly found in the literature. Characterization of the microstructure (i.e., porosity, fiber distribution and pore structure) and mechanical properties (i.e., stiffness, strength and failure mode) indicated that the multimodal scaffold had superior mechanical properties in comparison with the controls, despite the large porosity (approximately 90% on average). A biological assessment was conducted with bone marrow stromal cell type. While the new material compared favorably with the controls with respect to cell viability (on the outer surface), it outperformed them in terms of cell colonization within the scaffold. The latter result, which could neither be practically achieved in the controls nor expected based on current models of pore size distribution, demonstrated the greater openness of the pore structure of the bimodal material, which remarkably did not come at the expense of its mechanical properties. Furthermore, nanofibers were seen to form a nanoweb bridging across neighboring microfibers, which boosted cell motility and survival. Lastly, standard adipogenic and osteogenic differentiation tests served to demonstrate that the new scaffold did not hinder the multilineage potential of stem cells.

An integrated function of the abdominal wall segment is the interface with the intra-abdominal viscera. This function is fulfilled physiologically with a complex membrane covering both the intra-abdominal viscera as well as lining the abdominal wall, the peritoneal membrane. Nanotechnology was used in manufacturing different materials aiming to minimize the adhesions of intra-abdominal viscera to the abdominal wall reconstruction area.

Seprafilm is a bioabsorbable membrane that is composed of two chemically modified polysaccharides: hyaluronic acid and carboxymethylcellulose. It was proven by many studies to decrease the rate of postoperative adhesions [23]. As the Seprafilm membrane decreases the inflammatory process to minimize

adhesion formation, it also inhibits wound healing based on initial phases of inflammatory response. It is advised by the manufacturing company that Seprafilm should not be wrapped around an intestinal anastomosis as such usage may result in increased anastomotic leak related events, such as abscess or peritonitis.

Zong *et al.* [24] evaluated the efficacy of nonwoven bioabsorbable nanofibrous membranes of poly(lactide-co-glycolide) for prevention of post surgery-induced abdominal adhesions. In a rat model, the electrospun PLGA/PEG-PLA membranes (PLGA and poly(ethylene glycol)/poly(D,L-lactide) (PEG-PLA) blends) impregnated with 5 wt% cefoxitin sodium (equivalent to approximately 10% of the systemic daily dose typically taken after surgery in humans) completely prevented cecal adhesions after cecostomy.

16.7 Future Directions

The current data clearly shows that we are far from obtaining the ideal replacement material for abdominal wall reconstruction. The complexity of the role of the abdominal wall and its static, flexible and dynamic function that varies according to different abdominal wall segments, cannot be currently replaced by any of the above discussed artificial materials. It is also clear from reviewing the evolution of this field that there is progress on understanding the complexity of the abdominal wall and the role of autogenous host cellular contribution of the ideal replacement material.

Consequently, when complex abdominal wall reconstruction is needed, a completely synthetic mesh replaced by biomaterials and nanotechnology is currently applied to improve the biocompatibility of these biological materials and increase their important characteristics of promoting cellular adhesions and growth.

We believe that future directions should go beyond abdominal wall reconstruction to abdominal wall regeneration. This goal only will be achieved with the assistance of pluripotent stem cells able to differentiate into new musculoskeletal tissue. The future material will be the one with integrated pluripotent stem cells, or one able to attract the host stem cells to the new material and allow their growth and differentiation into a new abdominal wall. The future material should provide the necessary temporary strength of the abdominal wall till cellular differentiation is complete. After the regeneration of the new abdominal wall, the new nanobiomaterial should be completely resorbed once a new abdominal wall is regenerated.

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Poly(Polyol Sebacate)-based Elastomeric Nanobiomaterials for Soft Tissue Engineering

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Abstract

A number of elastomeric biomaterials have been developed in the last fifteen years for soft tissue engineering. This review provides a comprehensive update on the most recent developments of elastomeric nanobiomaterials. The article is organized as follows. An introduction on tissue engineering is first provided. Then biodegradable elastomers used in soft tissue engineering is reviewed, focusing on soft elastomers, poly(polyol sebacate) (PPS). This is followed by a review on nanocomposites, with a focus on biocompatible elastomeric nanocomposites, including PPS-based nano-Bioglass® and nano-halloysite filled composites. The studies conducted on each of these nanomaterials are reviewed in great detail in term of materials science, technology and engineering. Finally the limitations of presently developed elastomeric constructs are summarized, the materials with highest potential of successful application are identified, and future directions of research are discussed.

Keywords: Elastomer, nanocomposite, degradation, mechanical property, bioceramics

17.1 Introduction

Tissue engineering is a multidisciplinary research field, involving medicine, biology, materials science and engineering. A major goal of tissue engineering is through biomaterial scaffold design that allows for containment of cells and signalling molecules as a transplantable construct, to enable regeneration of functional tissue in the host as an alternative to conventional organ transplantation and tissue reconstruction. Tissue engineering has grown rapidly over the past 40 years, drawing widespread attention as an adjunct to medical treatment for most tissue types, in particular those damaged or lost following debilitating health problems such as cancer and other disorders [1].

Biomaterials play a critical role in the engineering of tissue constructs, working as an artificial extracellular matrix and three-dimensional support environment

for cells (entrained *in vitro* or migrating *in vivo* from the host tissue) to regenerate a wound site [2]. Biomaterials for tissue engineering can include polymers, ceramics, composites, and occasionally metals, either of natural or synthetic origin [3]. Due to their mechanical versatility and greater similarity to the structural characteristics of tissue, polymers are the most popular biomaterials in tissue engineering, except in orthopedics [4]. Biodegradable polymeric biomaterials, which help the host body to repair and regenerate the damaged tissue with minimal long-term inflammation and technical issues associated with revision surgeries [5], have become the paradigm for most medical and related applications [6–8].

Huge efforts have been invested in the development of polymeric biomaterials that mimic natural tissues. Despite much preliminary progress, currently there are few tissue engineering products, especially synthetic ones, available for practical clinical use that can substitute soft and mechanically functional tissues such as muscle and connective tissue [9]. The precise reasons for graft failure in experimental animal studies and preclinical trials for such tissues are not fully understood, but include mechanical incompatibility of biomaterials with the tissue to be engineered [10].

Polymeric biomaterials are currently dominated by thermoplastic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL) and their blends or copolymers [11]. Although these biomaterials have been well characterized and fabricated to match the biochemical properties of soft tissues, there is generally a lack of mechanical compatibility between thermoplastic polymer implants and living tissues. In living tissue, elasticity and tensile strength are provided by the naturally occurring structural macromolecules, which include collagens, elastin and fibronectin. Table 17.1 lists the mechanical properties of some tissues. The elastic stretchability is a major mechanical property of biological tissues, but one that cannot be provided by thermoplastic polymers, since they undergo plastic deformation almost immediately and have elongation at break of typically less than 3% [12]. Indeed, for the repair of most soft tissue types, one of the major problems encountered by biomaterials scientists is the need for replicating their innate elasticity.

Table 17.1 Mechanical properties of some human soft tissues.

Tissue	Young's modulus (MPa)	Strain at break (%)	Ref.
Relaxed smooth muscle	0.006	300	13
Contracted smooth muscle	0.01	300	13
Aortic valve leaflet	15 ± 6	21 ± 12	14
Pericardium	20.4 ± 1.9	34.9 ± 1.1	15
Cerebral vein	6.85	83	16
Myocardium	0.2–0.5 (at the end of diastole)	>20	17–18

Over the past ten years, there has been increasing research activity toward the development of synthetic, biodegradable elastomers as transplantable biomaterials for tissue engineering. Among these elastomers, poly(polyol sebacate) (PPS) is a family of crosslinked elastomers, developed recently for application in soft tissue repair and regeneration [19]. To further expand the spectrum of mechanical properties of this family of elastomers, PPS-based elastomeric nanocomposites have been developed by our group. This chapter aims to provide an updated review of PPS-based elastomeric materials.

17.2 Poly(polyol sebacate) Elastomers

Poly(polyol sebacate) (PPS) is a family of ester-bond crosslinked elastomers. Unlike thermoplastic elastomers, chemically crosslinked elastomers gain their elasticity from a chemical bonding process that occurs between polymer chains when the compound is subjected to heat and pressure. During this process (often called “curing”), special chemical agents within the compound react to the heat and pressure to vulcanize the molecules together. Once cured, thermoset compounds obtain the necessary physical properties. Reheating thermoset compounds will not cause them to melt as thermoplastics do.

17.2.1 Synthesis and Processing of Poly(polyol sebacate)

Poly(polyol sebacate) (PPS) has recently been developed for medical applications by Langer's group at MIT [19]. A polyol is an alcohol containing multiple hydroxyl groups. Sebacic acid is a dicarboxylic acid with structure $(\text{HCOO})(\text{CH}_2)_8(\text{COOH})$ (Figure 17.1). A subclass of polyols is represented by the sugar alcohols, which are commonly used in the food industry. Glycerol, maltitol, sorbitol, and xylitol represent the common types (Table 2). The synthesis of PPS involves polycondensation (i.e., esterification) between carboxylic acids and alcohol groups (Figure 17.2). The primary $-\text{OH}$ groups at the two ends of polyol monomers react first with carboxylic acids, forming polymer chains at the early stage of polymerization (Fig. 17.2a). At later stages, there is a lack of primary alcohol groups in the reacting system such that those secondary $-\text{OH}$ groups (at the middle of polyol monomers) start to react with $-\text{COOH}$ groups of carboxylic acids to form ester bonds (i.e., crosslinks) between the polymer chains (Fig. 17.2b).

The synthesis of PPS is typically conducted in two steps: prepolymerization and final crosslink reaction. The prepolymer is formed by heating a mixture of polyol and sebacic acid at an inert gas atmosphere, which is purged through the

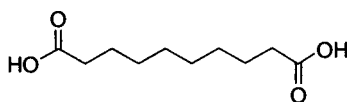


Figure 17.1 Molecular structure of sebacic acid.

Table 17.2 Some common types of sugar alcohols and their structures.

Sugar alcohol	Molecular structure
Glycol (2-carbon)	$\text{HO}-\text{CH}_2-\text{CH}_2-\text{OH}$
Glycerol (3-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Erythritol (4-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Threitol (4-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Arabitol (5-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Xylitol (5-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Mannitol (6-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$
Sorbitol (6-carbon)	$\text{HO}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}_2-\text{OH}$

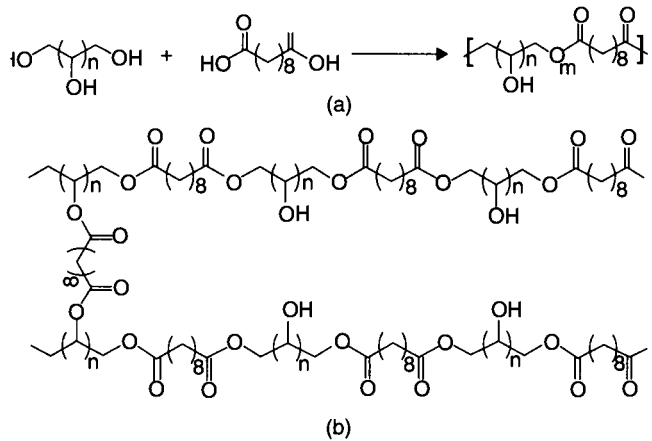


Figure 17.2 Synthesis diagram of poly(polyol sebacate) PPS. (a) Formation of a PPS polymer chain. (b) Crosslinking between two PPS polymer chains.

reacting chamber to prevent reactants oxidation and evaporation of the volatile polyol monomers. The prepolymerization conditions vary from 110–150°C and 1–14 hrs, depending on the polyol monomer. The combination of temperature and time of prepolymerization is such that the esterification reaction in the system is limited to the primary hydroxyl groups (with the carboxylic acids) to form polymer chains (Fig. 17.2a), with few secondary –OH groups available to cross-link chains. As such, the non-crosslinked prepolymer can melt at about 50°C or dissolve into solvents, such as methanol, acetone and tetrahydrofuran (THF). The prepolymerization is then followed by further curing for several days at the same temperature as the first step under vacuum. During this second-step synthesis, there is a lack of primary –OH groups in the reactant system such that the secondary alcohol groups on polymer chains begin to react with carboxylic acids, resulting in crosslinking between polymer chains (Fig. 17.2b).

Although the crosslinked PPS is neither soluble nor melted, rather it permanently remains in its final shape, the prepolymer of PPS (polymerized but uncrosslinked) can be processed into various shapes by being melting around 50°C or dissolved in organic solvents such as 1,3-dioxolane, tetrahydrofuran (THF), N,N-dimethylformamide, isopropanol and ethanol [20].

Among all PPSs, poly(glycerol sebacate) PGS is most well studied [20–22]. So far, PGS has been synthesized into sheets with printed microstructure (by lithography) [23], 3D porous scaffolds (by salt leaching technique) [24–25], and fibers [26]. Most recently, Freed and Engelmayer have successfully created an accordion-like honeycomb microstructure with PGS using an excimer laser microablation technique (Figure 17.3) [27]. This new process has produced a porous, elastomeric 3D scaffold that mechanically matches with the heart tissue in terms of stiffness.

17.2.2 Biocompatibility of PPS

As discussed, polyols of sugar alcohols are commonly used in the food industry. Sebacic acid is also a naturally occurring chemical derived from castor oil. In the industrial sector, sebacic acids have been used as plasticizers, aromatics, antiseptics, cosmetics and drug coatings. Polyol and sebacic acid are both endogenous monomers found in human metabolites [29–31]. Hence, It is not a surprise that PPSs generally have little toxicity to the host body [32]. Highly (or nearly fully) crosslinked PPSs also showed excellent cytocompatibility *in vitro* (Table 17.3).

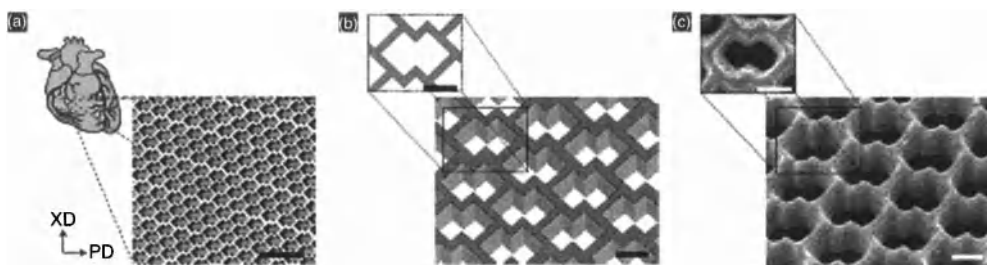


Figure 17.3 Computer design of accordion-like honeycomb scaffolds (a, b) and scanning electron micrographs of the synthetic accordion-like honeycomb design in PGS(c). Scale bars: (a) 1mm, (b, c) 200μm [28].

Table 17.3 Cytocompatibility of PGSs in vitro.

Polymer / molar ratio of polyol and sebacic acid	Synthesis conditions (Prepolymerization/crosslinking)	Cell type	Main results	Refs
PGS / 1:1	120°C, 1d / 120°C, 2d	NIH 3T3 fibroblast	The cells grew faster, with normal morphologies, when compared to PLGA as a positive control.	39
PGS / 1:1	150°C, 4h / 120°C, 3d 120°C, 8h / 120°C, 3d	Platelet (blood)	Relative to glass (100%), platelet attachment on ePTFE, PLGA and PGS was 61%, 100%, and 28%, respectively. Whole blood clotting profiles of PGS were comparable to or better than those of the reference polymers tested.	40
PGS / 1:1	120°C, 1d / 120°C, 2d	Schwann cells	PGS presented minimal cytotoxic effects and compared with PLGA.	41–42
PGS / 1:1	120–125°C, 1d / 120–125°C, 2–4d	• Mouse fibroblast • ESC-derived cardiomyocytes	• Soft PGS crosslinked for 2 days exhibited cytotoxicity • The lag phase of fibroblast proliferation was significantly longer on PGS than on PDLLA. • Cytotoxicity could be minimised by pre-conditioning. • Beating cardiomyocytes survived on preconditioned PGS.	21, 43–44
PGS / 1:1	160°C, 7.5 to 16 h	Neonatal rat heart cells	Accordion-like honeycomb PGS had mechanical properties more closely resembling native myocardium and promoted the formation of grafts with aligned heart cells and.	27

PGS / 1:1	120°C, 148h / 150°C, 15h	Bovine aortic endothelial cells	Cells cultured on substrates with smaller pitches exhibited a substantially higher frequency of cell alignment and smaller circularity index.	45
PGS / 1:1	120°C, 1d / 120°C, 2d	Vascular cells (Endothelial and smooth muscle cells)	Endothelial and smooth muscles cells both adhere to and proliferate on PGS films and in PGS scaffolds. Both cell types exhibit expected morphologic and phenotypic properties, and maintain their specific phenotypes under confluent conditions.	46–47
PGS / 1:1	120°C, 85h / 120°C, no accurate time	Mouse retinal progenitor cell (mRPC)	PGS held up mRPCs, which were retained through simulated transplantations. mRPCs adherent to PGS differentiated toward mature phenotypes as evidenced. Transplanted composites demonstrated long-term mRPC survival and migrated cells exhibited mature marker expression in host retina.	48–49
PGS / 1:1	120°C, 1d / 120°C, 2d	Chondrocytes	PGS supports cartilaginous matrix production by chondrocytes <i>in vitro</i> .	50
PXS / 1:1 and 1:2	150°C, 2h / 120°C, 4d	Human foreskin fibroblasts	Cells attached to and proliferated on PXS elastomers in a manner comparable to those on PLGA.	36
PSS / 1:1 and 1:2	150°C, 4–14h / 120°C, 5d	Human foreskin fibroblasts	Cells attached to and proliferate on PSS 1:2, but not on PSS 1:1 .	38
PMS / 1:1 and 1:2	150°C, 4–14h / 140°C, 5d	Human foreskin fibroblasts	Cells attached to and proliferate on PMS 1:2 and 1:4, but not on PMS 1:1 .	38
PMS / 1:4	150°C, 4–14h / 150°C, 5d			

17.2.2.1 *In Vitro Evaluation*

PGS is the most extensively evaluated member of the PPS family. The majority of *in vitro* data demonstrated that PGS has a good compatibility (Table 17.3). It is worthy of mentioning that the PGS scaffold of accordion-like honeycomb microstructure (Fig. 17.3) can guide the orientation of healthy cardiac cells [28], and under the electric field stimulation, heart muscle cells can grow and contract with directionally dependent electrical excitation thresholds [28].

Xylitol is a well-studied monomer in terms of biocompatibility and pharmacokinetics in humans [29–31, 33]. As a metabolic intermediate in the mammalian carbohydrate metabolism, xylitol enters the metabolic pathway slowly without causing rapid fluctuations of blood glucose levels [34–35]. Inspired by the good biocompatibility of xylitol and its multifunctional groups that allow formation of randomly crosslinked networks, Langer's group developed the xylitol-based elastomers PXS [36–37]. An *in vitro* evaluation on biocompatibility of PXS, PSS and PMS polymers showed that they supported the primary HFFs in terms of cellular attachment and proliferation with the exception of PSS and PMS that were synthesized at the ratio of 1:1 (polyol : sebacic acid) [38].

There are a few reports on the toxic effects of PPS on cells, as highlighted in Table 17.3. Apparently, the degree of cytotoxicity is associated with the crosslink density. For instance, soft PGS, which was prepared from equimolar monomers and crosslinked for a short time, was shown to be toxic to mouse fibroblasts, whereas the PGS of the same molar ratio but nearly fully crosslinked demonstrated excellent cytocompatibility [21, 43–44]. Under the same synthesis conditions, a high crosslink density can also be achieved by modification of molar ratio of the polyol and sebacic acid, such as in PSS (1:2) and PMS (1:2 and 1:4) [38]. This could explain why PSS (1:2) and PMS (1:2 and 1:4) have much improved cytocompatibility, compared with PSS (1:1) and PMS (1:1) [38]. It is possible that a highly crosslinked network degrades (via hydrolysis of ester bonds) slowly, and thus the concentration of potentially toxic degradation products in the environmental medium is sufficiently low to cause little cellular death.

Highly crosslinked PPSs tend to have compromised mechanical properties, such as brittleness. Hence there is a necessity to produce flexible PPS of low crosslink density. Actually it is hard to achieve a satisfactory balance of good cytocompatibility and compliance simultaneously in a pure PPS polymer. To improve cytocompatibility of soft PGS, as well as other properties such as the rapid degradation rate and suitable mechanical strength, PGS has been copolymerized with other polyesters [51–53], or made as a composite with bioceramics [43–44], which will be discussed further in Section 17.3 and Section 17.4, respectively.

17.2.2.2 *In Vivo Evaluation*

In vivo assessment of PGS was first conducted by subcutaneous implantation of 3mm-thick material in Sprague-Dawley rats [32, 39, 54] (Table 17.4). This evaluation showed that PGS induced an acute inflammatory response

Table 17.4 Biocompatibility of PPSs *in vivo*.

Polymer / molar ratio of polyol and sebacic acid	Synthesis conditions Prepolymerization/ crosslinking	Animal model	Main results	Refs
PGS / 1:1	120°C, 1d / 120°C, 2d	Subcutaneously in rats	Acute inflammation was observed, but no chronicle inflammation, whereas PLGS has both. Implants are absorbed completely within 60 days with restoration of the implantation sites to their normal architecture.	32, 54
PGS / 1:1	120°C, 42-144h	Subcutaneously in rats	Mild tissue responses were observed and the biocompatibility was excellent.	55
PGS / 1:1	120°C, 1d / 120°C, 2d	Sciatic nerve in Fisher rats	PGS showed a similar or lower level of fibrosis and inflammation, compared with PLGA.	41-42
PGS / 1:1	120°C, 1d / 120°C, 2d	Left ventricle of rats	No sign of inflammation caused by foreign bodies. PGS was completely absorbed in 6 weeks post implantation.	56
PGS / 1:1	120°C, 85h / 120°C, no accurate time	Subretinal transplantation in adult porcine eyes	The PGS component of the graft degrades without signs of inflammation. The remaining grafted retina survives well but often displays an atypical retinal architecture in the form of rosettes which can already be observed prior to transplantation <i>in vitro</i> .	48, 57
PXS / 1:1 and 1:2	150°C, 2h / 120°C, 4d	Subcutaneously in rats	PXS elastomers displayed a high level of structural integrity and form stability during degradation. The <i>in vivo</i> half-life ranged from approximately 3 to 52 weeks. PXS elastomers exhibited improved biocompatibility compared with PLGA implants.	36-37
PSS / 1:1 and 1:2	150°C, 4-14h / 120°C, 5d	Subcutaneously in rats	The acute inflammatory response is mild. The fibrous capsule formation (chronicle response) surrounding PXS is similar to or less than that surrounding PLGA.	38
PMS / 1:1 and 1:2	150°C, 4-14h / 140°C, 5d	Subcutaneously in rats	The acute inflammatory response is mild. The fibrous capsule formation (chronicle response) surrounding PXSis similar to or less than that surrounding PLGA.	38
PMS / 1:4	150°C, 4-14h / 150°C, 5d			

but no chronic inflammation, while PLGA caused both. The PGS implants in rats were completely absorbed after 60 days without scarring or permanent deformity in the histological structure [39]. Another *in vivo* investigation via subcutaneously implanted PGS films in the same species of rats has shown that PGS has an excellent biocompatibility, inducing only a mild inflammatory response [55]. *In vivo* applications of PGS in nerve [41], vascular [22–23, 40, 50] and myocardial [56] tissue engineering consistently shows little or mild foreign body responses in terms of both acute and chronic inflammations.

Subcutaneous implantation of PXSs in Lewis rats has shown improved biocompatibility when compared to PLGA implants [36]. Up to now, reports on PXS have indicated that these elastomers could be viable candidates as biodegradable medical devices that can offer structural integrity and stability over a clinically required period [37]. PSS and PMS polymers also exhibit better *in vivo* biocompatibility than PLGA, evidenced by mild acute inflammatory reaction and less fibrous capsules of chronic inflammatory response [38].

It must be mentioned that the soft PGS material shown to be cytotoxic *in vitro* (Table 17.3) did not demonstrate considerable toxicity *in vivo* [56] (Table 14.4), which may reflect differences in how the materials respond to static versus dynamic microenvironments, and with different cell types in an *in vivo* situation. Hence, the cytotoxicity revealed in a confined microenvironment *in vitro* does not necessarily lead to considerable toxicity *in vivo*, which is typically an open system. Nonetheless, the *in vitro* cytotoxicity matters when the biomaterial is intended to be used as a cell delivery vehicle, as the cells to be delivered will have to be cultured on the biomaterials substrate *in vitro* prior to implantation, to ensure their expansion and survival.

17.2.3 Biodegradation of PPS

Polymers containing hydrolyzable bonds (e.g., ester bonds) can be degraded by hydrolysis, a reaction that can be catalyzed by biological enzymes. PPSs are one of such degradable polyester system. In an *in vitro* study [20], PGS was reported to degrade up to 11–23% (in terms of weight loss) after 60 days agitation in PBS at 37°C. Similar degradation kinetics was also reported by Liang *et al.* who found that the weight loss of PGS and PGS/Bioglass® composites was around 10–25% after 60 days incubation in a standard tissue culture medium [44]. However, the above *in vitro* degradation rates of PGS-based materials are inconsistent with the degradation kinetics of PGS *in vivo*. Wang *et al.* report that PGS is completely resorbed after 60 days implantation in rats [32]. This comparatively faster degradation rate of PGS *in vivo* was also reported by Stuckey *et al.* who used PGS sheets as a pericardial heart patch [56]. They found that the PGS patch was completely resorbed after 6 weeks. These examples of *in vivo* degradation indicate that aqueous enzymatic action, combined with dynamic tissue movements and vascular perfusion, might enhance the enzymatic breakdown of ester bonds in PGS and thus facilitate the hydrolytic weakening of this material *in vivo*.

Since enzymatic complexes may have limited diffusion into a crosslinked polymer network, it is likely that enzyme-assisted degradation of PPSs *in vivo* occurs predominantly through surface erosion. This is indicated by steady and linear mass loss, similarity between percent size reduction and the percent weight loss [58], and preservation of implant geometry [59]. Hence, the degradation rate could be influenced by the surface area per mass of the medical device. Another important factor could be differences in degradation depending on the anatomic position of implantation in these two studies. In the work of Wang *et al.* the samples of PGS of 3 mm in thickness were implanted subcutaneously [32], whereas the PGS patches of 0.3 mm in thickness were epicardially grafted in the work of Stuckey *et al.* [56].

Most recently, an *in vitro* enzymatic degradation protocol was reported to be able to simulate and quantitatively capture the features of *in vivo* degradation of PGS-based materials [58]. In this study, PGS and PGS/Bioglass composites were subject to enzymatic degradation in tissue culture medium or a buffer solution at the pH optima, in the presence of defined concentrations of an esterase. The *in vitro* enzymatic degradation rates of the PGS-based materials were markedly higher in the tissue culture medium than in the buffered solution at the optimum pH 8. The *in vitro* enzymatic degradation rate of PGS-based biomaterials crosslinked at 125°C for 2 days was approximately 0.5–0.8 mm/month in tissue culture medium, which falls within the range of *in vivo* degradation rates (0.2–1.5 mm/month) of PGS crosslinked at similar conditions. Enzymatic degradation was also further enhanced in relation to cyclic mechanical deformation.

In a previous study [55], it was reported that the degradation rate of PGS materials is apparently not a function of the crosslink density, in contrast to our results. However, this conclusion is arguable. First, the PGS materials (crosslinked at 120°C for 42, 66, 90, 114 h following prepolymerization) used in that work seemed to have been fully crosslinked, except for the 42 h group. In our lab, PGS prepolymerized at 120°C for 24 h (with 70–80% water being condensed out) and cured for another 3 days or longer exhibited similar mechanical properties, indicating a saturated crosslink density. Second, the *in vivo* data reported in reference [55] showed reduced degradation rates in the groups cured for 66, 90 and 114 h, compared with the group cured for 42 h [55], and it is not surprising that there were no significant differences between the three groups cured for 66, 90 and 114 h, which might have a crosslink density similar to that in our study [3]. Finally, lipase was used in the *in vitro* investigation in reference [55], which is not the primary enzyme responsible for the degradation of polymeric implants *in vivo*. Rather esterase has been reported to play a key role in the degradation process of polymers *in vivo* [60–62].

In brief, PGS and the related PPS family are rapidly degrading polymers (several weeks) (Table 17.5). The rapid degradation is believed to limit their application as a scaffold material in engineering tissues that have a healing rate of several months or years (e.g., cardiac muscle). Hence, alternative chemistry approaches are needed to decrease the enzymatic hydrolysis rate of the ester bonds in PGS polymers [55]. The rapid kinetics do, however, match the healing rate of tissues like bone, with complete healing rates of 6–12 weeks [63].

Table 17.5 Biodegradation rates of PPSs and their copolymers in vitro or in vivo.

Form of polymer (molar ratio)	Synthesis conditions Prepolymerization/ crosslinking	<i>In vivo</i> or <i>In vivo</i> (animal model)	Degradation rate	Refs
PGS sheets of 0.5 mm in thickness(1:1)	125°C, 1d / 125°C, 2d	<i>In vitro</i> Enzyme-free PBS	Weight loss 17% after 60 days incubation in standard tissue culture medium	44
PGS sheets of ~ 1 mm in thickness (1:1)	125°C, 1d / 125°C, 2d or 7d	<i>In vitro</i> Esterase-mediated deg- radation in DMEM or boride buffer solution (pH 8)	- Degradation is significantly faster in DMEM than in the buffer solution. - Weight loss is 60–80% after one-month incu- bation in tissue culture medium, which is equivalent to 0.5–0.8 mm / month.	58
PGS blocks (1:1)	120°C, 1d / 120°C, 2d	<i>In vivo</i> (subcutaneously in rats)	Specimens of 3 mm in thickness were com- pletely absorbed within 60 days, which is equivalent to a rate of 1.5 mm / month.	32, 54
PGS sheets(1:1)	120°C, 42–144h	<i>In vivo</i> (subcutaneously in rats)	The mass loss rates of PGS were not a function of the degree of crosslinking in the network during the one-week implantation.	55
PGS (1:1)	120°C, 1d / 120°C, 2d	<i>In vivo</i> (sciatic nerve in Fisher rats)	N/A	41–42
PGS sheets of 0.3 mm (1:1)	120°C, 1d / 120°C, 2d	<i>In vivo</i> (left ventricle of rats)	Sheets of 0.3 mm in thickness were completely absorbed in 6 weeks after implantation, which is equivalent to a rate of 0.2 mm / month.	56

PGS / 1:1	120°C, 85h / 120°C, no accu- rate time	Subretinal transplantation in adult porcine eyes for one month.	N/A	48, 57
PXS / 1:1 and 1:2	150°C, 2h / 120°C, 4d	<i>In vivo</i> (subcutaneously in rats)	The <i>in vivo</i> half-life ranged from approxi- mately 3 to 52 weeks.	36–37
PSS / 1:1 and 1:2	150°C, 4–14h / 120°C, 5d	<i>In vitro</i> , PBS for 105 days	Mass loss: 2–22%, depending on the level of crosslinking. Generally, a PPS crosslinked to a higher level degrades more slowly than those crosslinked at a lower level. e.g, the degradation rate increases in the following orders: PMS1:4 < PMS 1:2 < PMS 1:1 PSS 1:2 < PSS 1.	38
PMS / 1:1 and 1:2	150°C, 4–14h / 140°C, 5d			
PMS / 1:4	150°C, 4–14h / 150°C, 5d			
PSS / 1:1 and 1:2	150°C, 4–14h / 120°C, 5d	<i>In vitro</i> , 0.1N NaOH solu- tion, for 50 days	Mass loss: PXS1:1, PXS 1:2, and PMS1: 4: 4%, PSS1:2: 25% PMS1:2: 80% PMS1:1 and PSS 1:1 : 100% in 20 days	38
PMS / 1:1 and 1:2	150°C, 4–14h / 140°C, 5d			
PMS / 1:4	150°C, 4–14h / 150°C, 5d			
PGS-co-LA	150°C, 6 h / 140°C, 30h	<i>In vitro</i> Enzyme-free PBS	After incubation in PBS for 80 days: PGS-0.25 molar ratio of LA: 40% mass loss; PGS and PGS-0.5 or 1 molar ratio of LA: 20% mass loss.	64

Note: polymers of different type have different crosslinking kinetics. Hence, it is not wise to compare any two types without quantified crosslink densities.

17.2.4 Mechanical Properties of PPS

The structure of PPS is analogous to vulcanized natural rubber and can provide mechanical stability and structural integrity to tissues and organs without mechanical irritation to the host. Their mechanical properties are directly related to the crosslink density in the network. In general, the Young's modulus and ultimate tensile strength (UTS) values of PPS network increase with increasing strand density, while elongation at break decreases with increasing strand density (Figure 17.4). In addition to their rubber behavior, these polymers generally have soft and flexible mechanical properties (Table 17.6) that make them suitable to work with soft tissue/organs in a mechanically dynamic environment, such as heart or lung [65].

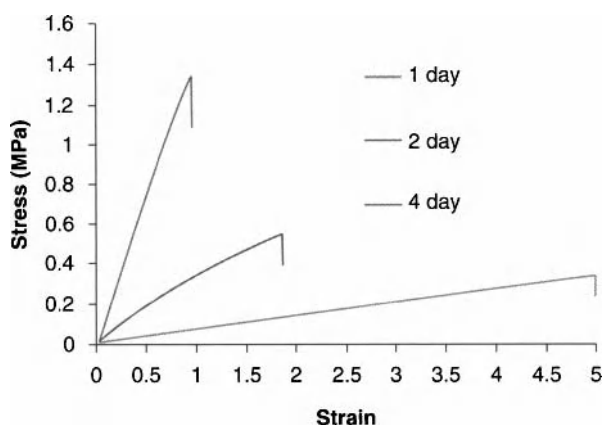


Figure 17.4 Typical stress-strain curves of PGS crosslinked at 125°C for 1, 2 and 4 days following prepolymerization at 125°C for one day.

Table 17.6 Mechanical properties of poly(polyol sebacate) and their copolymers.

Polymer	Young's modulus (MPa)	UTS (MPa)	Elongation at break (%)	Resilience	Ref.
PGS	0.05-1.5	0.3-1.5	40-500	> 98%	43-44, 66
PXS	0.8-5.3	0.5-1.5	30-200	N/A	38, 67
PMS	0.4-2.7	0.6-1.2	66-200	N/A	38
PSS	0.2-3.80	0.8-1.8	10-50	N/A	38
PGSA	0.05-1.38	0.05-0.5	42-189	N/A	52
PGS-co-LA	6-21	N/A	N/A	N/A	64
APS*	1.45-4.34	0.24-1.69	21-92	N/A	68

*APS = Poly (1, 3-diamino-2-hydroxypropane-co-polyol-sebacate)

17.2.4.1 *Poly(glycerol sebacate) (PGS)*

The Young's modulus of PGS is in the range of 0.056–1.5 MPa, and its elongation at break ranges from 40–450% depending on the synthesis conditions [69]. The above values of the Young's modulus of PGS cover those of many soft tissues, such as muscle (0.01–0.5 MPa) [70–71], skin (0.7–16 MPa) [70–71] and ligament (0.5–1.5 MPa) (72–74).

17.2.4.2 *Poly(xylitol sebacate) (PXS)*

PXS elastomers have displayed a Young's modulus range from 0.82–5.33 MPa and elongation at break between 33 and 205% by alteration of the stoichiometric ratios [67]. Except for PXS with a xylitol:sebacic acid ratio of 1:2, the mechanical properties of investigated PXS types decrease in an approximately linear manner with time [67].

17.2.4.3 *Other PPS Polymers*

The mechanical properties of another two members of this group, PSS and PMS have also been documented [38]. The PPS family collectively demonstrates a broad range of mechanical properties with ranges of Young's modulus of 0.5–380 MPa, UTS of 0.5–18 MPa and strain at break of 10–200% [38].

It must be noted that the stress-strain curves of synthetic elastomers are approximately linear at a low strain level (< 15%), while the stress-strain curves of biological tissues are nonlinear, showing an approximate J-shaped profile at the same level (Figure 17.5). Hence, the deformation profile of synthetic elastomers does not fit well with the J-shaped stress-strain curve of biological tissues.

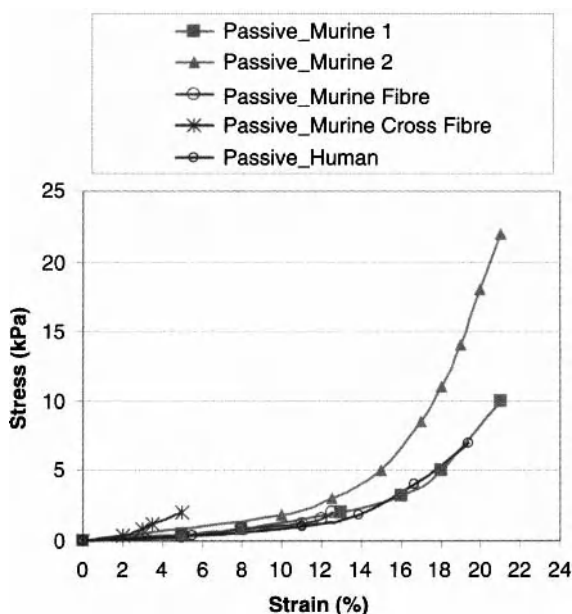


Figure 17.5 Typical passive stress-strain curves of human and murine heart muscle [75].

17.2.5 Applications of PPS in Tissue Engineering

The major reason why PPS is of major interest in tissue engineering and regeneration is that PPS polymers degrade by simple hydrolysis into metabolizable products, and have been reported to be nontoxic based on *in vitro* cell-based outcomes [23] and *in vivo* findings using animal models [76–77]. Although PGS (one of the PPS) has been found to produce leachates with significant cellular toxicity *in vitro* [21], the toxicity was not manifested *in vivo*. Acidic degradation products are not unique to PGS polymers; biodegradable thermoplastic polyesters have also been documented to behave in a similar manner, limiting their ability to serve as a vehicle for transplantable cells in many organ systems [78–79].

PGS is the most well-studied PPS elastomer, with specific benefits shown in cardiovascular tissue engineering [21, 40] and small diameter nerve grafting [80]. 45S5 Bioglass is one of few available degradable bioceramics [81]. Regarding concerns associated with calcification, studies have demonstrated that mineralization is unlikely to occur in a non-osseous tissue [82–84]. Implantation of Bioglass alone in rat muscle neither calcified the muscle nor caused abnormality in any of the major organs including heart, kidney and liver, in rat models [82–84].

17.2.6 Poly(polyol sebacate)-based Copolymers

In order to slow down degradation rates and improve the cytocompatibility of PGS, it has been copolymerized with acrylate and thermoplastic polyester lactic acids (LA). A series of PGS-co-LA polymers have been synthesized at different molar ratios, including 1:1:0.25, 1:1:0.5, 1:1:1 molar ratio of glycerol : sebacate : lactic acid [64, 85]. The copolymers are prepared by heating a mixture of the three monomers at 150°C under nitrogen for 6 h, followed by crosslinking at 140°C for 30 h, resulting in an elastomeric polyester.

Since the polymerization kinetics of lactic acid are relatively slow [86], it is not a surprise that the crosslinking kinetics of PGS-LA copolymers are markedly slower than the PGS counterparts. In addition to the intrinsic nature of LA, another reason behind the slow kinetics in the copolymer is the increased density of spaces between crosslinking positions [85]. The polymer chains in the PGS-co-LA system can have either -G-S-, -L-L- or -G-L-S- links, with crosslinking of the PGS polymer chains occurring between two secondary alcohol groups on the glycerol units (Figure 17.6a). Hence, the incorporation of lactic acid units in the polymerization reduces the frequency of glycerol-glycerol crosslinks between polymer chains simply due to the reduced number and availability of glycerol units in the chains (Fig. 17.6b).

Synthetic surgical sealants have also been developed from PGS and PGS-co-LA polymers [85]. The polycondensation of a glycerol and sebacic acid at a non-stoichiometric ratio of the triol, glycerol and the diacid (sebacic acid) produces a polymer with excess alcohol groups (33%). These additional polar groups endow the polymer with significantly improved adhesive properties. To produce a sealant that can be pasted as a liquid, it is crucial to achieve maximal polymerization before massive crosslinking occurs. The mechanism

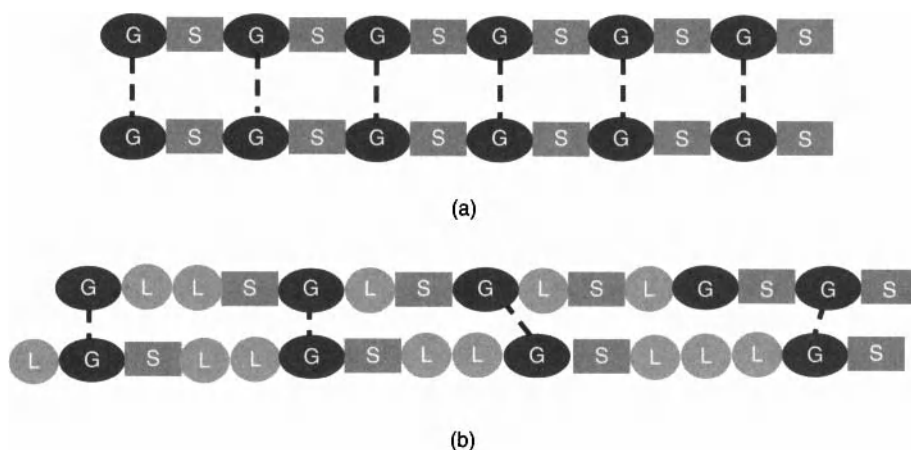


Figure 17.6 Schematic illustration of potential crosslinking points in polymers of (a) PGS of 1:1 molar ratio and (b) PGS-co-LA of 1:1:1 molar ratio.

to manipulate the level of polymerization versus crosslink density lies in the reaction kinetics of -OH groups of glycerol in the primary (at the two ends) and secondary (in the middle) positions (Fig. 17.6a). The primary -OH groups react much faster than the secondary ones at $100\text{--}150^\circ\text{C}$, such that the polymerization is dominated by the formation of PGS polymer chains. The crosslinking process, which involves the secondary -OH groups (Fig. 17.6a), become prominent only when there is a severe lack of primary -OH groups in the reactant system. Hence, a reasonably short synthesis time is the key to produce a virtually pure PGS polymer with few crosslinks. The incorporation of lactic acid monomers increases the spacing between glycerol monomers in polymer chains, thus retarding the crosslinking reaction and enhancing the maximal level of polymerization prior to massive crosslinking. As a result, a sealant with better adhesive strength can be achieved with PGS-co-LA than with PGS alone [85].

As expected, the incorporation of LA in PGS improves cytocompatibility of the PGS counterparts (at a similar level of polymerization) [85]. Moreover, PGS-co-LA elastomers inhibited platelet adhesion, prolonged whole blood clotting time, and activated partial thromboplastin time and prothrombin time [64].

Short-term degradation of PGS-LA copolymers in PBS results mainly surface erosion, but after long-term degradation *in vitro*, a combination of surface erosion and bulk degradation occurs. The degradation rate of PGS-co-LA at the molar ratio of 0.25 LA was shown to be fastest in PBS, and 40% mass loss occurred after 80 days. However, the degradation rate of PGS doped with LA is only slightly lower compared with that of pure PGS material, with mass losses of 18% (1:2 ratio) and 20% (1:1 ratio) after 80 days [64] (Table 17.6).

The Young's moduli of PGA-LA copolymers are higher than those of PGS polymers, being $6\text{--}21$ MPa [64]. Meanwhile, elongation of PGS-co-LA is reduced. However, the resilience of the copolymer remains as high as pure PGS polymer, being 98% or above [87].

17.2.7 Summary of PPS

The family of PPS elastomers generally possesses good biocompatibility and a broad range of mechanical properties. Their mechanical properties can be tuned by using a different polyol, altering monomer stoichiometry, changing curing conditions, and/or designing a PPS-co-polymer. Hence, PGS is regarded to be a very promising candidate biomaterial for many applications in soft tissue engineering. The challenges with PPS polymers include: 1) the development of methods of controlling their rapid degradation rates, 2) the achievement of a good balance between cytocompatibility and elasticity, and 3) the achievement of nonlinear-elasticity resembling that of soft tissues. The third issue is prevalent among all synthetic bioelastomers.

17.3 Elastomeric Nanocomposites

17.3.1 Introduction to Elastomeric Nanocomposites

From a biological perspective, it is a natural strategy to combine polymers and ceramics to fabricate scaffolds for osseous or ossified tissue engineering (e.g., bone, ossified tendon and ligament) because these tissues are the combination of a naturally occurring polymer and inorganic compounds, which imparts compressive strength, but compromises elasticity. From the materials science point of view, a single material type does not always provide the necessary mechanical and/or chemical properties desired for a particular application. For example, among PPS polymers with elongation to break of more than 20% (required for tissue engineering), the range of Young's moduli (0.05–13 MPa [78]) does not entirely cover that of living tissues, such as muscle (0.01–0.5 MPa), skin (0.7–16 MPa) or tendon (1.5 GPa) [88–89]. Although PPS polymer can be synthesized with a Young's modulus that is higher than the above 13 MPa, the elongation at break is compromised (< 11%) [78]. Thus it is difficult to achieve a high modulus without compromising the elongation to break and this is common to most materials, including elastomers.

In these instances, composite materials designed to combine the advantages of both materials may be most appropriate. Polymers and ceramics that degrade *in vivo* should be chosen for designing biocomposites for tissue engineering scaffolds. While massive release of acidic degradation products from polymers causes inflammatory reactions [90–92], the basic degradation of calcium phosphate or bioactive glasses can actually buffer the acidic by-products of polymers and may thereby help to avoid the formation of an unfavorable environment for cells due to a decreased pH. Mechanically, inorganic compounds are generally stronger than polymers and play a critical role in providing mechanical stability to constructs prior to synthesis of new extracellular matrix by cells. However, inorganic compounds are very fragile due to their intrinsic brittleness and flaw sensitivity. To capitalize on their advantages and minimize their shortcomings, inorganic compounds can be combined with various polymers to form polymer-based, inorganic filler-reinforced composite biomaterials.

For thermoplastics-based composites, reinforcement results in an increase in Young's modulus and hardness. The effect of inorganic filler, which is called Einstein's filler effect, is that they replace a part of the matrix and thus the Young's modulus of the composite material becomes higher. At the same time, however, deformation at break decreases [93].

The situation is very different for elastomers [94–96]. The addition of inorganic fillers, such as carbon black and silicate, increases Young's modulus and elongation at rupture, simultaneously. The replacement of a part of the deformation matrix by rigid filler particles does not reduce, rather increases its stretchability. The increase of these two antagonistic properties characterizes elastomeric composites [97]. Although the reinforcement of elastomers by particulate fillers has been extensively studied in the past, especially in the 1960s and 1970s, the reinforcement mechanism in elastomers remains largely undefined [97].

In contrast to the wide application of vulcanized natural rubber-based composites in the tire industry [93], the development of elastomeric composites used as biomaterials in tissue engineering began very recently. Most of the studies on rubber-based biocomposites aimed at the fabrication of 3D scaffolds from the composite biomaterial, and few have focused on the elastic properties of the composite. The first systematic investigation of mechanical properties of elastomeric biocomposites was reported on PGS-based bioceramic-reinforced composites in 2010 [44].

17.3.2 Thermoplastic Rubber-based Nanocomposites

There are a number of reports of polyurethane (PU) or Polyhydroxyalkanoate (PHA)-based bioceramics-filled composite scaffolds used for tissue engineering. However, most of these studies aimed at the development of 3D scaffolds from the material, and few investigations are focused on the material as an elastomeric composite. One of them is TiO_2 -reinforced poly(ethylene terephthalate)/dimer

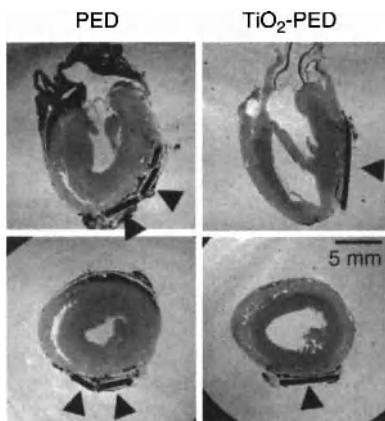


Figure 17.7 *Ex vivo* scaffold detection; MR microscopy images of PED and PED- TiO_2 scaffolds attached to the epicardium of rat hearts. Images are in the long-axis (top) and short-axis (bottom) orientation. Arrows indicate scaffold location [56].

fatty acid (PED-TiO₂) [98]. One of major drawbacks of thermoplastic elastomers is their heterogeneous degradation fashion [99–100], which is caused by the crystalline or high glass transition temperature regions of the thermoplastic that contribute to the strength but resist degradation. This heterogeneous degradation is presented as a localized, widespread loss of structure in a material with time (Figure 17.7) such that loss of mechanical strength is less predictable [56]. To address the issue associated with the premature mechanical failure is one of the driving forces behind the development of thermoplastic rubber-based composites.

Astypical elastomeric nanocomposites, the thermoplastic rubber composite PED/TiO₂ demonstrates the concurrent increases in mechanical strength (including Young's modulus and UTS) and rupture elongation upon the addition of inorganic filler (Figure 17.8) [101], which can be interpreted by Einstein's filler-effect and the bound-rubber theory. These strengthening mechanisms will be further discussed in more detail in Section 17.3 with respect to PGS/bioceramics composites.

The PED/TiO₂-based materials have been investigated as heart patches, and *ex vivo* trials using a rat model demonstrate that a good physical integrity absent in the PET rubber alone was achieved with the TiO₂-filled composite (Fig. 17.7) [56].

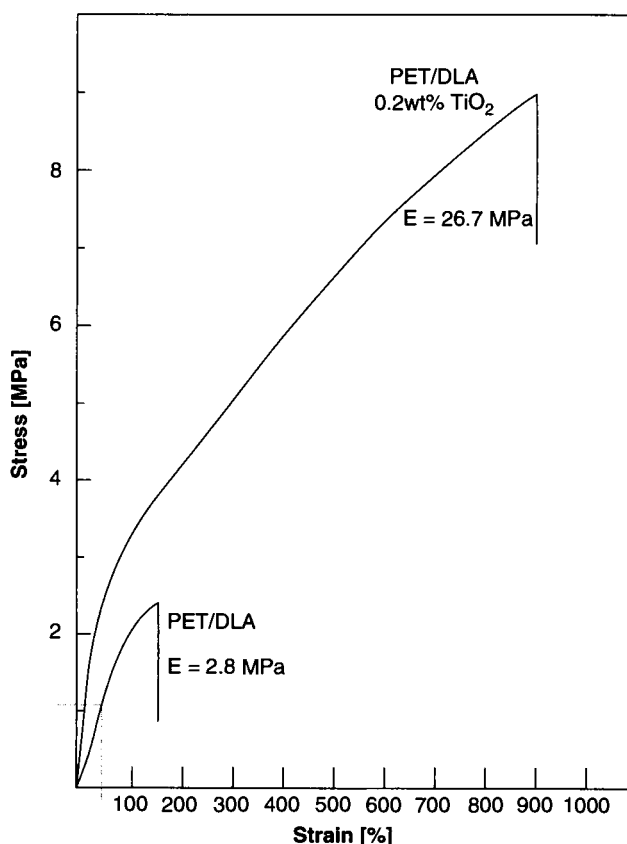


Figure 17.8 Stress-strain curves in tension for PET-DLA and PET-DLA/0.2 wt% TiO₂ biomaterials, Young's modulus values are 2.8MPa and 26.7MPa, respectively [101].

However, PED/TiO₂ composite was so rigid that it damaged heart muscle via surface friction, which resulted in increased chamber and scar size and reduced cardiac function. In contrast, mechanically compatible PGS effectively prevented postinfarction hypertrophy and reduced remodelling [56].

17.3.3 Crosslinked Elastomer-based Nanocomposites

17.3.3.1 *Synthesis of Nanocomposites*

PGS and POC have been composited with Bioglass [43–44] and hydroxyapatite [102], respectively. The crosslinked composites are prepared in two stages. Initially a prepolymer is synthesized by polycondensation under nitrogen gas at this stage, the reaction is incomplete and the prepolymer remains ungelled and could be dissolved in THF or melted. Various percentages of bioceramics (e.g., 45S5 Bioglass, hydroxyapatite, or halloysite) can be added to a solution or melt of the prepolymer at this stage, then mechanically stirred or extruded thoroughly. The slurry is further polymerized under vacuum to increase the crosslink density of the final material. For a filler of nanosized particles, melted prepolymer combined with an extrusion process is recommended, so as to minimize the incidence of bubble formation in the final composite product.

17.3.3.2 *Effects of Bioglass Filler on Crosslinking Kinetics of PGS Matrix*

The influence of bioceramic filler on the crosslinking rate of PGS matrix varies, depending on the acidity of the filler material and the ions released. In general, acidic fillers tend to slow down crosslinking progress, while alkaline fillers have the opposite effect if no other factors are involved. Silica, a weakly acidic filler, has been reported to reduce crosslink kinetics when incorporated in natural rubbers [103]. Another example is halloysite-filled PGS composite [104]. Halloyiste, 2SiO₂·2Al(OH)₂, is a biocompatible clay and has recently been investigated as a drug delivery vehicle [105–106]. The outer layer of halloysite nanotubes is dominated by SiO₂, while the inner layer of the cylinder core is Al₂O₃. Hence, the chemical properties of halloysite tubes are similar to that of weakly acidic SiO₂, and when halloysite is added to neutralized ferrihydrite, the pH of the mixture decreases down to 5.5–5.8 [107]. It has been reported that the addition of halloysite slows down the crosslinking rate in the PGS elastomeric network, and results in a lower crosslink density in the PGS matrix, compared with the pure PGS polymer [103].

Alkaline Bioglass filler, however, reduces the crosslink density in the PGS matrix compared with the pure PGS counterpart, due to metallic ions being released from Bioglass particles. A pure PGS system is crosslinked by ester groups. When the PGS prepolymer is heated at an elevated temperature with Bioglass, the esterification reaction occurs in competition with the formation of calcium dicarboxylate bridges [44] (Figure 17.9). This competition between esterification and metallic carboxylation lowers the concentration of ester bonds in the composites, compared with pure PGS. When exposed to an aqueous environment, the unstable calcium ionic linkages are dissociated in the presence of water, leaving a lower crosslink density in the composites [44].

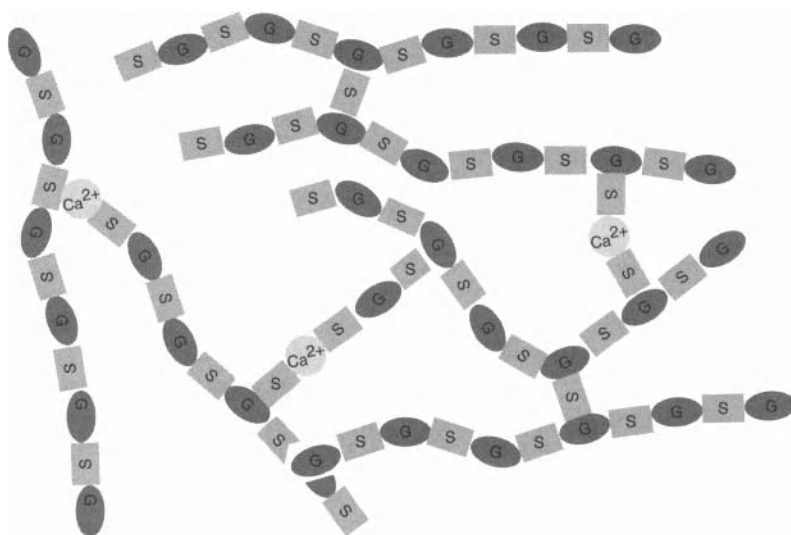


Figure 17.9 Schematic graph showing the crosslinks bridged by calcium carboxylate, as well as crosslinks by polyester bonds. G stands for glycerol, and S for sebacic acid.

17.3.3.3 *Effects of Bioglass Filler on Biocompatibility*

The basic degradation of bioactive glasses would buffer the acidic by-products of polymers and may thereby help to minimize the formation of an unfavorable environment for cells due to a decreased pH. This concept has been proved by the work on PGS/Bioglass composites [43–44], in which the acidity caused by the PGS degradation was alleviated by the dissolution of alkaline Bioglass filler, and as a result, the cytotoxicity of PGS/Bioglass is significantly lower than that of the pure PGS material.

17.3.3.4 *Effects of Bioglass Filler on Biodegradability*

The addition of Bioglass has two opposing effects on the degradation rates of PGS/Bioglass composites [58]. First, as mentioned above, Bioglass filler tends to reduce the ester-bond crosslink density in the PGS matrix during the crosslink treatment [44]. Therefore, the extent of enzymatic degradation of the ester bonds required to break up the network is less in the composites, so they degrade faster. Second, the Bioglass filler can also decrease the hydrolysis rate of the composites by buffering the pH of the surrounding medium, through the release of alkaline ions that neutralize the acidity of carboxylic acids.

If the first effect (crosslink density) is more prominent than the second one (pH), the degradation rate of the PGS/Bioglass composites will increase with increasing amounts of Bioglass filler. This is the situation with the PGS/Bioglass composites in a buffer solution, because the pH value of the surrounding environment is virtually unchanged in the buffer solution. In these circumstances, the decrease in ester group concentration and thus the crosslink density caused by the addition of Bioglass prior to immersion in the buffer lead to the increase in degradation rates with increasing amounts of Bioglass filler [58].

For the PGS/Bioglass materials soaked in the culture medium, the pH values of the medium were strongly influenced by the materials due to the ability of Bioglass filler to neutralize the acidity, which decreases the degradation rate of the composite. This explains why the degradation rate of PGS/Bioglass composites decreases with the amount of Bioglass filler added [58].

17.3.3.5 Effects of Bioglass Filler on the Mechanical Properties

As a typical elastomeric composite, PGS modified with the addition of Bioglass fillers increases its Young's modulus, UTS and elongation at rupture, simultaneously [43–44] (Figure 17.10). An interesting feature of PGS/Bioglass composites is that the mechanical strength of composite materials drops suddenly after one-day incubation in an aqueous solution, almost back to the strength level of pure PGS material. The dissociation of weak calcium dicarboxylate bonds in water is responsible for the rapid drop in mechanical strength. The significance of this mechanical behavior lies in that the improvement of biocompatibility can be achieved without compromising the compliance of elastomeric materials, as has been a challenge for pure elastomeric materials. Highly crosslinked elastomers have good compatibility but are rigid, while low crosslinked elastomers are soft but tend to be cytotoxic.

17.3.3.6 PGS/Halloysite Nanocomposites

In another work [108], nanotubes of halloysite, $2\text{SiO}_2 \cdot 2\text{Al}(\text{OH})_2$, which has been investigated as a drug delivery vehicle [105–106], is used as the reinforcing agent in a PGS matrix (Figure 17.11). As with bioglass filler, it is again found that the addition of nanotubular halloysite do not compromise the extensibility of material, compared with the pure PGS counterpart; instead the elongation at rupture is increased from 110 (in the pure PGS) to 225% (in the 20 wt% composite). Meanwhile, the Young's modulus and resilience of 3–5 wt% composites are ~ 0.8 MPa and $> 94\%$ respectively, remaining close to the level of pure PGS, which is desired for applications in soft tissue engineering.

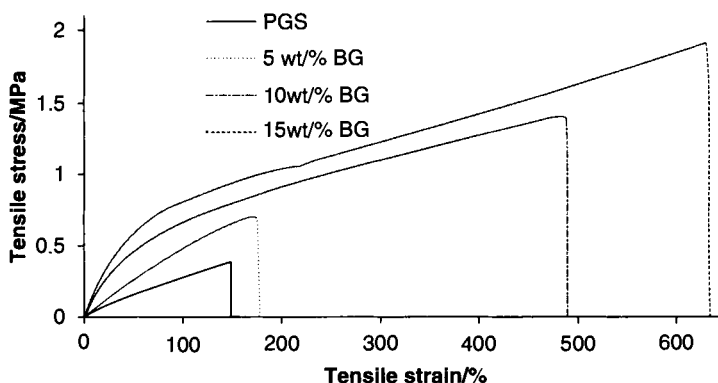


Figure 17.10 Typical tensile stress-strain curves of pure PGS and PGS composites of 5, 10 or 15 wt% Bioglass[®]. Note the mechanical strength and strain at rupture increased simultaneously with the addition of Bioglass filler.

Perhaps, one of important features of the 1–5 wt% composites is their stable mechanical properties over an extended period (Figure 17.12), which can allow the provision of reliable mechanical support to damaged tissues during the lag phase of the healing process. Hence, the good compliance, enhanced stretchability, and stable mechanical behavior over an extended period make the 3–5 wt% composites promising candidates for application in soft tissue engineering.

17.3.3.7 Bound-rubber Theory of Elastomeric Nanocomposite

The primary reason for the stiffening of the PGS composites by incorporation of ceramic microparticles is due to the Einstein's filler effect in which rigid particles hinder the movement of polymer chains and reduce the amount of readily extendable material in the specimen [109]. It is generally observed in metals, ceramics and polymers that an increase in modulus or strength usually occurs at the expense of elongation to break. However, for some elastomer-based

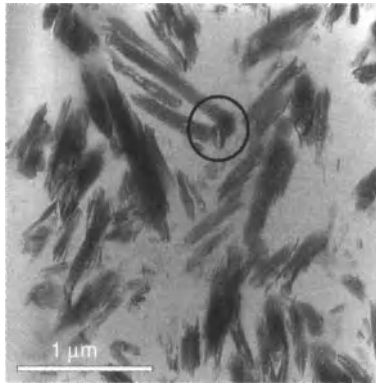


Figure 17.11 Representative distribution (by TEM) of halloysite nanotubes in the PGS/halloysite composites of 5 wt% concentration.

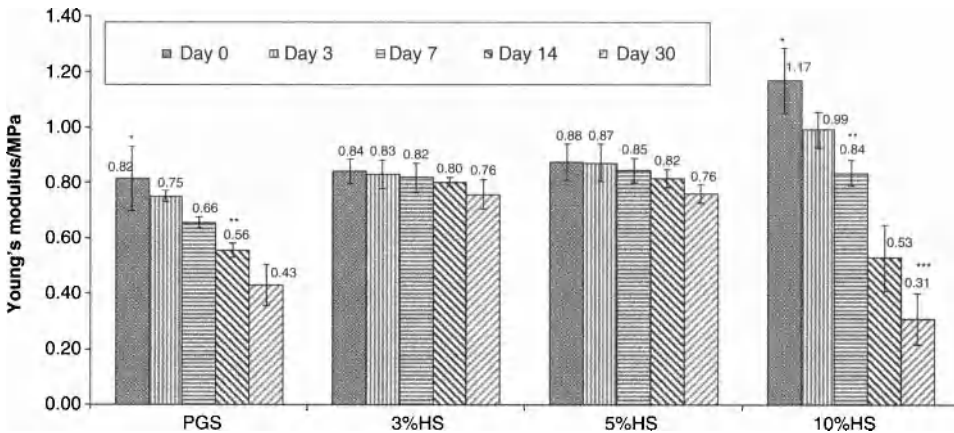


Figure 17.12 Young's modulus of PGS and its halloysite composites before and after incubation in tissue culture medium at 37°C under 5% CO₂ atmosphere. The data of 1 and 20 wt% showed a similar changing profile to that of PGS and the 10 wt% composite, respectively. The difference between (*) and (**) or (**) and (***) is significant ($p < 0.05$).

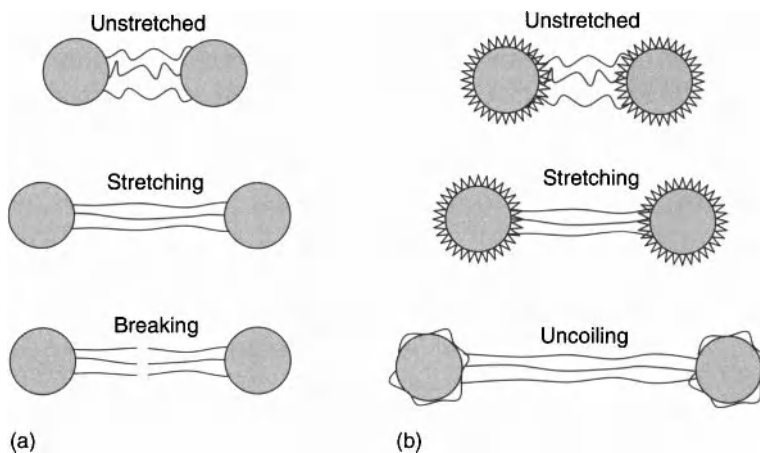


Figure 17.13 Schematic illustration of slippage mechanism of bound-rubber theory. The stretching behavior of polymer chains between two particles (a) without a bound-rubber layer; or (b) with polymer chains coiled on the surface.

nano-particulate-reinforced composites [110–111] a concomitant increase in elongation at break and Young's modulus has been reported. This is typically explained using the theory of bound rubber, in which macromolecules adsorb onto the filler's surface [112–113]. According to this mechanism, the adsorbed macromolecules can be uncoiled at higher stress, thus acting as an extra deformation source that can be activated at a high stress, resulting in additional elongation in bound-rubber composites (Figure 17.13). The bound rubber effect is influenced by the surface area of filler particulates, with smaller particles and larger ratio of surface area-to-volume producing a greater breaking strain, explaining why the bound rubber effect is seen most clearly in composites filled with nanoparticles [111].

17.4 Summary

Because the elastic stretchability is a major mechanical property of biological tissues, an increasing number of elastomers have been developed and examined for applications of tissue engineering over the past four decades. Pure elastomes have a limited spectrum of mechanical properties and degradation rates which, however, can be expanded by the incorporation of bioceramic fillers. Despite all the progress reviewed in this chapter, ideal elastomeric biomaterials with optimal properties which allow for an appropriate host response to ensure functional repair of damaged or diseased tissue and organs are still not available for most clinical applications, and significant challenges remain to be addressed:

1. Ideal degradation profile

The degradation kinetics of tissue engineering scaffolds must be a reasonable match to the healing rates of injured tissue. Too rapid a degradation

would lead to a mechanical failure of tissue repair under load-bearing conditions, whereas too slow a resorption process would create a barrier to the innate remodelling and fusion processes at the wound site. The time course of healing tissue exhibits three stages: lag, log and plateau phases [114–116]. Accordingly, ideal degradation kinetics of scaffolds that match the healing rate of growing tissue should possess three stages as well, i.e., steady state, rapid degradation and end of degradation, respectively. Such a three-stage degrading, mechanically suitable biomaterial has not yet been developed.

The challenge is two-fold. On the one hand, the healing rates of many tissues are unknown; on the other hand, there is only limited knowledge on the *in vivo* degradation kinetics of many biomaterials. Currently, most studies of the degradation kinetics of biomaterials are conducted *in vitro* in enzyme-free solutions [117], such as various buffer solutions, solvents or tissue culture media. However, the resorption of biomaterials, especially polymers, is frequently an enzyme-mediated process [118–119], with complexity that has yet to be satisfactorily predicted by any *in vitro* studies. Hence, to establish an *in vitro* experimental protocol that can simulate and thus predict *in vivo* enzymatic biomaterial degradation kinetics, or those induced by other biological processes, could greatly enhance their development.

2. Mechanical compatibility with host tissue

Despite much progress, the utilization of artificial substitutes to engineer tissue has been rather disappointing, with no successful examples of human applications to date [120]. The precise reasons for graft failure in experimental animal studies and preclinical trials are not fully understood, but include mechanical incompatibility of synthetic biomaterials with the tissue to be engineered [2]. To closely resemble a natural muscle, for example, the artificial substitute should ideally deform and reshape in the same manner as the muscle. However, the stress-strain curves of synthetic elastomers and their elastomeric composites are nearly linear at small strains (<15%), whereas those of muscles are J-shaped. The different mechanical deformation behaviors are attributed to the polymer chain structures, synthetic polymer chains being randomly tangled while macromolecules in biological tissues are generally well aligned and nanofibrous.

Hence, an approach to achieve nonlinear elasticity of biological tissues is to fabricate nanofibrous elastomers, with electrospinning being one of the most prevalent fabrication techniques toward this end. A major technical hurdle in electrospinning crosslinked elastomers is that these polymers cannot dissolve into any solvents for spinning once crosslinked, and any fibers spun from uncrosslinked prepolymers would be melted in subsequent crosslink treatment. To tackle this hurdle, core/shell electrospinning techniques have been applied [26]. However, many process parameters remain to be optimized.

3. Synthesis and processing of elastomeric nanocomposites

While providing mechanical support to injured tissue or organs and physical clues to guide the regeneration of tissues, drug delivery function is equally important in the design of a tissue engineering scaffold. Currently, the synthetic processes of most, if not all, elastomers are conducted at elevated temperatures ($> 37^{\circ}\text{C}$), a condition that is too harsh to incorporate drugs inside the material. Hence, to develop new synthetic techniques with the ability to incorporate and deliver proteins and growth factors without being denatured represents another challenge. Enzymatic synthesis could potentially make a breakthrough in this regard.

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Electrospun Nanomatrix for Tissue Regeneration

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Abstract

Recently electrospun nanofiber matrices have received a lot of attention and shown great potential in biomedical applications (e.g., in sutures, tissue engineering matrices, biosensors, and drug delivery devices). Nanofiber-based materials have several advantages compared to conventional fibers. In particular, they represent a very large surface area-to-volume ratio, high porosity and variable pore-size distribution. It is possible to modify the surface functionality and various morphologies can be achieved (i.e., nanotubes, etc.). The three-dimensional synthetic biomimetic scaffolds designed utilizing nanofibers serve as an excellent framework for cell adhesion, proliferation and differentiation. Physiochemical properties of nanofiber scaffolds can be governed by manipulating electrospinning parameters to meet the demands of a specific application. Many attempts have been made to modify nanofiber surfaces with several bioactive molecules to allow cells with the requisite chemical cues and a more *in vivo*-like environment. Therefore, electrospun nanomatrices have been used as scaffolds for musculoskeletal tissue engineering (e.g., bone, cartilage, ligament, and skeletal muscle), vascular tissue engineering, neural tissue engineering, skin tissue engineering and cardiac tissue engineering. Although in recent years there have been many scientific reports about the use of electrospun nanomatrices in biomedical application, this chapter will mainly focus on the recent developments and challenges for fabricating biomimetic, extracellular matrix (ECM) such as nanomatrices, that are useful for tissue engineering application.

Keywords: Electrospinning, nanomatrix, nanofibers, biomaterial, scaffold, extracellular matrix, tissue engineering

18.1 Introduction

Electrospun nanomatrices have gained a lot of attention, and show great potential in biomedical applications over the past several years. These nanofiber scaffolds represent a unique class of materials due to their three-dimensional

(3D) features that closely mimic the structure of the natural extracellular matrix (ECM) [1–3]. Nanofibrous systems may be suitable candidates as direct implantable biomedical devices for tissue regeneration due to their high surface-to-volume ratio features which also enable enhancement in cellular attachment, proliferation and efficient mass transport from the scaffolds. For examples, nanofibrous scaffolds have been manifested as desirable substrates for tissue engineering [4–7], wound dressing [8,9], artificial blood vessels [10,11], and immobilized enzymes [12–14]. They have also been used as vehicles for controlled delivery of therapeutic molecules [15–22]. The nanofibrous scaffold must demonstrate suitable physical and biological properties closely matching the desired necessities for a successful application to a specific target. For example, in tissue engineering, the electrospun scaffold should physically match the nanofibrous features of extracellular matrix (ECM) with desirable mechanical properties. It should also be able to promote cell adhesion, spreading and proliferation. The nanofibrous scaffold should not only serve as a substrate for tissue regeneration, but also should be used as a carrier for the delivery of suitable bioactive agents (drugs, proteins, DNA), within a controlled manner during healing.

Electrospinning is the most popular, simple, cost-effective way to produce continuous nanofibers of various materials from polymers to ceramics. Furthermore, electrospinning seems to be the only method which can be further formulated for large-scale production of continuous nanofibers for industrial applications.

In recent years the electrospinning technique, which was invented a long time ago, has emerged as a popular technique due to its potential use in the biomedical arena [23]. Pure polymers as well as composites and blends of both natural and synthetic polymers have been successfully electrospun into nanofiber scaffolds. The produced fiber's diameter is in the range of ~ 3 nm– $6\text{ }\mu\text{m}$ and several meters in length. So, nanomatrices have shown great potential in tissue engineering because of their high surface area-to-volume ratio, high porosity and variable pore-size distribution with structures mimicking natural extracellular matrix (ECM) [23–27].

18.2 Electrospun Nanomatrix

Electrospinning is one of the fanciest methods to fabricate nanofibers and was first patented by Cooley [28] and Morton [29] in 1902. An electrospinning device fundamentally consists of a high-voltage power supply, which creates an electric field between a collector and a syringe with a metal spinneret of small diameters filled with polymer solution as presented in Figure 18.1. Briefly, polymer jet is ejected from the surface of a charged polymer solution when a sufficiently high voltage is applied to liquid droplet, and the applied electric potential overcomes the surface tension of the polymer. Then, the ejected jet under the influence of applied electrical field travels rapidly to the

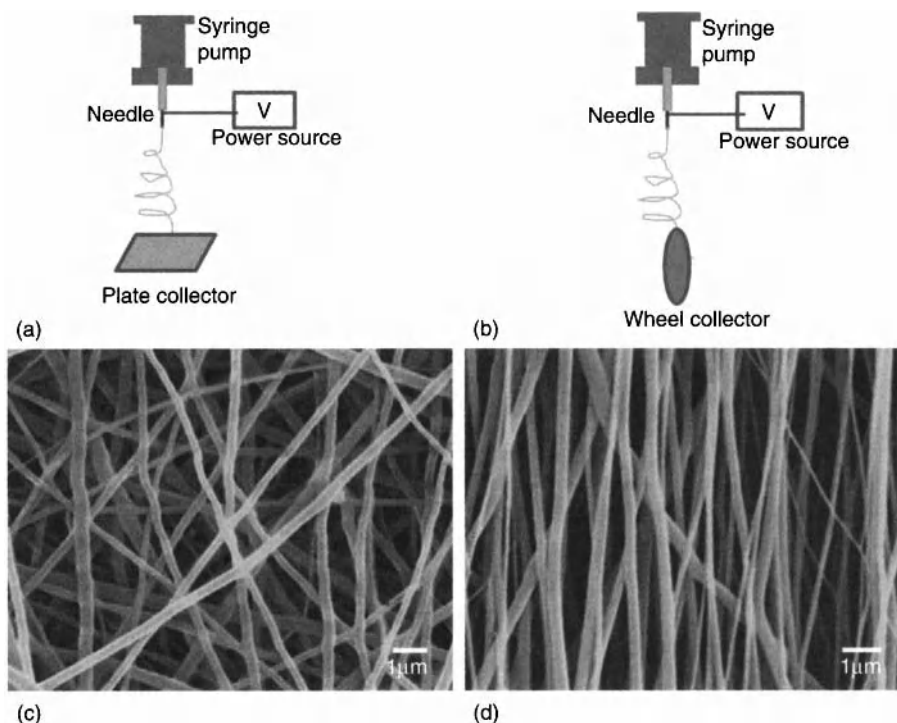


Figure 18.1 Schematics illustration of electrospinning setup and SEM morphology of nanofibers: (A) electrospinning setup with plate collector for fabrication of random nanofibers; (B) electrospinning setup with wheel collector for fabrication of aligned nanofibers; (C) SEM morphology of random PLGA nanofibers; (D) SEM morphology of aligned PLGA nanofibers.

grounded collector. The solution jet which is applied with electrostatic charges undergoes stretching before reaching the collector. Finally, the jet is eventually solidified on the collector in the form of nanofibers with evaporation of solvents (Fig. 18.1).

Usually nonwoven two-dimensional (2D) sheet are produced by electrospinning because the rate at which the polymer solution is ejected by the orifice must be controlled at a low value if nanoscaled fiber diameter is desired. But, if the electrospinning time is prolonged, 3D nonwoven fibrous sheet can be obtained. One efficient method to increase the thickness of the nonwoven sheet is to use more than one orifice simultaneously, in which the electrospinning speed will be proportional to the number of orifices. In the future this method may be helpful to develop 3D-nanofibrous scaffold in a short time period [31]. Kidoaki *et al.* prepared multilayer structured nanofiber mat by electrospinning different polymer solutions layer by layer [32].

Various polymers have been used in electrospun nanofibers. Biodegradable polymers such as poly(lactic-co-glycolic acid), PLGA or PCL and water soluble

biomaterials such as poly(ethylene oxide) (PEO) and polyvinyl alcohol (PVA) can be easily electrospun into nanofiber, utilizing water or organic solvent [33–36]. Natural polymers such as collagen [37, 38], silk protein [39–41], elastin-mimetic peptide [42], fibrinogen [43], casein and lipase enzyme [44], and DNA [45], have also been electrospun into nanofibers. Electrospinning of naturally occurring biomaterials is much more disputed compared with synthetic polymers because of the difficulties in finding suitable solvents. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) is a commonly used solvent for electrospinning of proteins [37, 45]. Silk fibroin electrospun nanofibers have been produced with formic acid as solvent [40, 41]. Silk fibroin can also be electrospun by mixing with PEO in water [42, 43]. Huang *et al.* applied a genetic engineering method to synthesize polypeptides containing the repeated elastomeric peptide sequence of elastin, (Val-Pro-Gly-Val-Gly)₄(Val-Pro-Gly-Lys-Gly). The polypeptide was dissolved in water and electrospun into nanofibers with diameters from 200–300 nm under appropriate conditions [42]. Fang and Reneker reported the electrospinning of calf thymus sodium-DNA aqueous solutions (concentration, 0.3–1.5%) into nanofibers with diameters of 50–to 80 nm [45].

18.3 Polymeric Nanomatrices for Tissue Engineering

18.3.1 Natural Polymers

Several natural polymers are used as biomaterials, such as chitosan, collagen, gelatin, hyaluronic acid, elastin, and silk [3, 37, 38]. Collagen nanofibers have been demonstrated to show compatibility with a number of cell types, including myoblasts and chondrocytes [3]. Huang *et al.* produced the nanofibers blend of type I collagen with poly(ethylene oxide) (PEO) by electrospinning. Their results indicated that the mechanical strength of the nanofiber system was significantly increased due to a high number of intermolecular interactions between collagen and PEO [42]. Their studies showed the promising role of collagen in tissue engineering.

Hyaluronic acid is an important component of the ECM of tissue and has been used as a biomaterial. Um *et al.* developed nanofibers of hyaluronic acid using the technique of electrospinning [46]. They reported that electrospinning of hyaluronic acid does not allow the uniform production of high-quality nonwoven nanofibers. Another important natural biomaterial that has been well studied is gelatin. Zhang *et al.* produced gelatin/PCL composite fibrous scaffolds using the electrospinning technique, and discovered that the composite nanofibers have improved mechanical strength and wettability compared with gelatin or PCL alone [105]. Furthermore, gelatin-PCL nanofibrous scaffold shows good cell attachment, growth, and migration of bone marrow stromal cells. Therefore, composite nanofibers of natural and synthetic materials could be a good selection for improving the mechanical properties of natural biomaterials for tissue engineering applications. Silk fibroin is another potential natural biomaterial for nanofibrous scaffolds because of their high

porosity and cytocompatibility. Therefore, a wide variety of natural polymers have been utilized for the formulation of nanofibers-based scaffolds for tissue engineering applications.

18.3.2 Synthetic Polymers

A wide variety of synthetic polymers have been utilized for the nanofibers formulation by electrospinning technique. These include poly(lactic-co-glycolic acid) (PLGA), which is an important polymer used to fabricate nanofibers for bone and cartilage tissue engineering and controlled delivery of therapeutic molecules [17]; PCL in neural and cartilage tissue engineering [35, 36], poly(lactic acid) (PLA) [47]; and poly(ethylene terephthalate) (PET) for blood vessel. Various copolymers such as poly(L-lactic acid-co-epsilon-caprolactone) (PLLA-CL) are used as a biomimetic ECM for smooth muscle and endothelial cells; poly(ethylene-co-vinylacetate) (PEVA) nanofibers for controlled drug delivery; and PLGA-poly(ethylene glycol) (PLGA-PEG) block copolymeric nanofibrous scaffolds as a matrix for DNA delivery.

18.4 Biocompatibility of the Nanomatrix

Although a large variety of biodegradable and nonbiodegradable polymers have been electrospun into nanofibrous scaffolds, many of them have low biological compatibility with cells, which has limited their use in advanced therapeutic applications. So, various biodegradable and nonbiodegradable synthetic nanofibers have been biofunctionalized with bioactive molecules for advanced therapeutic application. Synthetic polymers are more controllable and much easier to process in electrospinning than natural polymers. Hydrophilic polymers and natural polymers are frequently considered to be difficult to directly process into nanofibers due to their unstable nature to processing conditions and weak mechanical property [49]. A wide variety of natural polymers having unique biological functions can be immobilized onto the nanofibrous surface of synthetic polymers without compromising bulk properties due to the processing benefits of synthetic polymers. Such biofunctionalized synthetic nanofibers can directly enhance cellular interactions and organization, because the tissue regeneration process is strongly involved in various biochemical cues on the cell contacting surface.

Various surface modification techniques are being employed to synthetic polymer nanofibers to tissue engineering and drug delivery. Plasma treatment has usually been utilized to introduce the desired functionality onto the nanofibrous surface of an electrospun matrix. Plasma treatment improves the surface adhesion and wetting properties of the matrix [50–53]. Ideal selection of the plasma source enables the introduction of various functional groups on the target surface to improve biocompatibility or to allow subsequent covalent immobilization of various biomolecules. For example, various electrospun nanofibers made of polystyrene, poly(epsilon-caprolactone)

(PCL), PCL/ hydroxyapatite, and silk fibroin were surface-modified by air or argon plasma, resulting in an improved cell attachment and proliferation [51–54]. Poly(ϵ -caprolactone) (PCL) electrospun nanofibers were prepared and type-I collagen was then immobilized on the nanofibers after surface modification by plasma treatment. Collagen immobilization enhanced the adhesion, spreading and proliferation of human dermal fibroblasts cells [55]. Gelatin grafting onto plasma-treated PCL nanofibers improved endothelial cell proliferation as well as cell orientation [56].

Most of the synthetic biodegradable polymers retain their hydrophobic surface nature, frequently requiring hydrophilic surface modification for desired cellular responses. So, surface graft polymerization has been applied to introduce the surface hydrophilicity and the multifunctional groups on the surface for covalent immobilization of bioactive molecules for the purpose of enhanced cell attachment, proliferation, and differentiation [57–60]. Electrospun nanofibers of polyethylene terephthalate (PET) were modified with poly (methacrylic acid) by graft polymerization in a mild condition without any structural changes in the bulk phase [61].

Since most of the biomolecules are charged cationic or anionic polyelectrolytes, the immobilization of these biomolecules onto 3D-nanofibrous scaffolds can be carried out by a layer-by-layer self-assembly process [62–64]. For instance, nanofibrous PLLA scaffold was activated in an aqueous poly(diallyl dimethyl ammonium chloride) (PDAC) solution to obtain positively charged pore wall surface. The scaffold was subsequently immersed in a solution of negatively charged biomolecules (e.g., gelatin). Positive or negative charges are developed on the surface by alternative immersion in the two different solutions. Thus self-assembly approach is carried out in aqueous solutions under mild conditions and offers a controlled way to govern the surface charge type and the thickness of the surface modification layer [65]. Growth factors and DNA can be self-assembled onto nanofibrous scaffolds as a gene-delivery strategy to regulate cellular response and gene expression.

Functional polymer segments and nanoparticles can be directly exposed on the surface of nanofibers by coelectrospinning with bulk polymers [66–69]. For instance, when PLLA solution was blended with hydroxyapatite (HAp) nanocrystals, HAp was exposed on the surface of the resultant electrospun fibers, giving rise to high surface free energy and low water contact angle [70]. These composite fibers indicated a retarded degradation rate as compared to pure PLLA fibers.

18.5 Electrospun Nanomatrices for Tissue Engineering

Scaffolds are generally recognized as an essential element in tissue engineering and regenerative medicine strategies. They are temporarily used as guides for cell seeding, migration, proliferation and differentiation prior to the regeneration of biofunctional tissue or natural extracellular matrix (ECM). Preferably, to create an artificial tissue engineered construct capable of regenerating a fully

functional tissue, morphological similarity with the native tissue is important. Nanofibrous scaffolds with fiber diameter down to nanometer size are explored for their potential for tissue engineering by virtue of their high porosity, high surface area and the similarity of their 3D architecture to the natural ECM. Such physical cues enhance the cell adhesion, proliferation and differentiation, and consequently neo-tissue formation on nanofibrous meshes of both natural and synthetic polymers [71,72]. Several nanotopographical features have been created and employed as new generation tissue engineering scaffolds and biomedical implant surfaces [73]. The remarkable nanotopographic features of the nanofiber matrices allow cells the necessary physical cues similar to the nanotopographical features of a natural basement membrane.

Polymeric nanofiber matrices can also play a role as carriers for a variety of bioactive agents, such as growth factors, DNA, siRNA, etc. [74]. Core-shell nanofibers have been successfully designed to release the desired therapeutic agents at therapeutic concentrations in both a spatial and temporal pattern [74–76]. Recently Jayasinghe *et al.* have successfully demonstrated the capability of encapsulating living cells within core-shell microfiber matrices [77, 78] and they noticed that the electrospun fiber matrices showed significant numbers of viable cells over long periods of time. So, nanofiber matrices encapsulated with suitable growth factors, cells or bioactive agents have a great potential for use in tissue regeneration by providing cells with essential physical and chemical cues. Therefore, nanofibrous systems have been pursued as scaffolds for the regeneration of various tissues such as skin, bone, tendon/ligament, cardiac, blood vessel and nerve.

18.5.1 Bone Tissue Engineering

Although nanofibrous scaffolds have been well studied for multiple tissue types, musculoskeletal tissue is the most likely type studied. Various synthetic and natural materials have been utilized for bone tissue engineering. The physical properties of bone tissue such as mechanical strength, pore size, porosity, hardness, and overall 3D architecture are very important factors to design the scaffolds for bone tissue engineering. Scaffolds with a pore size in the range of 100–350 μm and porosity greater than 90% are favored for better cell/tissue ingrowth and hence enhanced bone regeneration [79, 80]. Yoshimoto *et al.* formulated nonwoven electrospun PCL scaffolds for the purpose of bone tissue engineering [81]. Bone marrow derived MSCs from neonatal rats were seeded on the nanofibrous scaffold to understand the influence of mesenchymal stem cells (MSCs) on nanofibers. The results concluded that the MSCs migrated inside the scaffold and abundant extracellular matrix was produced in the scaffold. Shin *et al.* investigated the PCL nanofibers along with MSCs *in vivo* in a rat model in a continuation to this study. They noticed the ECM formation throughout the scaffold along with mineralization and type I collagen synthesis [82]. These studies indicated that PCL-based electrospun nanofibrous scaffolds are potential candidates for bone tissue engineering.

Recently, Araujo *et al.* reported the potential application of electrospun PCL nanofibers that was surface-modified with calcium phosphate in bone tissue engineering. They noticed that calcium phosphate layers on nanofibers showed similar characteristics of human bones and the nanofibers strongly enhanced the osteoblastic differentiation and proliferation for a prolonged period of cell culture [83].

Venugopal *et al.* discovered that PCL provided mechanical stability, collagen supported cell adhesion and proliferation, and nanoHA (nHA) provided the mineralization of osteoblasts for bone regeneration. The osteoblast proliferation rate was increased by 35% and mineralized by 55% upon addition of nHA and collagen [84]. In another study, Meng *et al.* developed electrospun scaffolds of type I collagen and poly(3-hydroxybutyrate-cohydroxyvalerate) (PHBV). PHBV is a well known biodegradable, biocompatible, nontoxic, thermoplastic polyester produced by bacteria. The study suggested that the PHBV-collagen nanofibrous scaffold accelerated the adhesion and growth of NIH3T3 cells more effectively than PHBV nanofibrous scaffold [85]. Zhang *et al.* noticed that by incorporating gelatin with PCL, bone marrow stromal cells spread better and migrated deeper inside the gelatin/PCL scaffold. The cells infiltrated up to 114 μm in gelatin/PCL scaffold compared to 48 μm in only PCL scaffold [86]. Recently Shao *et al.* fabricated conductive random and aligned PLA/MWCNTs nanofiber meshes. Their results indicated that osteoblasts grew along the axis of aligned nanofibers and cells proliferation was slightly better on the aligned PLA/MWCNTs nanofiber meshes than those on random oriented nanofiber meshes (Figure 18.2). They also noticed that random oriented nanofiber meshes electrical stimulation significantly promoted elongation of osteoblasts, while on aligned nanofiber meshes the stimulation only slightly enhanced elongation along the nanofiber direction [87].

18.5.2 Cartilage Tissue Engineering

Over the last few years, nanofibers have been used as new functional structures for cartilage tissue regeneration [88]. Various synthetic and natural materials have been used to produce electrospun nanofibers for cartilage tissue regeneration, such as PCL, PLGA, chitosan-based materials, or starch-based materials [89–92]. Cartilage is an avascular tissue made up of chondrocytes entrapped in a rich extracellular matrix of collagen and proteoglycans [93]. Cartilage has a small capacity of self-regeneration potential, due to the absence of vascular networks and progenitor cells in the tissue [94]. Chondrocytes have been utilized to generate engineered cartilage tissue, [95, 96] and are generally isolated from articular cartilage tissues. Stem cells are also commonly proposed for cartilage tissue engineering, since chondrocytes are developmentally derived from stem cells [96]. Stem cells are easier to obtain and manipulate compared to adult chondrocytes, as they can undergo various passages before loosening their differentiation potential [97]. Selection of the ideal source of cells and the choice of the scaffolds is a demanding and challenging task for cartilage tissue engineering.

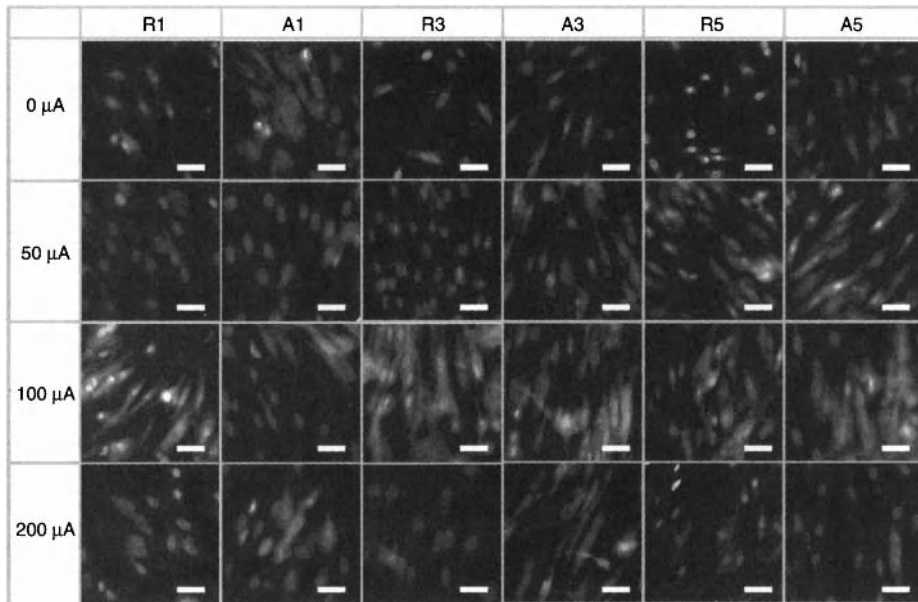


Figure 18.2 Fluorescence microscope images of osteoblasts cultured on random (R1, R3, R5), and aligned (A1, A3, A5)PLA/MWCNTs nanofibers meshes for 5 days with DC electrical stimulation 0 μA (ES-0), 50 μA (ES-50), 100 μA (ES-100), and 200 μA (ES-200). The nucleus was stained by DAPI (blue) and the cytoplasm was stained by Rhodamine 123 (red). All the scar bars means 50 μm [87]. Reprinted with permission from Elsevier.

A recent study evidenced the dissimilar chondrogenic differentiation behavior of MSCs derived from different tissues, namely, human embryonic stem cells, bone marrow, and adipose tissue. Furthermore, their behavior also differs among the tested silk and chitosan scaffolds [97]. Electrical and mechanical properties of the scaffolds are very important for cartilage reconstruction. So, conductive nanofibrous scaffolds were fabricated by electrospinning of biodegradable poly(lactic acid) (PLA) mixed with single-wall carbon nanotubes (SWNT) [98]. It was noticed that the SWNT incorporated nanofiber scaffold still allows cells to grow with no hostile influence on cell proliferation [98]

Wise *et al.* developed electrospun oriented polycaprolactone (PCL) scaffolds (500 or 3000 nm fiber diameter) and cultured human mesenchymal stem cells (hMSCs) on oriented nano and microfibrillar electrospun PCL scaffolds as well as a random porous PCL film to achieve a similar structure to articular cartilage [99]. Cell viability, morphology, and orientation on the fibrous scaffolds were decided as a function of time for biological investigations. They described that engineering an oriented extracellular matrix environment to regulate tissue alignment could be optimized by oriented electrospun nanofibers. Creating the superficial zone of articular cartilage may be dramatically improved by a combination of stem cells and nanofibrous scaffolds [99]. Recently, electrospun polycaprolactone (PCL) and starch compounded PCL (SPCL) nanofibers were

utilized to evaluate extracellular matrix (ECM) formation by bovine articular chondrocytes (BACs) [100]. Da Silva *et al.* investigated the suitability of PCL and SPCL nanofiber webs in chondrocytes cultures, and their potential to produce ECM when seeded onto these nanofibrous materials. They also investigated the effect of culture conditions (static vs. dynamic) on ECM formation and found that an extensive cell colonization of the entire nanofiber web was found for both materials. Some degree of cell infiltration inside the nanofiber webs was noticeable for both materials. PCL and SPCL nanofiber webs have potential for ECM formation and therefore are adequate for cartilage tissue engineering [100].

18.5.3 Ligament Tissue Engineering

The anterior cruciate ligament (ACL) is a vital ligament for movement and very important for knee stabilization. The ACL has inferior healing potential due to poor vascularization, and needs to be replaced after damage has occurred. Treatments that are presently available, grafts and prostheses, have host limitations, such as donor scarcity, donor-site morbidity, tissue rejection, disease transmission and poor long-term performance. Sahoo *et al.* investigated the supplementation of PLGA nanofibers on knitted PLGA scaffold for tendon/ligament tissue engineering. They found that knitted PLGA displayed the favorable mechanical properties and integrity for replacing a tendon/ligament while PLGA nanofibers possess a larger surface area and better hydrophilicity [101]. Therefore, nanofibers deposition on a knitted substrate would facilitate cell adhesion, new ECM deposition, and tissue formation without compromising mechanical integrity. Porcine bone marrow stromal cells proliferation was faster in the hybrid scaffolds compared to the control (knitted) scaffolds. Cells in the hybrid scaffolds also showed a higher expression of collagen I, decorin, and biglycan genes as well as an abundant production of ECM on the hybrid scaffold [101].

18.5.4 Skeletal Muscle Tissue Engineering

Skeletal muscle consists of approximately 48% of the body mass and controls voluntary movement of the body and maintenance of its structural shapes [6, 102]. Riboldi *et al.* investigated the use of degradable polyester urethane (PEU) electrospun scaffolds, DegraPol®, for skeletal muscle tissue engineering [6]. Cellular adhesion and proliferation of murine myoblast cell line (C2C12) on electrospun scaffolds is comparable with tissue culture plastic, which is implicative of no toxicity and excellent cell compatibility. Their results indicated that the electrospun microfibers of PEU showed satisfactory mechanical properties and advancing cellular response in terms of cell adhesion and differentiation. Based on these studies, the electrospun polyester urethane (PEU) shows potential to be further explored as a scaffolding system for skeletal muscle tissue engineering. Huang *et al.* seeded C2C12 cells onto a gelatin- or fibronectin-coated nonwoven electrospun PLLA fiber mesh (fiber diameter 500 nm) and found the disordered actin filament arrangement on randomly oriented fiber mesh; whereas actin fibers were aligned along the fiber direction

on aligned fiber meshes [103]. Myotubes attachment and formation of multi-nucleated myofibers were noticed along the nanofiber direction globally after seven days in differentiation media. Myotubes were extremely organized on the aligned mesh and significantly longer than those on randomly oriented scaffolds [103].

18.5.5 Skin Tissue Engineering

Tissue engineering is an alternative treatment for excessive skin loss compared to the traditional autografts and allografts. Out of the two layers of skin, the epidermis has less capacity to heal, but the dermis has enormous capacity to regenerate. Nevertheless, when large areas of the epidermis need to be replaced, normal regeneration is lacking. Scar tissue forms due to the absence of dermis lacking in elasticity, flexibility, and strength of the normal dermis [104]. Thus, scar tissue limits movement, causes pain, and is cosmetically undesirable. So, engineered skin tissue would be an excellent alternative, not only for wound dressings, but also to induce the regeneration of the dermis. Several other natural and synthetic polymers have been utilized for skin tissue engineering [104]. Min *et al.* utilized nonwoven silk fibroin nanofibers developed by electrospinning for skin tissue engineering and they noticed that type I collagen-coated fibroin nanofibers promoted keratinocytes/fibroblast adhesion and spreading due to their high porosity and high surface-to-volume ratio. Thus, the silk fibroin nanofibers showed potential as a scaffold for skin tissue engineering [39]. Khil *et al.* utilized polyurethane nanofiber matrices for wound dressing materials in a rat skin defect model that showed increased rate of epithelialization with well-organized dermis which allowed good support for wound healing [4]. Zong *et al.* noticed that PLAGA nanofiber matrices in a rat model depicted an excellent anti-adhesion effect [105].

Various natural and synthetic polymers, alone or in combination, were successfully electrospun into nanofiber scaffolds, and assessed as dermal substitutes with cells. Patel *et al.* investigated *in vitro* wound healing on heparin-coated poly(L-lactide) nanofibers, which was then used to bind laminin (an ECM protein) and bFGF, both of which promote growth and migration of dermal fibroblasts [106]. An artificial wound was created in a monolayer of human dermal fibroblasts, with the underlying scaffold either random or oriented parallel or perpendicular to the wound. Random scaffolds were furnished adequate wound coverage after 48 h. Nevertheless, wound filling was enhanced by perpendicular fiber alignment. In particular, the number of cells near the center of the wound was significantly higher and cells were hindered from entering the wound region for parallel fibers. Powell *et al.* produced bovine collagen nanofibrous scaffolds using either freeze-drying (FD) or electrospinning (EC) processes [107]. They noticed that no significant differences were detected in cell proliferation, surface hydration, or cellular organization between the freeze-dried collagen and electrospun collagen skin substitutes (ECSS). They also reported that electrospun scaffold was a better choice for skin substitutes than the freeze-dried one [107].

In another study, Rho *et al.* reported producing electrospun collagen type I nanofibrous matrix for the application of wound dressing. They studied three groups of nanofibrous scaffold: collagen nanofibers treated with collagen type I, collagen nanofibers treated with laminin and uncoated collagen nanofibers [108]. They examined the cytocompatibility effect, cell behavior, cell and collagen nanofiber interactions, and open wound healing in rats. They found relatively low cell adhesion on uncoated collagen nanofibers, whereas collagen nanofibrous matrices treated with laminin or type I collagen were functionally active in response in normal human keratinocytes. Rho *et al.* concluded that the treated ones with collagen or laminin showed better results among 3 types of scaffolds of electrospun collagen nanofibers [108]. Recently Liu *et al.* utilized the PLGA/collagen nanofibrous membrane for the wound healing application in rats [109]. They noticed that after three week of post surgery, the wound treated with PLGA/collagen nanofibers was almost healed with newly synthesized fibrous tissue and covered by re-epithelialized epidermis (Figure 18.3). On the contrary, the wounds treated with commercial dressing or gauze depicted incomplete re-epithelialization and inflammatory cell infiltration.

18.5.6 Vascular Tissue Engineering

Although attempts have been made to develop a small-diameter vascular graft (<6 mm), it is challenging due to thrombosis and intimal hyperplasia [110]. The seeding of endothelial cells onto the luminal surface of the graft is believed to be a possible route to improve the potential of small-diameter prostheses [111]. Endothelialization of a graft surface efficaciously depicts the surface to become antithrombogenic. The fabrication of biomaterials analogous to ECM can assist the cells adhesion, proliferation, and the phenotypic maintenance of endothelial cells on vascular scaffolds, since the endothelial cells adhesion occurs on the surface of an extracellular matrix (ECM) in nature [110, 111]. Nowadays, electrospinning is the most useful technique to develop the nanofibrous structures that mimic the tissue structures such as blood vessel. Mo *et al.* developed an aligned biodegradable PLLA-CL (75:25) nanofibrous scaffold using a rotating collector disc, and these aligned nanofibers were explored to fabricate tubular scaffolds that could be used for engineering blood vessels [112, 113]. Their results manifested that the nanosized fibers formed a well-defined architecture for smooth muscle cell adhesion and proliferation, and provide mechanical properties comparable to human coronary artery. Aligned fibers furnished the structural integrity and sufficient mechanical strength necessary to sustain the high pressure of the human circulatory system. They also found that smooth muscle cells cytoskeleton organization was along the direction of the nanofibers. These results indicated that aligned nanofibers might provide for a good scaffolding system for vascular tissue engineering [112–115].

Ma *et al.* developed electrospun PET nonwoven nanofibrous mat, and its surface was modified by grafting gelatin [63]. Their study showed enhanced spreading and proliferation of endothelial cells on the modified PET nanofiber

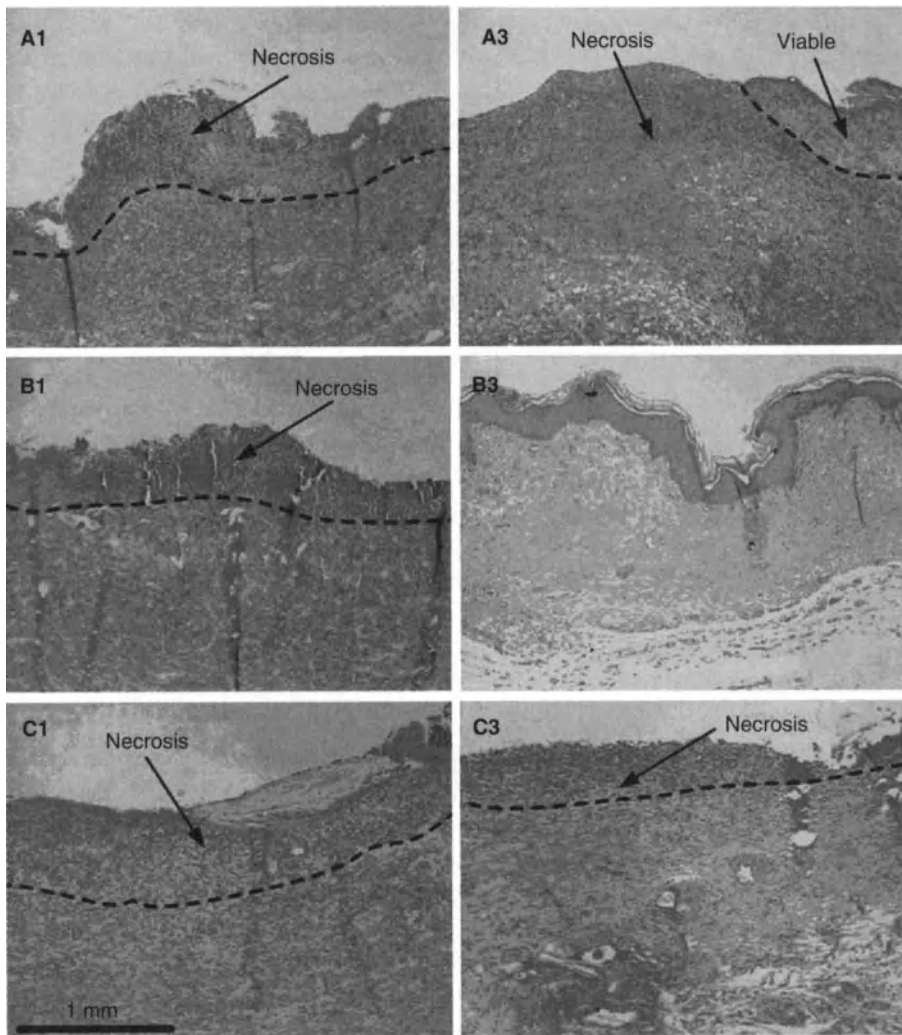


Figure 18.3 Histological images of (A) gauze group, (B) 50/50 PLGA/collagen nanofiber group, and (C) commercial dressing group, at 1 week (left) and 3 weeks (right). (No necrosis was observed in B3.) [109]. Reprinted with permission from Elsevier.

mats, while preserving their phenotype. Therefore, gelatin-modified PET nanofibers could be potential candidates for the engineering of vascular grafts [63]. Boland *et al.* formulated electrospun micro- and nanofibrous-scaffolds from natural polymers such as collagen and elastin with the aim of developing constructs for vascular tissue engineering [116]. Their results showed that electrospun collagen and elastin nanofibers were able to mimic the complex architecture needed for vascular constructs, and were also able to provide effective mechanical properties that were desirable in the environment of the blood stream [116]. Recently, Xu *et al.* prepared aligned poly(L-lactide-co-caprolactone) (PLLA-CL) copolymer nanofibrous scaffold for engineering blood vessel [114]

using a technique proposed by Theron *et al.* [117]. Their results demonstrated that the adhesion and proliferation rate of SMCs on the aligned nanofibrous scaffold were significantly improved as compared to using polymer films. They also noticed the attachment and migration of SMCs were along the direction of aligned nanofibers [117].

One of the major problems in blood vessel engineering is graft occlusion and failure. Luong-Van *et al.* embedded heparin in PCL electrospun fibers to prevent the vascular smooth muscle cell (VSMC) proliferation that can lead to graft occlusion [118]. They observed that fiber diameters decreased significantly with increasing heparin concentration. They studied the effect of heparin concentration on the diameter and water contact angles of electrospun PCL fibers. The result indicated that a sustained release of heparin from the fibers could be achieved over 14 days with controlled release diffusion during this time, and nanofibers did not induce an inflammatory response in macrophage cells *in vitro*. It suggested that electrospun PCL fibers are a promising candidate for delivery of heparin to the site of vascular grafts [118]. In another study, Stitzel *et al.* fabricated a biological vascular substitute from collagen, elastin and PLGA blend nanofibers using electrospinning [119]. The results showed the scaffolds possessed tissue composition and mechanical properties similar to native vessels, and it was concluded that the electrospun vessel matrix was biocompatible and did not elicit local or systemic toxic effects when implanted *in vivo* [119].

Williamson *et al.* prepared a new scaffold suitable for small-diameter vascular grafts that advanced strong attachment of endothelial cells [120]. They developed the scaffold in two steps, first the luminal surface was formed by wet spinning polycaprolactone (PCL) fibers, and then the vessel wall substitute was prepared by electrospinning porous polyurethane (PU) onto the back of the PCL fibers. Cell culturing evidenced strong attachment between human endothelial cells and the composite PCL-PU scaffold, and human endothelial cells were proliferated to form a monolayer with strong PECAM-1 expression. It was observed that the composite scaffold may also deliver bioactive molecules [120].

Jeong *et al.* produced a hybrid scaffold from marine source collagen and PLGA using freeze-drying and electrospinning as a vascular graft [121]. The hybrid scaffolds were fabricated by freeze-drying collagen to form a porous collagen matrix and using the electrospinning method to form a fibrous PLGA layer. This fibrous layer improved the mechanical strength of the collagen scaffolds in both the dry and wet states. These results indicated that the co-culturing of SMCs and ECs, using collagen/PLGA hybrid scaffolds under a pulsatile perfusion system, lead to the improvement of vascular EC development, as well as the retention of the differentiated cell phenotype. In a recent study, Mo *et al.* fabricated collagen-chitosan and poly(L-lactic acid-co-epsilon-caprolactone), (P (LLA-CL)) nanofibers by the electrospinning method for tissue engineering application such as blood vessel grafts. Results of the experiments showed that the mechanical properties of the collagen-chitosan complex nanofibers varied with the collagen content in the complex. It was also found that the biodegradability of P(LLA-CL) nanofibers was faster than its membrane and that smooth muscle cells (SMC) grew faster on collagen nanofibers than on P(LLA-CL) nanofibers [122].

Zhang *et al.* evaluated the electrospun silk fibroin scaffolds for vascular cell growth *in vitro*. Human aortic endothelial (HAEC) and human coronary artery smooth muscle cell (HCASMC) were cultured on electrospun silk fibroin scaffolds. Results demonstrated the formation of ECM for the HCASMCs based on the quantification of collagen type I expression at protein and transcription levels [123]. The authors found that results indicate a favorable interaction between vascular cells and electrospun silk fibroin scaffolds. Electrospun silk fibroin scaffolds supported the growth and expansion of human aortic endothelial cells and human coronary artery smooth muscle cells based on cell proliferation, morphology and phenotype studies *in vitro* [123].

Tillman *et al.* prepared electrospun polycaprolactone collagen scaffolds for vascular tissue engineering [124]. They evaluated the *in vivo* stability of the resulted scaffolds in a rabbit aorta-iliac bypass model. Their results showed that electrospun scaffolds supported adherence and growth of vascular cells under physiologic conditions, and that endothelialized grafts resisted adherence of platelets when exposed to blood. It was also noticed that when the scaffolds implanted *in vivo*, they were able to retain their structural integrity over 1 month of implantation as evidenced by serial ultrasonography. The authors proposed that electrospun nanofibrous scaffolds combined with vascular cells might become an alternative to prosthetic graft for vascular regeneration [124]. Recently Lu *et al.* suggested that aligned PLLA fibers could be excellent scaffolds for vascular tissue engineering [125]. They observed that the aligned PLLA fibers promoted and guided the sustained proliferation of outgrowth endothelial cells (OESc).

18.5.7 Nerve Tissue Engineering

Neural tissue repair is much disputed in tissue engineering, since almost all neural injuries lead to an irreversible loss of function. Peripheral nerve injuries, diseases or tumor surgery demand grafts to bridge the proximal and distal nerve ends. Autografts are the present available treatments for nerve tissue repair, however it is often limited in supply, requires a prolonged surgical time, and can cause donor site morbidity. Recently, a variety of conduit materials both natural and synthetic have been used as a nerve graft. Various studies have been carried out to see the effect of nanofiber diameter and alignment in terms of neurite outgrowth and neural stem cells (NSCs) differentiation.

Yang *et al.* investigated the efficacy of aligned PLLA nanofibrous scaffolds in nerve tissue engineering by utilizing neonatal cerebellum C17.2 stem cells [126]. They reported that NSC's elongation and its neurite outgrowth were in the direction of PLLA aligned nanofibers, and the rate of NSC differentiation was higher on PLLA nanofibers than on PLLA microfibers [126]. Patel *et al.* noticed that surface-modified PLLA electrospun aligned nanofiber matrices with ECM proteins and bFGF growth factor simulated the natural ECM environment by providing necessary physical and chemical cues [106]. They also observed that the aligned nanofibers dramatically induced the neurite outgrowth compared to randomly oriented nanofibers [106].

Schnell *et al.* reported that both random and aligned nanofiber scaffolds of PCL and collagen/PCL (C/PCL 25:75 wt%) blend supported cell attachment, Schwann cell migration and axonal regeneration [127]. Ahmed *et al.* discovered that glass coverslips coated with polyamide electrospun nanofibers in the diameter range of 100–800 nm supported neuronal attachment and neurite outgrowth *in vitro* [128]. They also noticed that surface modification of these nanofibers with neuroactive peptides derived from human tenascin-C significantly enhanced the ability of the nanofibers to facilitate neuronal attachment, neurite generation and neurite extension *in vitro* compared to poly-L-lysine-coated coverslips [128]. Itoh *et al.* employed electrospun chitosan nanofiber mesh tubes and tendon-chitosan tubes for bridge grafting in the rat sciatic nerve model, and autografts were used as control [129]. Histological evaluations after four and eight weeks showed significantly improved performance for chitosan nanofiber mesh tube groups over both the tendon chitosan tube and the autografts [129]. Yang *et al.* observed that aligned nanofibers scaffolds were better suited for culturing nerve stem cells *in vitro* than scaffolds of random microfibers [7]. Recently, Xia *et al.* investigated neurite outgrowth on nanofiber scaffolds with different orders, structures, and surface properties and noticed that aligned electrospun nanofibers had been demonstrated to provide guidance for neurite extension. Nevertheless, neurite outgrowth plays a critical role for nerve regeneration and guidance of neurite extension. However alone it is insufficient for promoting nerve regeneration. It can be efficiently enhanced utilizing electrospun nanofibers functionalized with bioactive molecules such as ECM proteins (e.g., laminin and fibronectin), neuroactive peptides, or growth factors (e.g., basic fibroblast growth factor, bFGF, and nerve growth factor, NGF) [130].

18.6 Status and Prognosis

In summary, mimicking the architecture of ECM is one of the great challenges of tissue engineering. Nanofibers have shown the most anticipated results amongst all the approaches used to prepare synthetic ECM. Nanofiber matrices have been used as scaffolds with a high large surface area, porosity, surface functionality, and superior mechanical properties. These amazing properties impart nanofiber matrices a structural and functional similarity to the native ECM structure and show significant cell functioning both *in vitro* and *in vivo*. Hence nanofibrous scaffolds are currently being explored for tissue engineering, including bone, cartilage, ligament, skin tissue engineering, vascular tissue engineering, neural tissue engineering, and controlled delivery of therapeutic molecules (drugs, proteins, and DNA). Currently, although numerous studies have evidenced the great potential of electrospun fibrous materials for tissue engineering applications, few *in vivo* studies have been carried out. Efforts are required to address a few challenges such as improving mechanical properties, avoiding use of organic solvents and scale up production. In general, several fluorinated and organic chlorinated toxic solvents are utilized in electrospinning to dissolve polymers and bioactive agents during nanofiber fabrication. These toxic solvents might affect the structural conformation changes of various polymers

and bioactive agents, and result in undesired cellular response. Nevertheless, complete removal of traces of organic solvents from the nanofiber matrices is doubtful. So, substituting these toxic organic solvents with less hazardous and aqueous-based solvents is essential. Another important attempt is required to improve the surface properties of nanofiber matrices to elicit better cell response that may include immobilizing several bioactive agents. Furthermore, it is also critical to understand the cellular mechanism of electrospun materials with different composition, fiber orientation and microstructures. Addressing these issues can encourage the efficacy of the nanofiber-based product development, and can lead directly to the development of commercially viable biomedical products that may improve the quality of life in the near future.

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Conducting Polymer Composites for Tissue Engineering Scaffolds

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Abstract

Tissue engineering is a novel technique used to replace or regenerate any damaged part of the body or the body's tissue. The main challenge in tissue engineering is the development of a scaffold which can mimic the extracellular matrix and help in cell growth and proliferation. Different kinds of materials have been tried so far for the development of scaffolds including natural and man-made biological molecules. Nowadays the use of conducting polymers and carbon materials like carbon nanotubes (CNTs) is also a matter of great interest. Conducting polymers such as polypyrrole, polyaniline, poly(3,4-ethylenedioxythiophene) (PEDOT) and polythiophene and its derivative provide good electrical conductivity, whereas CNTs provide high mechanical strength. Certain tissues like that of the heart and brain require electrical stimulus. At these sites the use of conducting polymers as scaffolds act as promising materials. The CNT, on the hand, due to its mechanical strength helps in bone tissue regeneration. Different types of scaffolds, in the form of films, foams, hydrogels and fibers have been tested so far, and a variety of studies ranging from *in vitro* to clinical applications have been carried out. Of all these different forms, nanofibers are of the greatest interest because they provide 3D support for cell growth and attachment. Electrospinning is a novel technique to fabricate such nanofibers which help in cell support and proliferation. In the future it is also reasonable to expect the development of composite nanofibers of CNT with conducting polymers and biological materials for use in tissue engineering scaffolds.

Keywords: Conducting polymers, carbon nanotubes, smart polymers, composites, tissue engineering, regenerative medicines, biocompatibility

19.1 Introduction

Tissue engineering deals with an interdisciplinary field of science and technology which applies the principles of biological, chemical and engineering for the replacement or regeneration of a tissue in the biological system. Earlier tissue engineering was considered a part of biological science, but due to much demand in recent years, tissue engineering now has its own branch. A scaffold is a substrate which provides better surface for cell attachment and helps cell growth and its differentiation. Hence, in overall tissue engineering scaffold is the development of such a substrate that helps cell attachment, growth, and differentiation and which can easily replace or regenerate a damaged tissue.

The main challenge in tissue engineering is to develop a scaffold which can provide a better surface for cell attachment, growth, and differentiation, and when implanted into the biological system, would not affect normal functioning of the cell or other body part. The other condition is that it should be biocompatible and should not leach any cytotoxic substance inside the body. It should be biodegradable so that it can be easily removed from the body. Biomaterials play an important role in the development of such a scaffold to help in cell growth and attachment. But nowadays conducting polymers and their composites with biomaterials or biological compounds are also used in tissue engineering for the development of these scaffolds. In this chapter we will discuss the conducting polymers, carbon nanotubes (CNTs) and thermo responsive polymeric materials used as tissue engineering scaffolds. The future prospects of these materials will also be discussed later in the chapter.

19.2 Conducting Polymers

Conducting polymers (CPs) are a special class of polymeric materials which have high electronic and ionic conductivity, porous structure and good process ability. Due to these properties they are used in a variety of applications in the dry or wet state [1, 2]. Conducting polymers, as their name suggests, were used in the field of energy storage and electrical applications earlier. But now there are varieties of biomedical applications for conducting polymers including the development of artificial muscle [3], controlled drug delivery [4, 5], neural recording [6] and the stimulation of nerve regeneration [7]. Different conducting polymer like polypyrrole (PP), polyaniline (PANI), poly(3,4-ethylene dioxythiophene) (PEDOT) and polyacetylene (PA) have been used up till now for various biological applications. Table 19.1 explains some biological applications of various conducting polymers. Out of the various applications, the use of conducting polymers in the field of tissue engineering is very important because the electrical stimulation of conducting polymers help in cell growth and attachment. For the tissue which requires electrical response like the brain, heart and other skeletal tissue, the use of conducting polymers at that site are highly promising because of their good conducting properties.

Table 19.1. Various biological applications of some conducting polymers.

Conducting polymers	Applications
Polyaniline	Biosensors, Drug delivery, Bioactuators, DNA recognition, Cardiovascular applications, Tissue engineering scaffolds.
Polypyrrole	Biosensors, Drugdelivery, bioactuators,Neural prosthetics, Cardiovascular applications, Tissue Engineering, Cell Culture and Stimulation.
Poly (3,4- ethylene dioxythiophene)	Biosensors, Antioxidants, Controlled Drug delivery, Neural Stimulation.

19.3 Synthesis of Conducting Polymers

Generally there are two methods of synthesis of conducting polymers, the chemical and the electrochemical method.. Electrochemical synthesis is carried out in a beaker type three electrode system. The electrochemical method results in the synthesis of thin polymer film over a working electrode which may be carbon electrode or metal electrode. In case of electrochemical method the monomer of conducting polymer is dissolved in a proper solvent which acts as electrolyte. Organic or aqueous solvent are used as electrolyte. A dopant is used in the synthesis which helps in doping of the conducting polymer. A working electrode is used for deposition of thin polymer film. The polymerization is carried out against a reference electrode. Generally saturated calomel electrode or silver/silver chloride act as reference electrode. The third electrode is counter electrode. Platinum may be used as counter electrode. The polymerization is carried out on a voltage window. During the course of polymerization, a thin film of polymer gets deposited over the surface of the working electrode. The electrochemical polymerization method is limited in terms of mass production and there is a requirement of a conducting working electrode for deposition of thin layer. It is also limited in terms of mass production.

On the other hand, chemical polymerization generally produces a large amount of polymers. In this method there is a requirement of a strong oxidizing agent like ammonium peroxy disulfate, hydrogen peroxide or ferric chloride, etc. In the chemical polymerization method the monomer is directly added in the solvent and is oxidized using oxidant. This results in the formation of insoluble polymer solid which can be obtained by filtering the reaction mixture.

19.4 Application of Conducting Polymer in Tissue Engineering

There are various forms of a material which can be used in tissue engineering such as nanoparticles, hydrogels, nanofibers, etc. The nanoparticles of a conducting polymer can be synthesized by using the chemical or electrochemical method of polymerization. However the best way to use the conducting polymer is in the nanofibrous form which can be prepared by using the electrospinning technique. Here we will discuss the role of three conducting polymers in tissue engineering application.

19.5 Polypyrrole

Polypyrrole is one of the most studied conducting polymers in the field of tissue engineering. Many researchers have worked on polypyrrole and produced some good results regarding biocompatibility of the polymer. Some researcher used the polymer with some biologically active compounds, and others used it without any biological compound.

A study on human umbilical vein endothelial cells was carried out by Garner *et al.* [8] on Polypyrrole-heparin films. The heparin was chosen because of its biocompatibility and also because it is a component of the extracellular matrix of blood vessels and has anticoagulant properties. They showed that the PPy-heparin composite provide good substrate for growth of endothelial cells. Another work on the polypyrrole composite was carried out by Collier *et al.* [9]. They studied the composite in both *in vitro* and *in vivo* conditions. They prepared the composites of polypyrrole and hyaluronic acid. They used PC-12 cell line (derived from transplantable rat pheochromocytoma) on the surface of polypyrrole-hyaluronic acid. *In vitro* cell study showed that the polypyrrole-hyaluronic acid composites supported cell attachment and cells showed good viability on their surface. Figure 19.1 shows the *in vivo* investigation of polypyrrole-hyaluronic acid composite implanted in rat subcutaneous pouches for two and six weeks. In this study polypyrrole-polystyrene sulphate was used as control. In the study it was discovered that compared with a polypyrrole-polystyrene sulphate control, the vascularization around polypyrrole-hyaluronic acid composite was significantly higher. Generally, it should be described as a small molecular weight hyaluronic acid that is considered to be the angiogenesis promoter, and this study suggests that the hyaluronic acid retains the ability of angiogenesis during the polypyrrole-hyaluronic acid composite.

Wang *et al.* [10] evaluated the biocompatibility of polypyrrole prepared from chemical as well as electrochemical method. They carried out some toxicity tests by applying polypyrrole extract solution to cell cultures and animal models which shows that the extract solution is highly biocompatible. They also showed that the Schwann cells cultured on electrochemically polymerized

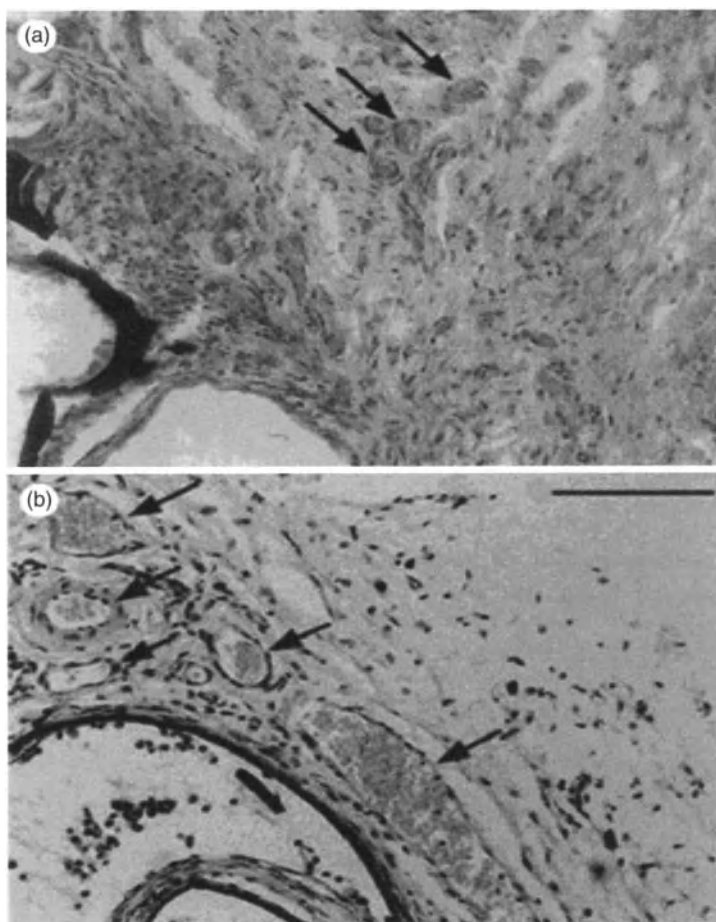


Figure 19.1 *In vivo* culture response of polypyrrole-hyaluronic acid. (a) Polypyrrole-polystyrene sulphonate (PPy/PSS) control and (b) Polypyrrole-hyaluronic acid films implanted into subcutaneous pouches in rats. Scale bar, 100 mm. The figure suggests that hyluronic acid retains the ability of angiogenesis during the polypyrrole-hyluronic acid composite because many more blood vessels were seen around this implant compared to the polypyrrole-polystyrene sulphate control. Reprinted from Collier *et al.* [9] with permission from John Wiley & Sons, Inc.

PPy have good growth compared to bare glass substrates. In an experiment carried out by Li *et al.* [11], PC12 cells showed good attachment and growth on porous PPy-tetraethylammonium perchlorate-polyvinyl alcohol composites compared to tissue culture polystyrene (TCPS). This was due to the presence of pores in the PPy composite. The pores generally help in transfer of signal and thus enhance the cell growth. Work on the electrical conductivity of polypyrrole related to biomaterials was carried out by Cui *et al.* [12]. They worked on the PPy-synthetic peptide composite. In their work they reported that when PPy-synthetic peptide coated electrodes were implanted in guinea pig brain they showed excellent recordings of electrical activity over two weeks with better nerve cell integration than the electrodes which were uncoated.

19.6 Poly(3,4-ethylene dioxothiophene)

Poly(3,4-ethylene dioxothiophene) is a conducting polymer with an importance of its very own. They are used in a variety of biological applications in various forms. The electrospun nanofibrous form is of high importance in the case of tissue engineering. A great work on PEDOT was carried out by Maria Bolin *et al.* [13]. They used polyethylene (terephthalate) (PET) electrospun nanofibers. They were coated with PEDOT-tosylate composite in two different forms. One using the chemically polymerized PEDOT-tosylate composite and another was vapor phase polymerization of the PEDOT over the surface of PET nanofibers. Figure 19.2 is the SEM micrographs of various types of composites prepared by them. These nanofibers were used to stimulate Ca^{2+} signalling in SH-SY5Y neuroblastoma cells.

The nanofibrous surface was tested for cell growth and adhesion and it was found that the nanofibers provide 3D surface for cell growth and attachment. Thus, it can be concluded that the nanofibers' surface act as biological extracellular matrix for cell growth and provide better surface for cell attachment and growth. Figure 19.3 shows the SEM image of cell grown on the nanofibers surface. The arrow shows the neurite.

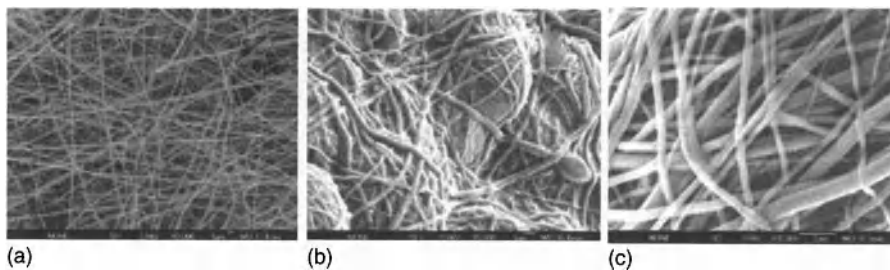


Figure 19.2 SEM images of PET nanofibers (a) uncoated electrospun (b) coated by chemically polymerized PEDOT, and (c) coated by vapor phase polymerized PEDOT[13].

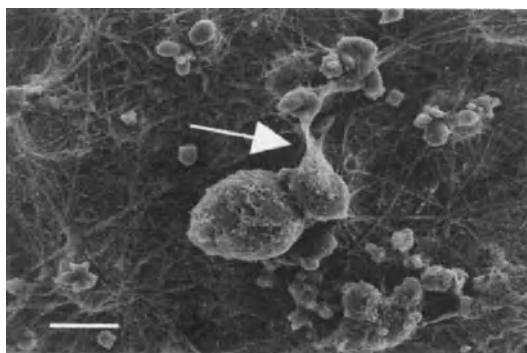


Figure 19.3 SEM micrograph of SH-SY5Y cell grown on the surface of nanofibers. The arrow in the image indicates the neurite. Scale bar is 10 μm [13].

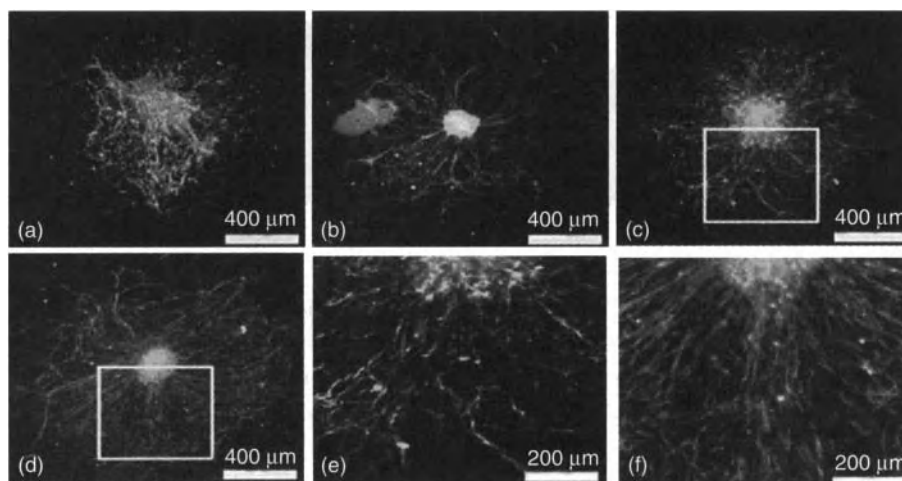


Figure 19.4 DRG explants cultured on conducting-polymer films and nanotubes. Ganglia on PPy film (a) and PPy nanotubes (c) degraded and had shorter and more branched neurites than PEDOT film (b) and PEDOT nanotubes (d), respectively. The extent of branching is best observed at higher magnification of PPy nanotubes (e) and PEDOT nanotubes (f) [14].

Recently a comparative work on biocompatibility of PEDOT and polypyrrole was carried out by Abidian *et al.* [14] in the film form and nanotube form. They cultured the neuronal cells using an *in vitro* dorsal root ganglion (DRG) model. The growth of the cell was excellent on the surface of nanotube and film. However, the growth on nanotube was somewhat higher than film. DRG cells had better attachment and less branched and longer neurites on PEDOT nanotubes. It suggests that the PEDOT has excellent biological properties and can be used in the tissue engineering applications. Compared with polypyrrole the PEDOT shows higher cell growth exploring the biological applications of the PEDOT. Figure 19.4 shows the growth of ganglia on the surface of polypyrrole and PEDOT film and nanotubes.

19.7 Polyaniline

Out of different conducting polymer used up till now, polyaniline (PANI) is considered to be very important in various applications, including biological, because of its ease of synthesis by chemical and electrochemical method, environmental stability, good processability, low cost, chemical stability, better control over film formation, good conductivity, and better electrical and electronic properties. Although there is little work that has been done on PANI regarding its biocompatibility, currently much research is going on concerning this conducting polymer.

An exciting work on the electroactive conducting polyaniline was done by Li *et al.* [15]. They demonstrated the potential for using PANI in biological applications. They cultured H9c2 myoblast cells differently by covalently attaching

oligopeptides to PANI and electrospinning PANI-gelatin blend nanofiber scaffold. These scaffolds were analyzed in the cardiac tissue engineering applications. Figure 19.5 shows the morphology of the cells grown on the gelatine fibers and PANI-gelatin nanofibers blend which was electrospun. (Li *et al.* 2006). Because of their good conducting power the polyaniline stimulate the cell growth, and hence cell proliferation as well, on the surface of the polyaniline.

Bidez *et al.* [16] studied the biocompatibility of thin films of polyaniline, in both conductive and nonconductive forms, by using H9c2 rat cardiac myoblasts. For the cell culture experiment thin film of PANI was used in both conducting and nonconducting form and 10^4 cells/cm² were seeded on their surface. Figure 19.6 shows the cell proliferation on the surface of TCPS, in both conductive and nonconductive PANI forms.

From Figure 19.6 it can be concluded that cell grows very well on the surface of PANI, and hence PANI acts as good substrate for cell growth. However it can also be seen in the figure that the conducting form of PANI shows slightly delayed action in the cell growth which can be attributed to the presence of some acid dopant ion in the polymeric structure even after washing which

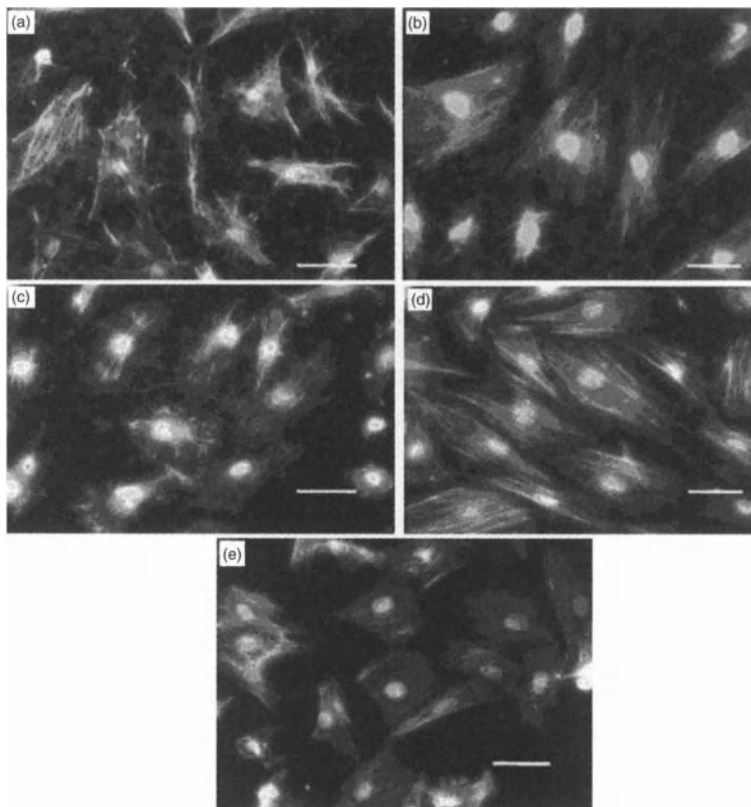


Figure 19.5 Morphology of H9c2 myoblast cells at 20 h of post-seeding on: (a) gelatin fiber, (b) 15 : 85 PANI–gelatin blend fiber, (c) 30 : 70 PANI–gelatin blend fiber, (d) 45 : 55 PANI–gelatin blend fibers, and (e) glass matrices. Staining: nuclei, bisbenzimidide; actin cytoskeleton, phalloidin; fibers, autofluorescence; original magnification 400X (Li *et al.* 2006). Scale bars, (a–e) 50 mm [15].

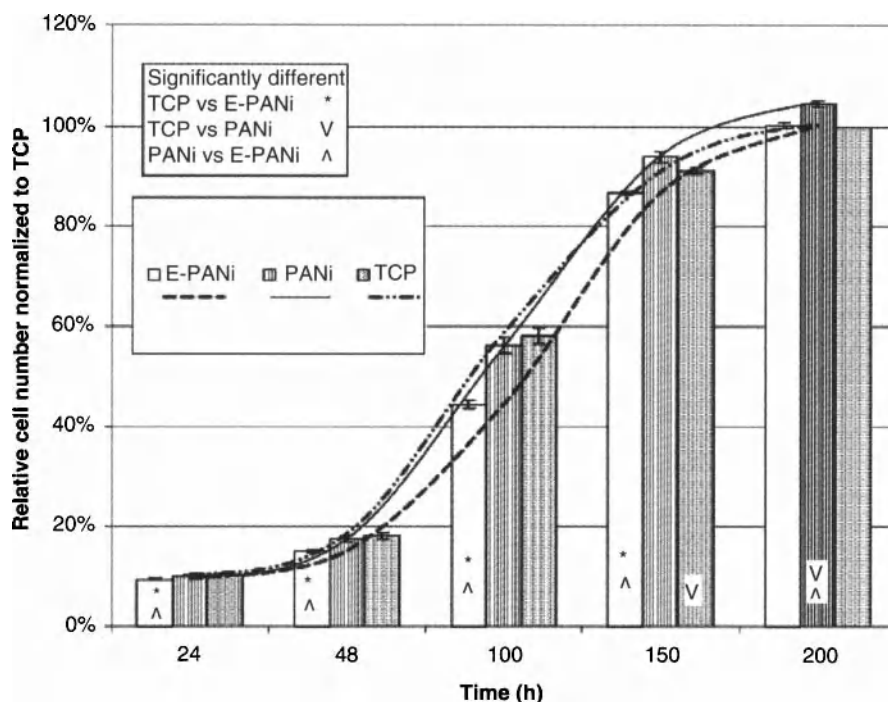


Figure 19.6 H9c2 myoblast proliferation on PANi, E-PANi and TCP controls. Cells were seeded at 10^4 cells/cm² and allowed to proliferate for up to 200 h. Cell numbers were assessed by measuring AB fluorescence at the time points indicated. The data are normalized to the maximal fluorescence signal of cells growing on Thermanox™ tissue culture plastic (TCP) control surfaces and expressed as means $\pm \sigma$, x ($n = 18$). The symbols (*, V and ^) denote statistically significant differences between various groups, as indicated in the figure ($P < 0.05$, one-way ANOVA, Tukey post-test) [16].

leach some toxicity to the cells and prevent their growth. But if the same surface is tested after 200 hours it shows somewhat comparative growth with the nonconducting form pointing out that the cytotoxic ion is not in the polymer any more.

Hence, overall the PANi has good biocompatibility and has potential applications in the field of tissue engineering applications.

19.8 Carbon Nanotube

Carbon nanotube is a form of carbon that was discovered by Iijima and Ichihashi [17]. After their discovery they have attracted considerable interest and importance in different areas and various applications due to their unique structural, mechanical and electronic properties. Up to now CNT were used only in energy storage applications and as filler in various polymeric materials for providing mechanical strength to the composite. But recently the CNTs were also used as biocompatible material. This investigation opened a new field of application for the CNTs which otherwise was considered for the energy storage only.

The use of CNTs in biological applications can be attributed because of their thermal stability and electronic properties. The CNTs play an important role in tissue engineering where much higher mechanical strength is required such as in the case of bone tissue engineering. It also reasonable to use a biodegradable polymer to form a composite with CNTs to further increase the biological properties of the CNTs.

Abarrategi *et al.* [18] studied *in vitro* and *in vivo* biocompatibility of CNT-chitosan composite scaffolds. The composite was prepared and the icesegregation induced self-assembly (ISISA) process by using C2C12 cell line (myoblastic mouse cell). It was concluded that the CNT composite is a biocompatible material and cell grows very well on the composite surface. Also, the good mechanical power of the CNT provide better cell support and area for cell proliferation. They also implanted the MWCNT/CHI scaffold in muscle tissue of mice. The implantation period was for three weeks after which the experiments were carried out. It was observed that the composite scaffolds are biocompatible even in the *in vivo* condition and can be used for bone tissue engineering. Figure 19.7 shows the *in vivo* cell growth. The composite is also degradable and can be eliminated from the body easily.

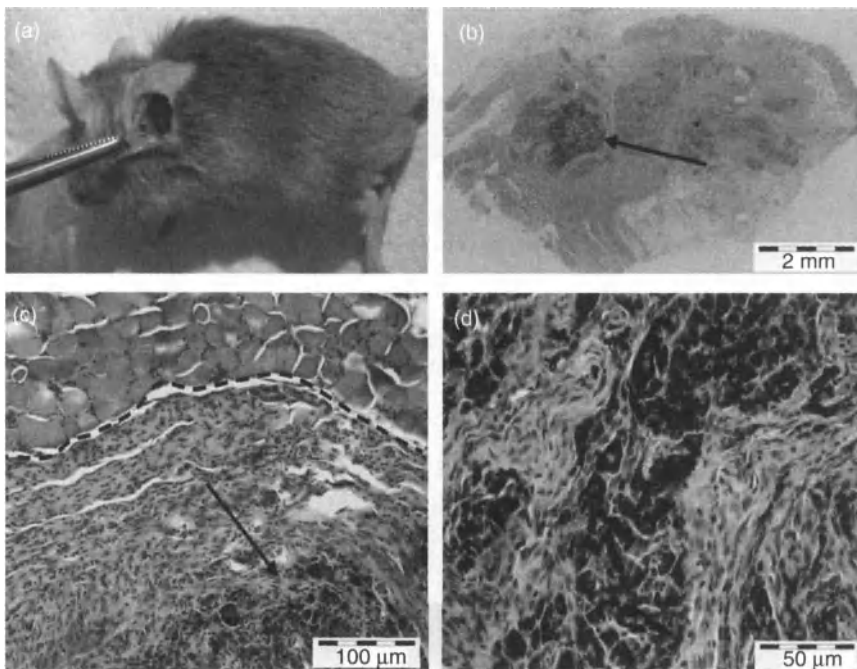


Figure 19.7 Picture (a) shows the surgery implantation of rhBMP-2 adsorbed MWCNT/CHI scaffolds into mouse subcutaneous muscular pocket. Optical microscope micrograph (b) shows regenerated bone tissue and a minor fraction of remaining MWCNT/CHI scaffold. Optical micrograph (c) shows a detail of regenerated bone tissue (collagen expressing cells, blue-green colored) after major disassembly of the MWCNT/CHI scaffold, surrounded by muscle tissue (pink colored). It is remarkable the well-limited interface between adjacent tissues (see black dash line). The remaining MWCNT/CHI scaffold (black colored) is pointed to by black arrow. Optical micrograph (d) shows a detail of remaining scaffold with plenty of fibroblasts (purple colored), prior to its disassembly and colonization by collagen expressing cells (blue-green colored) [18].

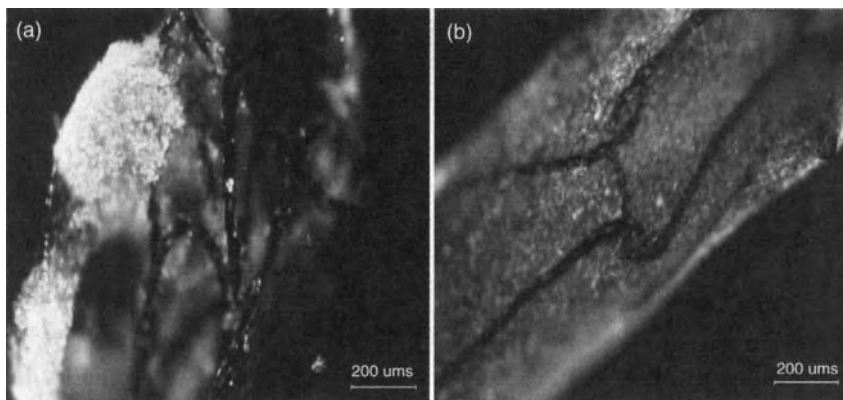


Figure 19.8 (a) Fluorescent optical micrographs of viable (green) and dead (red) fibroblast cell attachment and proliferation on MWCNT knitted scaffolds on day 13 and (b) Fluorescent optical micrographs of viable cell attachment and proliferation on MWCNT/PLGA composite scaffolds on day 17 [19].

Edwards *et al.* [19] also produced the same biocompatible results for the CNT composites in the tissue engineering applications. They used the composite of CNT with Poly(lactic-co-glycolic acid) (PLGA). The CNT was obtained by chemical vapor deposition method and then were spun in the form of yarn. The spun yarn of CNT were fabricated by PLGA and the composite was prepared. The composite was subjected to Mouse NR6 fibroblast cells for tissue engineering. They concluded that the CNT act as biological active material and retain their ability of mechanical strength and electrical conductance in the composite form and work towards the cell growth. Figure 19.8 shows the cell grown on the surface of CNT and the CNT-PLGA composite after 17 and 13 days of cell seeding, respectively. The overall results indicate the biocompatibility of CNT which opens up a new field of investigation in the exploration of CNT and its composites.

19.9 Future Prospects and Conclusions

Tissue engineering is the topic of interest of today because of increasing complexity in life style. The main challenge for tissue engineering is the development of a scaffold that mimics the function of extracellular matrix in the body and helps in cell growth and proliferation. Conducting polymers and CNTs are materials of interest in this field because of their mechanical, electrical and electronic properties. Particularly in the field of bone tissue regeneration and tissue that requires stimuli response, the conducting polymers and CNTs are key components to be used as scaffolds.

As the conducting polymers provide high conductivity and the CNTs provide better mechanical strength, it is reasonable to develop a composite of conducting polymers and the CNTs so that the composite can behave in an enhanced manner taking advantage of conductance from conducting polymers and mechanical strength from CNTs. The best way to develop such a

composite is the electrospinning technique by which fibers of nano to micro scale can be prepared. The fibers generally provide good 3D scaffolds for the cell growth and proliferation.

Acknowledgements

The authors wish to thank the European Commission (PIIF-GA-2009-254955), LIST, IGEN Linköping University and MEXT, NIMS, JST-CREST for their generous financial support.

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Cell Patterning Technologies for Tissue Engineering

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Abstract

Native body tissues are complex assemblies of cells of various types which lie in close contact. The direct interaction between those cells often determines the function of the tissue. Indeed, regenerating or repairing those tissues requires recovering a cellular micro environment suitable for hosting a heterotypic population of cells. Bottom-up approaches enable engineering tissues with controlled histoarchitecture in various scales with great potential to engineer biomimetic tissue constructs. This chapter focuses on recent advances in the engineering of patterned co-cultures of various cell types, and their applications in restoring biological function of corresponding tissues.

Keywords: Patterned co-culture, cellular interaction, bottom-up, top-down, biomimetic

20.1 Introduction

Tissue engineering is a multidisciplinary field that combines the principles of engineering and biology for the purpose of repairing or regenerating damaged biological tissues [1]. The ultimate goal of tissue engineering is to bypass the limitations in connection with organ transplants, such as immunogenicity, by producing functional tissue constructs for clinical applications. In this regard, two engineering approaches have been employed for manufacturing tissue constructs: the top-down and the bottom-up tissue engineering strategies.

The top-down approach involves utilizing scaffolds of either synthetic or natural origin and culturing them with suitable cells for tissue development. The scaffolds used for this purpose should be biocompatible, and provide appropriate surface chemistry and mechanical properties to promote cell attachment and proliferation. Although the top-down approach has led to

successes for engineering of thin or avascular tissues, such as skin, it has had limited applications in engineering of vascularized or complex tissue constructs due to difficulties in accurate colonization of the scaffold regions by the appropriate type of cells [2–4]. Therefore, the top-down approach for generating 3D tissue constructs is challenged by difficulties in nutrient transport to all the cells in the construct and waste removal from them [2, 3].

The bottom-up approach involves engineering of tissue building blocks and assembling them into larger tissue constructs [5–7]. The purpose of bottom-up tissue engineering approach is to accurately determine the localization of the biological components of one tissue, such as various cell types, toward engineering of a functional tissue, mimicking the native histoarchitecture [8–11].

The bottom-up approach assists with the transfer of nutrients and waste removal in engineered tissues, and the formation of an accurately controlled histoarchitecture in which various cell types can interact through direct contact.

Biological tissues are comprised of multiple cell types, which are surrounded by their appropriate extracellular matrix (ECM) and blood vessels. These heterogeneous ensembles of cells and their ECM components are formed by local microenvironments during tissue development and remodeling, which are induced by spatial and temporal heterogeneity of biological soluble factors [12, 13]. This highlights the significance of engineering multicellular constructs to regenerate functional histoarchitecture of live tissues and organs and to promote proper intercellular communication, which occur in tissue formation, as well as repair processes [14–16]. In the absence of such cellular interactions, some cell types lose their functional capabilities outside the body [17]. Considering that *in vitro* cell culture for tissue engineering involves tissue digestion and isolation of specific cell populations, the native heterotypic cellular interactions are lost. Therefore, patterned co-cultures of multiple cell types have been used as useful tools to study cellular interactions and engineering tissue constructs which better mimic the histoarchitectural complexity of the native tissues by increasing control over the localization of cells in culture. This enables accurate tuning of the degree of homotypic and heterotypic cellular contact, and preserves biological function of cell types outside the body.

Cell patterning, as a bottom-up tissue engineering strategy, has been developed with various scales and dimensions. In this chapter, recent meso- and micro-scale technologies for generating 2D and 3D patterned co-cultures are discussed. In addition, the applications of those systems in the engineering of functional biological tissues are explained.

20.2 Patterned Co-culture Techniques

A plethora of methods have been developed for generating patterned co-cultures to control the degree of interaction between cells of same type (homotypic) and different types (heterotypic). In this section, some of the available methods of generating patterned co-cultures are reviewed. Common approaches in this regard include substrate patterning with ECM components,

patterned cell seeding in microfluidic devices, and cell repulsion/adhesion switching of substrates, etc.

20.2.1 Substrate Patterning with ECM Components

Patterning of cell culture substrates with cell-adhesive ECM components has been used as a tool for selective adhesion of cells to substrates. Various cell types adhere to different surfaces in a manner which is proportional to their expression levels of adhesion molecules, such as integrins. The differences in cells adhesiveness to substrates make it possible to seed multiple types of cells on a substrate which is patterned with cell-adhesive ECM components. In one approach, a substrate is micropatterned with a cell-adhesive ECM component, such as collagen, which enables the adhesion of one cell type under serum-free condition onto the patterned regions. Then, a second type of cell is seeded on the substrate in the presence of serum, which makes them adhere nonspecifically to the unmodified regions of the substrate.

This technique has been used to localize cells efficiently within patterned co-cultures. Elloumi *et al.* employed microcontact printing of fibronectin on temperature-responsive culture dishes and seeded primary rat hepatocytes on them under serum-free conditions, which allowed hepatocytes to attach exclusively onto fibronectin-printed regions [18]. Then, in the presence of serum in the culture medium, the authors seeded endothelial cells onto the regions of the dishes, which were not modified with fibronectin (Figure 20.1). Bhatia *et al.*

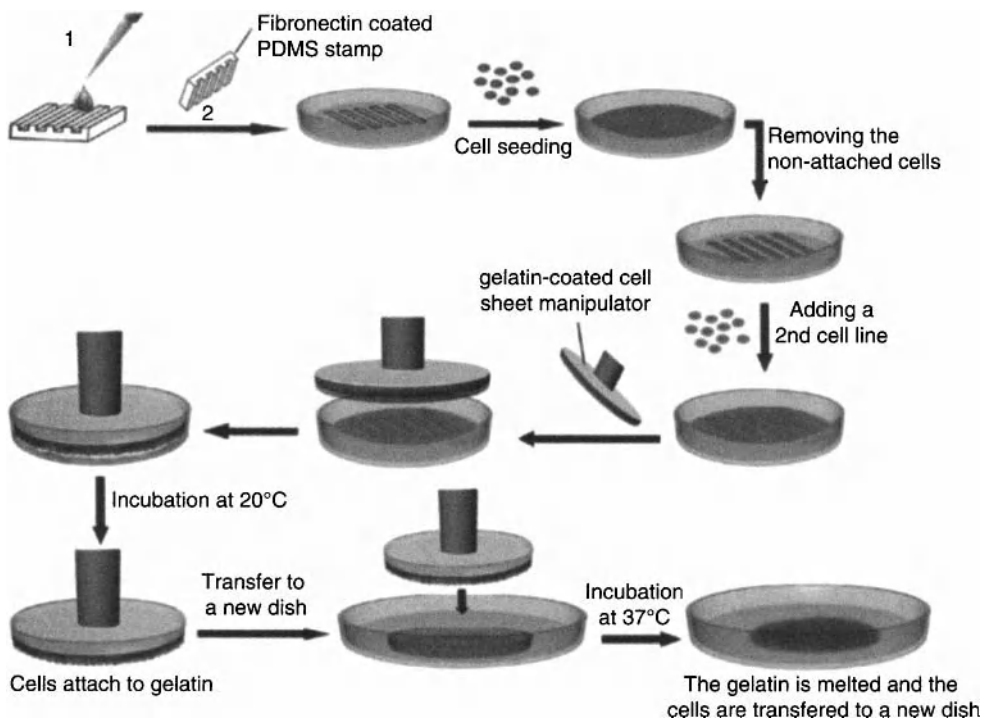


Figure 20.1 Procedures for fabrication and transfer of micropatterned-co-cultured cell sheets [18].

utilized a similar method to generate a patterned co-culture of rat hepatocytes and fibroblasts for studying the role of the interaction between these cell types in maintaining liver function [19].

This is an easy, fast, and versatile approach, which only utilizes developed techniques such as microcontact printing. Attempts at automating this technique have been described and reviewed elsewhere [20]. However, several issues, such as specific cell–cell and cell–substrate adhesiveness of each cell type, need to be considered for successfully implementing this method. Furthermore, the choice of substrate and cell type is limited as some cells exhibit strong adhesiveness to commercially available culture dishes even in serum-free condition [21].

20.2.2 Microfluidic-based Patterning

Characteristics of fluids flow in microfluidic channels have made it possible to pattern the substrate with cells and biomolecules. Due to the laminar flow of fluids in microchannels, several parallel flow streams can be flown in them without considerable convective mixing, which allows the localized delivery of cells or biomolecules to the regions of the substrate corresponding to each stream flow [22–25].

To further improve the method, microfluidic channels with microwells on the substrate have been fabricated to capture cells in the flow [26]. In this approach, cells are immobilized in the microwells, which imposes a lower shear stress on cells, and thus facilitates cell adherence to the substrate (Figure 20.2).

Fabrication of microchannels by photolithography is a well-established technique. Therefore, microfluidic-based methods of generating co-cultures

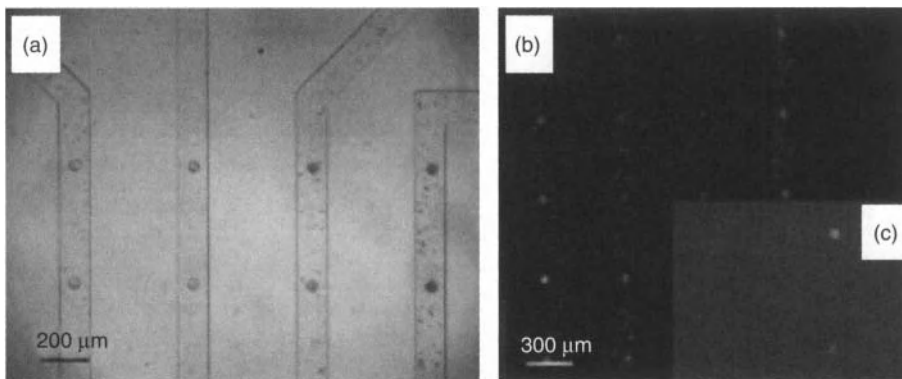


Figure 20.2 Cell docking within microchannel arrays: (a) the light microscopy image of ES cells flowing within an array of micro-channels; (b) is the fluorescent image of cells (right to left: ES/AML12/ Saos-2/PC3/NIH-3T3 cells) labeled with membrane dyes CFSE (green) and SYTO (red) as they flow through the channels; (c) Once the cells had docked in the microwells, a cell-free solution was flowed through the channels to remove any remaining non-adhered cells. Reprinted with permission [26].

are versatile and straightforward. However, they are limited by the uniqueness of pattern shape (i.e., parallel stripes) that they can generate.

20.2.3 Switchable Surface-based Patterning

This method involves utilizing stimuli such as light [27–32], voltage [33–37], and heat [18, 38–41] to turn surfaces from cell-repulsive to cell-adhesive. These techniques can be used for the sequential patterning of multiple cell types and control over the adhesion and motility of individual cell types.

Yousaf *et al.* fabricated an electroactive monolayer of alkanethiolates on gold substrate which was cell-repulsive at the initial stage, but acquired cell-adhesiveness upon electrical oxidation of hydroquinone groups and subsequent conjugation of RGD peptide [36, 37]. The authors used this dynamic substrate to co-culture two different cell types by culturing a first population of cells on the regions of the monolayer which were micro-contact printed with fibronectin, and then switching the other regions of the monolayer to being cell-adhesive by applying an electric potential for culturing a second cell type. This technique enables temporal, as well as spatial, control over cell positioning on a substrate. However, it involves a complicated chemistry.

Yamato *et al.* utilized thermally responsive polymers to generate patterned co-culture of cells [40]. The authors grafted a thin layer of poly(*N*-isopropylacrylamide) (PIPAAm) covalently onto tissue culture dishes by electron beam radiation. This polymer is hydrated at temperatures lower than its lower critical solution temperature (LCST, 32°C), and thus is cell-repulsive. However, at higher temperatures, the polymer network of PIPAAm is dehydrated and therefore becomes cell-adhesive. It is possible to synthesize copolymers of PIPAAm and other monomers to vary its LCST. Using this technique, patterned co-cultures of cells have been generated by selectively seeding a first cell type onto tissue culture plates with PIPAAm polymer patterns at lower temperatures followed by seeding a second type of cells on the polymer surface at higher temperatures (Figure 20.3). Once cells are seeded on PIPAAm at temperatures above its LCST, lowering the temperature switches the polymer back to being cell-repulsive. Therefore, this method can be used to detach the entire patterned co-culture from the polymer as a “cell sheet” and transfer it to a new culture environment [18, 42].

To further improve this technique, Eda Hiro *et al.* synthesized a PIPAAm material with spiropyran chromophore side chains, which enabled more accurately localized cell culturing by using light to turn the polymer cell-adhesive [30].

20.2.4 Mechanical and Stencil-based Patterning

Patterned co-culture of cells have been generated by simpler methods such as mechanical juxtaposing of different cell cultures, as well as patterned blocking of culture substrate by stencils.

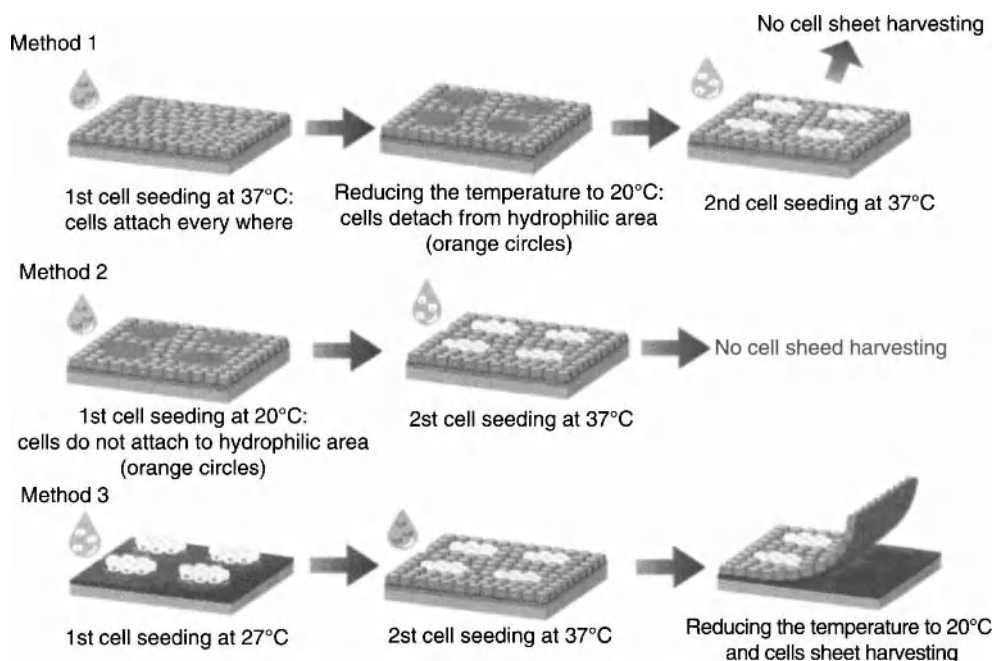


Figure 20.3 Schematic diagram of three methods of cell seeding for patterned co-culture. In method 1, the first cell types are seeded at 37°C and adhered onto the entire dish surface. By reducing temperature below the LCST, spread cells are detached only from PIPAAm-grafted domains (hydrophobic area). Finally, the second cell types are seeded and adhere onto only PIPAAm-grafted domains without cells at 37°C. In method 2, the first cell types are seeded at 20°C and adhere only onto non-grafted regions of tissue culture plate. Then, the second cell types are seeded and adhere onto only PIPAAm-grafted domains without cells at 37°C. In method 3, the first cell types are seeded at 27°C and adhere only onto PIPAAm-BMA-co-grafted domains (orange circles). Then, the second cell types are seeded at 37°C and attach to the rendered-hydrophobic-PIPAAm. Finally after reducing the temperature to 20°C, cells are harvested as a patterned-co-cultured-cell sheet. Reprinted with permission [42].

Hui *et al.* constructed a silicon substrate consisting of two interlocking parts for the dynamic control of cell–cell adhesion [43]. The authors utilized this device to study the interaction between hepatocytes and supportive stromal cells by culturing them on the interlocking substrates and placing the parts in full contact or within a controlled gap.

Microengineered polydimethylsiloxane (PDMS) stencils with patterned holes have been used for localized cell culture on substrates [44]. In this method, a PDMS membrane with microengineered holes is attached reversibly to a culture substrate, and then the first cell type is seeded onto the substrate through membrane holes. By removing the membrane, a micro-patterned array of cells is achieved. Finally, the second cell type is cultured on the substrate regions, which are not covered by the first cell type.

In addition to PDMS, parylene has been used to fabricate stencil membranes for generating patterned co-cultures due to its mechanical robustness and ease of use [45]. Moreover, parylene surface can be modified to host cells

for creating a dynamic co-culture; however, it requires a more complicated fabrication process than PDMS membranes.

20.2.5 3D Patterned Co-cultures

Although the methods described above have been successful in creating patterned co-culture of cells in 2D structures, it is crucial to develop 3D structures containing heterogeneous cell types, which better mimic the complex organization of native body tissues. Several bottom-up approaches have been taken to implement cell–cell interaction in 3D structures.

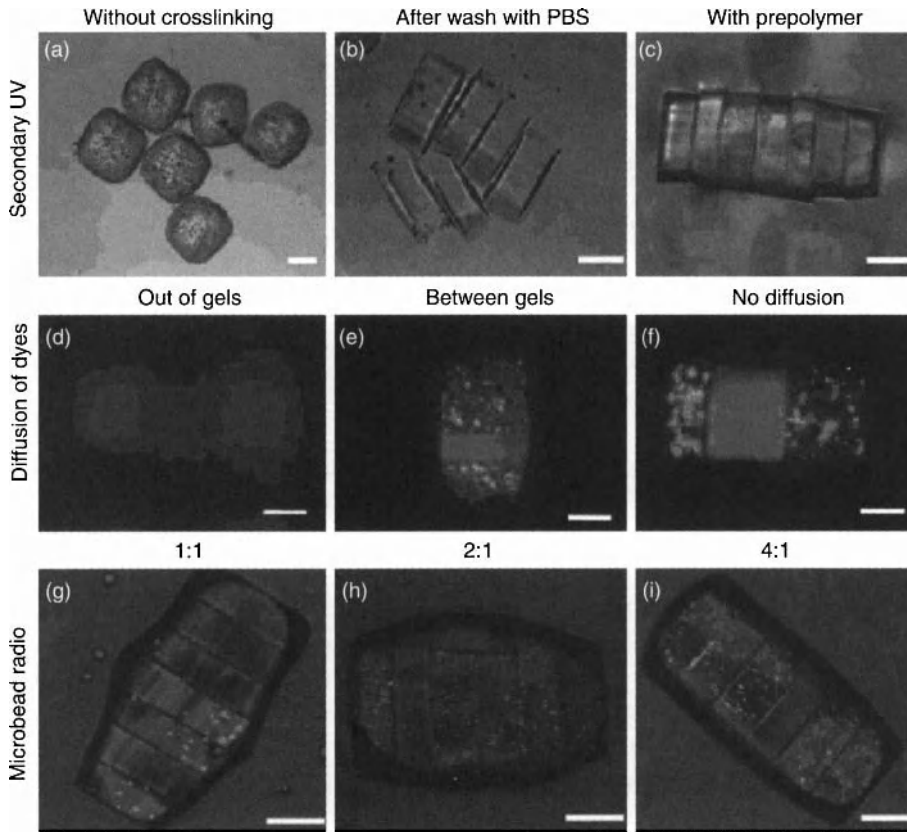


Figure 20.4 Secondary cross-linking of the microgel assemblies. (a) Dissociated microgel assemblies after replacing mineral oil with culture medium without secondary cross-linking; (b) Stabilized microgel assemblies in culture medium after secondary cross-linking; (c) Dissociated microgel assemblies after secondary cross-linking with removed residual prepolymer; (d–f) Diffusion of dyes through the hydrogel; (d) Free diffusion of rhodamine-dextran ($M_r = 10$ kDa) out of the hydrogel into PBS; (e) Two groups of microgels labeled with rhodamine-dextran or FITC-dextran ($M_r = 2,000$ kDa) were assembled, and the rhodamine-dextran diffused throughout the entire hydrogel assembly; (f) Microgel assemblies with FITC-dextran- or Nile red-stained microgels; (g–i) Microbead-containing and plain microgels were used to model microgels containing two types of cells by varying the initial mixing ratios of these two types of microgels (1:1, 2:1, and 4:1, respectively). (Scale bars, 200 μm). Reprinted with permission [49].

For example, previously described cell sheets cultured on PIPAAm-grafted thermo-responsive culture dishes were stacked to generate a double-layered co-culture system [46]. Using this approach, Harimoto *et al.* produced sheets of endothelial cells, which were directly placed on a layer of hepatocytes after detaching the endothelial cell sheet from the thermo-responsive culture dishes [46]. Moreover, same group generated complex overlaid cultures of micropatterned endothelial cells and fibroblast monolayer sheets [47]. In a different approach, magnetic labeling of cells has been used to control localization of cells in 3D tissue structures [48]. In this method, cells are labeled by up-taking magnetite cationic liposomes which possess a positive surface charge. Then, the labeled cells move and adhere to the culture regions where a magnet is positioned.

Hydrogels are gaining increased attention for generating 3D cultures due to their ECM-like viscoelastic properties. A number of efforts have been made to enable controlling cell-cell interaction in hydrogel 3D constructs. In a bottom-up approach, cell-laden microengineered gels were directly assembled to generate 3D constructs [49]. The authors fabricated cell-laden rectangular hydrogels through photopolymerization with a photomask. Then, they created aggregates of those microgels in a hydrophobic mineral oil. The resulting aggregates were solidified through a secondary UV polymerization step (Figure 20.4). This is a versatile technique to generate 3D structures of cell-laden hydrogels with different aspect ratios for optimizing nutrient transfer to cells.

20.3 Applications of Co-cultures in Tissue Engineering

Homotypic and heterotypic cellular interactions play crucial roles in the body. Therefore, it can be helpful to develop micropatterned co-cultures for maintaining biomimetic environments for engineering functional tissues by controlling cellular interactions *in vitro* [50]. In this section, some of the applications of patterned co-culture systems in tissue engineering and cellular interactions investigations are discussed.

The role of fibroblasts in tissue angiogenesis was investigated by co-culturing human fibroblasts together with endothelial cells [51]. The authors achieved an increase in the angiogenesis rate by controlling the surface topology and heterotypic interactions between fibroblasts and endothelial cells.

In a study of neural differentiation, it was shown that co-culturing neural stem cells with vascular endothelial feeder cells promoted their differentiation into neural cells as evidenced by the expression of neural progenitor markers, as well as self-renewal of neural stem cells [52]. Interestingly, in addition to inducing neural differentiation, co-cultures of neural cells and endothelial cells have also been shown to enhance angiogenesis [53]. In the same context, Takano *et al.* co-cultured mouse astrocytes and neurons to control cell growth and localization to elucidate cell-signaling events in neural cells [54]. The authors demonstrated the application of spatially separated co-cultures in this study for controlling and investigating cellular interactions.

Liver is an important organ consisting of several cell types, which are difficult to maintain *in vitro*. Several studies have demonstrated that co-culturing hepatocytes with other types of cells, such as fibroblasts and endothelial cells, improves the maintenance of their liver-specific function [55–59]. For example, Yamato *et al.* used thermo-responsive polymers to generate patterned co-cultures of primary rat hepatocytes and human diploid lung fibroblasts [40]. The authors demonstrated the formation of confluent co-cultures, which mimicked native tissues.

In addition to co-culturing two different types of cells, attention has been paid to generating more biomimetic microenvironment for tissue engineering applications by co-culturing more cell types since there are multiple types of cells present in native tissues. In this regard, Wright *et al.* utilized a dynamic micropatterning technique to culture multiple types of cell in a temporally and spatially controlled manner [45]. Using this technique, the authors investigated temporal effects on cellular interactions among mouse fibroblasts, mouse hepatocytes and mouse ES cells.

The experimental examples described in this chapter clearly show the significance of utilizing patterned co-cultures in more accurate generation of functional tissues *in vitro* for tissue engineering applications, as well as more basic studies of heterotypic cellular interactions.

20.4 Concluding Remarks

In this chapter, some of the techniques of generating patterned co-cultures of cells and their applications in tissue engineering were discussed.

Native tissues in the body consist of multiple cell types, which are organized in a complex histoarchitecture. Therefore, the cellular interactions in these tissues can be most accurately studied by creating *in vitro* co-cultures of multiple types of cells. In this regard, patterned co-cultures offer the advantage of controlling the degree of homotypic or heterotypic cellular interactions over traditional methods of random co-cultures. Patterned co-cultures have contributed to elucidating the cell-cell and cell-matrix interactions, which may lead to designing biomimetic structures for tissue engineering.

However, there is limited data available for 3D and multiple-typed patterned co-cultures of cells. Future studies should be focused on designing and developing better biomaterials to be used for 3D co-cultures under physiological conditions, which would enable accurate cell positioning as well as nutrient transfer to cells.

Acknowledgements

The first author would like to thank Professor Murugan Ramalingam for the opportunity of contributing a chapter in this book, and also Dr. Imen Elloumi-Hannachi for the critical reading of the manuscript.

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