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Functional Chitosan

Drug Delivery and Biomedical Applications

Springer

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ISBN 978-981-15-0262-0 ISBN 978-981-15-0263-7 (eBook)
https://doi.org/10.1007/978-981-15-0263-7

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

The book focuses on functional chitosan for the purpose of drug delivery and biomedical applications. Chitosan is composed of α-1, 4-linked 2-amino-2-deoxy-α-d-glucose (N-acetyl glucosamine). It is a nontoxic, biodegradable, biocompatible natural amino polysaccharide with versatile applications. According to the United States Food and Drug Administration (USFDA), it is a GRAS (Generally Recognized as Safe) material and, hence, is widely used in pharmaceutical and biomedical fields, including drug delivery, food technology, and tissue engineering. It has a cationic character owing to its primary amino groups ($-NH_2$). These primary amino groups are important for synthetic modifications for controlled drug release, in situ gelation, mucoadhesion, permeation enhancement, and transfection properties. Due to its chemical modifications, most of these characteristics can even be further improved. Due to its fast dissolution in gastric fluid, its use is limited as oral sustained drug release carriers. Considering the importance and convenience of the oral route, the drug delivery properties of chitosan carriers was improved by modification of functional groups or with the use of other polymer in combination with cross-linker. By this way rigid matrix structure could be prepared to control the premature drug release. Due to the short biological half-life of the drug substance, it often requires frequent dosing, which may ultimately lead to toxicity due to the accumulation of excessive degradative products from the drugs. So, functional chitosan is promising in the area of drug delivery and biomedical engineering.

This book consists of different chapters emphasizing drug delivery and biomedical application of chitosan.

Chapter 1, "Chitosan and Its Derivatives: A New Versatile Bio-polymer for Various Applications," discusses the preparation, characterization, and various modifications of chitosan and its biomedical applications.

Oral drug delivery is the most important route of drug administration due to its safety, convenience, and cost-effectiveness. However, some drugs cannot be administered orally, mainly due to drug degradation at acidic pH in the digestive system. Chapter 2, "Application of Chitosan in Oral Drug Delivery," focuses on chitosan-based systems for oral drug delivery carriers of therapeutic molecules and drugs.

Transdermal drug delivery has been attracting attention for last few decades in the field of drug delivery and biomedical research, in compared with other

administration routes. This approach is generally accepted owing to its ease of application, allowing the drug carriers to directly enter into systemic circulation via transdermal delivery to avoid hepatic metabolism, protect from acidic pH, the enzymes effect of GIT and the fluctuating plasma drug concentrations associated with the oral delivery. Chapter 3, "Transdermal Delivery of Chitosan-Based Systems," discusses various chitosan-based systems for transdermal drug delivery.

Chitosan-based ocular drug delivery systems are widely investigated to improve the bioavailability at the anterior/posterior pole of the eye due to its mucoadhesive property that helps in increasing the efficacy of existing ocular drugs, affords stimuli-responsive/targeted-based delivery regimen, enhances the corneal permeability, and improves the accumulation of drugs in the corneal/conjunctival epithelium for an extended period of time. Chapter 4, "Chitosan-Based Ocular Drug Delivery Systems," summarizes the major ocular diseases affecting the eye, novel ocular drug delivery systems, intraocular drug transport barriers, and ocular transporters. This chapter also discusses ocular drug delivery systems, such as stimuli-responsive systems, targeted delivery systems, and gene-based delivery systems.

Using chitosan as matrix and/or coat material can protect drugs from chemical and enzymatic degradation during oral administration. It binds strongly to mucus and show a mucosal permeation enhancement property that promotes drug absorption through the intestinal epithelial cells. Oral colon-specific delivery systems have been explored for targeted drug administration for the treatment of colon cancer, ulcerative colitis, Crohn's disease, irritable bowel syndrome, Hirschsprung's disease, antibiotic-associated colitis, and other colon diseases. Chapter 5, "Functional Chitosan Carriers for Oral Colon-Specific Drug Delivery," provides an overview of the relevant physicochemical and biological properties of chitosan and its derivatives and novel formulations with respect to their use as oral colon-targeted drug delivery system.

Chitosan-based hydrogels play an important role in the development of new biomaterials for biomedical applications. Many cross-linking (or polymerization) approaches have been developed to convert chitosan into smart hydrogels, with the aim of obtaining new drug delivery devices. Such hydrogels can also undergo changes in their physicochemical properties in response to environmental changes, such as pH, ionic strength, temperature, and magnetic field. Chapter 6, "Chitosan-Based Hydrogels for Drug Delivery," focuses on the most recent progress made regarding preparation, properties, and their salient characteristics in drug delivery.

Various delivery systems, such as micelles, liposomes, or nanoparticles, are a major line of investigation to improve chemotherapeutic treatment. Chapter 7, "Recent Advances of Chitosan-Based Systems for Delivery of Anticancer Drugs," discusses chitosan-based drug delivery systems and different strategies for the treatment of cancer.

Gene therapy is a relatively new branch of medical science with huge therapeutic potential for a disorder at its genetic root. The success of gene therapy greatly depends on the vector's or vehicle's ability to selectively and efficiently deliver gene to the target site with minimal or no side effects. Chapter 8, "Chitosan-Based

Systems for Gene Delivery," highlights the chitosan-based systems for the delivery of gene.

Interpenetrating polymeric network (IPN) has gained great attention in the last decades, which involves a blend of two or more polymers in a network with at least one of the systems synthesized in the presence of the other. The development of IPN is interesting as it generates free volume space for the easy encapsulation of drugs in the three-dimensional framework, which are obtained by cross-linking of two or more polymer networks. Chapter 9, "Chitosan-Based Interpenetrating Polymer Networks: Drug Delivery Application," discusses IPN based on chitosan for drug delivery and biomedical applications.

Chitosan biomaterial attains immense interest in the field of tissue engineering owing to its biocompatibility and biodegradation. Besides, it exhibits bactericidal and fungicidal properties along with enhanced immune response. Chitosan-based materials are mainly used for fabricating the scaffolds for tissue engineering, which have been discussed in Chap. 10, "Chitosan-Based Systems in Tissue Engineering." Chapter 11, "Chitosan-Based Nanoformulation as Carriers of Small Molecules for Tissue Regeneration," focuses on nanoformulation of chitosan as carriers of small molecules for tissue regeneration.

Nowadays theranostic approach has been widely used for diagnosis and treatment with accurate targeting of cancer-specific cells. Theranostic system is very interesting and useful due to its drug targeting and molecular imaging in a single platform. Chitosan-based systems for theranostic applications are discussed in Chap. 12.

The modification of chitosan by physical or chemical methodologies is important for controlled drug delivery. Grafted chitosan is interesting as it increases active functional groups, which may react with metals, metal oxides, or other materials, such as graphene and carbon nanotube for the drug target to specific sites along with prolonged release of drug. Chapter 13, "Grafted Chitosan Systems for Biomedical applications," highlights the need for grafted chitosan and synthesis techniques to obtain the desired properties and its biomedical applications.

Chapter 14, "Chitosan-Based Systems for Controlled Delivery of Antimicrobial Peptides for Biomedical Application," discusses chitosan-based antimicrobial peptides (AMPs) and their biomedical applications. The last chapter, "Antibacterial Activity of Chitosan-Based Systems," discusses the latest development of chitosan-based systems for antimicrobial activity.

The book is useful for students, researchers, scholars, industry personnel, and scientists in the field of pharmaceuticals, material sciences, and biomedical engineering.

We express our sincere gratitude to all authors for their contributions to this book. We also thank the publisher for the continuous support for the publication of this edited book.

Asansol, India Sougata Jana
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About the Editors

Sougata Jana is a B.Pharm (Gold Medalist) from West Bengal University of Technology, Kolkata, and M. Pharm (Pharmaceutics) from Biju Patnaik University of Technology, Odisha, India. He worked as an Assistant Professor at Gupta College of Technological Sciences, Asansol, West Bengal, India, and is currently working at the Department of Health and Family Welfare, Directorate of Health Services, Kolkata, India. He has been engaged in pharmaceutical education and research for the last 11 years. He was awarded the "M. N Dev Memorial Award" by IPA Bengal branch, Kolkata, India, for securing the highest marks in the state of West Bengal in 2005. He received the "Best Poster Presentation Award" at the 21st West Bengal State Science and Technology Congress, 2014, and "Outstanding Paper Award" at the 1st Regional Science and Technology Congress, 2016, organized by DST, Govt. of West Bengal, India. He has 30 publications in different national and international peer-reviewed journals. He edited books in Springer, Elsevier, and Pharmamedix India Publication Pvt., Ltd., and has contributed more than 35 book chapters to Elsevier, Springer, Wiley VCH, CRC Press, and Taylor & Francis. His research area of interest includes modification of synthetic and natural biopolymers, microparticles, nanoparticles, semisolids, and interpenetrating polymer network system for controlled drug delivery.

Subrata Jana is presently working as an Associate Professor at the Department of Chemistry, Indira Gandhi National Tribal University (Central University), Amarkantak, Madhya Pradesh, India, and his current research focuses on the design and synthesis of artificial receptors for the recognition of anions, cations, and *N*-methylated protein residue. His other area of research interest is biodegradable polymeric-based carrier systems for the delivery of drug molecules. So far he has published ~40 research papers in peer-reviewed international journals and contributed more than 10 book chapters to different edited books published by internationally renowned publishers. He is also an editorial board member in the *Journal of PharmaSciTech* (ISSN: 2231 3788) and the *International Journal of Scientific and Engineering Research* (ISSN: 2229-5518) and a reviewer in the *International Journal of Biological Macromolecule* (Elsevier), the *Journal of PharmaSciTech*, and *Current Pharmaceutical Design* (Bentham). He has obtained his PhD in organic chemistry from Indian Institute of Engineering Science and Technology (IIEST), Shibpur, India. Then he moved to the University of Victoria, Canada, to work with Professor (Dr.) Fraser Hof on supramolecular and medicinal chemistry as a postdoctoral fellow. He then worked further with Dr. Kenneth J Woycechowsky at the University of Utah, USA, on protein engineering and enzyme catalysis as a postdoctoral research associate. Overall he extensively studied on the supramolecular behavior of the host–guest interaction and synthesis of heterocyclics, such as pyrimidines, naphthyridines, quinoline, and diazepines, by exploiting microwave protocol for green chemical synthesis.

Chitosan and Its Derivatives: A New Versatile Biopolymer for Various Applications

1

Deepali Rahangdale, Neha Joshi, and Anupama Kumar

Abstract

Chitosan is a nontoxic, biodegradable, biocompatible natural aminopolysachharide with diverse applications. Chitosan can be easily modified into different forms such as membranes, sponges, gels, scaffolds, microparticles, nanoparticles, and nanofiber for drug delivery, gene therapy, tissue engineering, and wound healing in biomedical application. Recently, chitosan-based molecularly imprinted polymers have gained considerable attention and showed significant potential in fields, such as environmental remediation, medicine, as well as various industrial applications. However, the performance of the chitosan-based products in various applications is influenced by many factors including the source of chitin, extraction process, molecular weight, degree of deacetylation, pH, ionic strength, concentration, and temperature. This chapter will provide a brief overview of chitosan in molecular imprinting technique as a functional polymer or supporting matrix because of its low cost and high content of amino and hydroxyl functional groups as well as the computational modeling for the designing of chitosan-based material for desired application. Rational designing of chitosan-based derivatives using computational modeling is not only fast and economic but also a greener approach, which helps understanding various thermodynamic and spectroscopic aspects at molecular level. This chapter also discusses diverse applications of chitosan for biomedical, industrial, and environmental applications.

Keywords

Chitin · Chitosan · Computational modeling · Graft copolymer · Tissue engineering

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_1

1.1 Chitin and Chitosan: General Characterization

Chitin is the second most abundant natural polymer after cellulose. Braconnot (1811) was the first to describe "Chitin", a hard, white, elastic and nitrogenous polysaccharide (β-(1-4)-N-acetyl-D-glucosamine) derived from many lower plants and animals especially in coastal regions (Honarkar and Barikani 2009; Crini 2005). Most common and commercial sources of chitin are crab and shrimp shells; however, it is also found in the cell walls of fungi and yeast (Rinaudo 2006). The production of chitin by any chemical method is not feasible and therefore, it is mainly obtained through natural sources only. Chitin has high percentage of nitrogen and can be used as a chelating agent. Almost all the naturally occurring polysaccharides are acidic in nature. However, chitin is highly basic which gives it a special feature and thus it outperforms other natural polysaccharides (Kumar 2000).

Chitin has been extensively used in tissue engineering and as a wound dressing material and is now flourishing as an agent for biomedical purposes. It is also used for rapid wound healing in surgical and medical applications (Khor and Lim 2003). However, instead of using the raw form of chitin it is very much preferable to improve the characteristics of chitin and then use it for specific purposes. For the same reason, physical as well as chemical modification of chitin is done and is currently one of the most triggered fields for research.

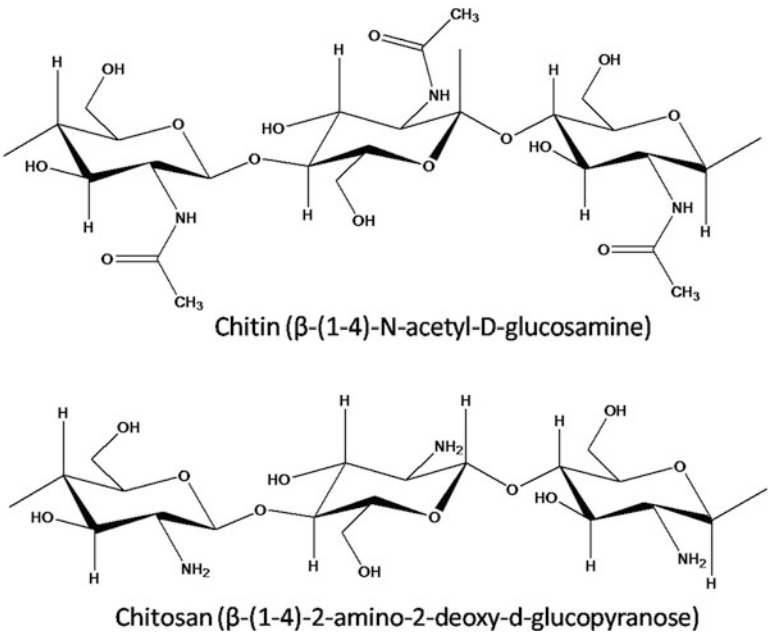

Fig. 1.1 Structure of chitin and chitosan

The most common derivative of chitin is its N-deacetylated form called chitosan as shown in Fig. 1.1. Chitosan can be easily obtained from chitin and has improved properties. Chitosan is extensively used as fertilizers, pesticides, etc., and is increasingly being used in food packaging and fishery industry.

Thus chitosan has wide domain of applications.

The function performed by chitosan is governed by its degree of deacetylation (DDA), viscosity and structure. The degree of deacetylation of chitosan depends upon the extent of deacylation achieved. Even the atmospheric condition in which the deacetylation reaction is performed decides the behavior of chitosan (Prashanth et al. 2002).

One of the reasons why the use of chitosan is limited, is its insolubility in basic solutions. So, in order to overcome this, chemical as well as physical modification is done on chitosan. The various derivatives of chitosan reported in literature can make it suitable for use in any type of medium (Sashiwa and Aiba 2004).

Along with the mentioned applications, chitosan is now widely used in diverse fields like agriculture, cosmetics, textile, etc. It can even be used as a catalyst in chemical reactions.

1.2 Sources and Extraction of Chitosan from Raw Materials

Globally, the shrimp processing industry generates over 7,000,000 tons of waste shell (Jeyasanta et al. 2017). According to Pal et al., around 65–85% of the shellfish waste is processed. The shrimp production in India was 4.34 lakhs million tons in the year 2014–2015 (Pal et al. 2014). The waste generated from shrimp processing needs to be sustainably managed in order to obtain the value-added products like proteins, carotenoids and chitin. Annual production of chitin in India from shrimp is 3560 tons (Varun et al. 2017). Apart from shrimps, other major sources of chitin/chitosan are crabs, lobsters, crayfish, krill, woodlice, and barnacles. This crustacean waste including shrimps contains approximately 10–60% chitin (Amar 2001). The production of chitin/chitosan from crustacean shell is economically feasible, because along with chitin/chitosan, other useful carotenoids can be obtained, which have not been synthesized yet; however is widely used fish food in aquaculture (Kumar 2000; Kyzas and Bikiaris 2015). Many Asian and European countries including India have started producing chitin and chitosan commercially as research on sustainable use of chitin and chitosan can bring economic and academic prosperity to the nation (Wang et al. 2016; Dutta et al. 2004; Rinaudo 2006).

The major sources of chitin are natural and it is obtained in high percentage in grooved tiger prawn, jinga shrimp, blue swimming crab (male as well as female), cuttlefish, scyllarid lobster, etc. To obtain chitin, the shells of these species are made free of loose tissues and then washed, dried, and sieved followed by demineralization and deproteinization. Deacetylation is performed on the obtained chitin to get chitosan (Al Sagheer et al. 2009). Depending on the source and arrangement of polymeric chain, chitin can be classified as α-chitin, β-chitin, and γ-chitin. α-Chitin

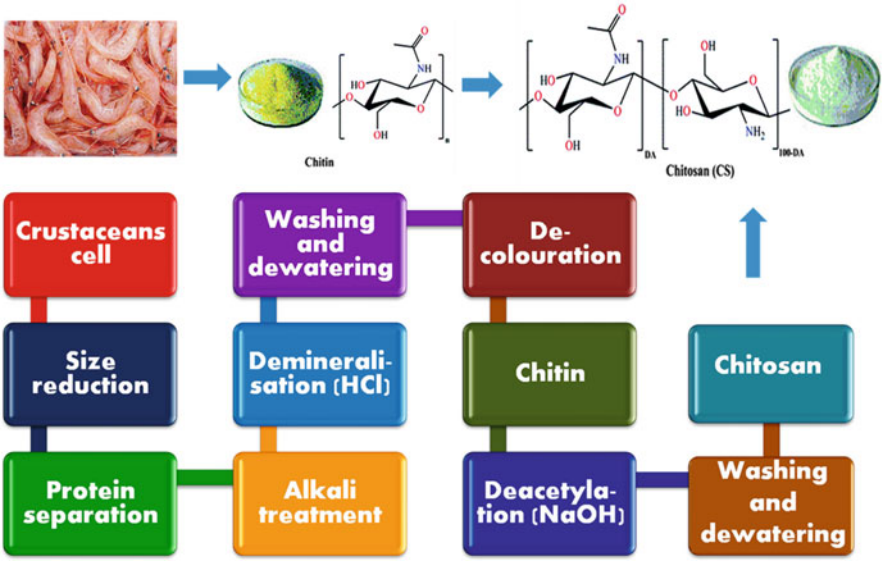

Fig. 1.2 Basic steps involved in extraction of chitosan from crustacean cell

is the most abundant form of chitin. Further, the α-chitin and β-chitin were isolated by reaction with HCl (demineralization) and NaOH (deproteinization).

The basic steps involved in the extraction of chitin from any of its sources are removal of inorganic material, demineralization in acidic medium, and then, finally, deproteinization in a basic medium (Fig. 1.2). The demineralization can also be done using biological methods taking the support of some microorganisms. This can replace the chemical demineralization. Often, decolorization is also done after the process of deproteinization.

Abdou et al. produced chitin as stated below. The raw material being in solid form was washed, cut, and then desiccated. The demineralization was done at room temperature using 1M HCl. The deproteinization was performed using 1M NaOH at 105 °C. After repeating the above steps several times, the product is washed and dried. Further, the decolorization was done. Thus the total process describes production of chitin. The above steps can be considered as a thumb rule for chitin production (Fig. 1.2). Pal et al. reported biological method for the production of chitin from shrimp waste using lactic acid.

Aline Percot et al. reported the optimization of various parameters for obtaining chitin in pure form from the raw material. The effect of temperature was also observed. Specific attention was given on the time optimization in the process of demineralization and deproteinization. The residual content of calcium in chitin after this optimized process was negligible. The calcium release depends upon the pH value. The optimum time for the demineralization step was just 15 min. The optimum temperature and time for deproteinization was reported to be 70 °C and

24 hours, respectively. β-Chitin was extracted from squid pens by Chaussard and Domard. The similar study for optimization, viscosity, and crystallinity was also performed by the authors.

Rhazi et al. concluded that squid pens are the largest sources of chitin. Crayfish, spiny lobster, and squilla were also among the main sources along with crabs and shrimp shells. Molecular weight determination of the obtained chitin was also done in the same study.

Deacetylation is the nonenzymatic process whereby chitosan is obtained from chitin by removing R-$NHCOCH_3$ residue and treating it with strong alkali at high temperatures. When the degree of deacetylation is greater than 50%, the biopolymer becomes soluble in acidic solutions and behaves as a cationic polyelectrolyte due to the protonation of amine groups in the presence of hydrogen ions (Stamford et al. 2013). Other ways to obtain chitosan are by using enzymatic processes. However, they are not used on an industrial scale owing to the high commercial cost of enzymes (deacetylases) and their low productivity, while nonenzymatic chemical processes are widely used because the processes is economical and efficient (Younes et al. 2014). Thus, although there are ample of well-defined sources of chitin, these sources need to be processed before we get chitin. At the same time well-defined methods are now available to obtain chitin and similarly chitosan from their respective raw materials.

1.3 Structure and Properties of Chitosan

Chitosan, the primary derivative of chitin, is comprised of linear β-(1-4)-2-amino-2-deoxy-d-glucopyranose repeating units where the N-acetylglucosamine residues in chitin macromolecular chain are fully deacetylated to become N-glucosamine residues. In general, chitosan occurs as a copolymer of N-acetylglucosamine and N-glucosamine units randomly throughout the biopolymer chain. The percentage of N-glucosamine units is also defined as the degree of deacetylation (DDA) of chitosan.

The discovery of chitosan was made by the French physiologist Charles Rouget in 1859. As compared to its parent polymer chitin, chitosan can be processed into different forms at much milder conditions due to its solubility in dilute acid solutions, making chitosan a more attractive biopolymer for a variety of applications.

Chitosan possesses very interesting chemical and biological properties as mentioned in Table 1.1 and, therefore, has been used in many applications, mainly in the medical and pharmaceutical fields.

Table 1.1 Properties of chitosan

Chemical properties	Biological properties
Solubility in various aqueous media/solution viscosity, multifunctionality, polyelectrolyte behavior, polyoxysalt formation, flexibility; ability to form gel, membrane, film, beads, etc.; metal chelation, optical properties	Nontoxicity, biodegradability, biocompatibility, cytocompatibility, antimicrobial, antioxidant, anticholesterolemic, anti-inflammatory, analgesic, hemostatic, mucoadhesion, adsorption enhancer, granulation and scar formation, macrophage activation

1.4 Factors Affecting Physicochemical Properties of Chitosan

1.4.1 pH

Chitosan is an amino polysaccharide consisting of free amino group; therefore pH substantially alters the charged state and the properties of chitosan. At pH < 6.5 the amino groups of chitosan get protonated and thus make it soluble in acidic solution. However, at pH greater than 6.5 chitosan solution exhibits phase separation and becomes insoluble under basic condition. At pH between 6.0 and 6.5 the amino groups of chitosan become less protonated and thus the hydrophobicity among the polymeric chitosan chain increases resulting into its self-aggregation in acetate buffer solutions via intra- and intermolecular hydrophobic interactions. At low pH (< 6) chitosan can electrostatically interact with negatively charged molecules or polymers, e.g., anionic glycosaminoglycans, proteoglycans, and other negatively charged molecules. At higher pH (above about 6.5) chitosan's amino groups are deprotonated and undergo hydrophobic interactions with several substrates (e.g., fatty acids and cholesterol) (Dash et al. 2011).

These pH-dependent properties of chitosan influence its biomedical activity and potential applications. One of them is the antimicrobial activity of chitosan (Kong et al. 2010). The antibacterial mechanism of chitosan is generally considered to be due to its positively charged amino group at the C-2 position of the glucosamine residue, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Singh and Dutta 2011). The presence of a large number of non-protonated amino groups as well as the poor solubility of chitosan at pH 7 means that chitosan's bactericidal activity is minimal (Aiedeh and Taha 2001; Sudarshan et al. 1992; Papineau et al. 1991). Helander et al. reported that chitosan displayed antibacterial activity in acid environment. It exhibited stronger inhibitory effect at lower pH, which decreased with the increasing pH. Kong et al. and Yang et al. observed that the antibacterial activity of the *N*-alkylated chitosan derivatives against *E. coli* increased as the pH rose from 5.0 reaching a maximum around pH 7.0–7.5. Also, the investigation of the antibacterial property of chitosan microspheres in a solid dispersing system showed that under neutral conditions, of the three tested chitosan microsphere samples with degree of deacetylation 2.5, 16.5,

and 37.4%, respectively, the highest inhibitory effect was observed for the chitosan microsphere sample with degree of deacetylation of 37.4% (Kong et al. 2008).

The metal ion adsorption efficiency of chitosan-based material is greatly influenced by pH. Kyzas et al. reported that at acidic pH 2 the adsorption capacity of carboxybenzyl grafted chitosan for metal cation Cu (II) and Ni (II) was low, which may be due to competitive binding of H^+. A slight increase in adsorption efficiency for Cu (II) and Ni (II) was observed at pH 2–4 and maximum adsorption efficiency was reported at pH 5 which may be due to the deprotonation of amino group making it available for uptake of Cu (II) and Ni (II) through chelation mechanism. However, at basic pH, the decrease in adsorption efficiency of carboxybenzyl grafted chitosan was observed due to the precipitation metal cation Cu (II) and Ni (II) as their hydroxides ($Ni(OH)_2$, $Cu(OH)_2$). Thus the pH 5 was selected as optimum value for the adsorption study (Kyzas et al. 2013).

1.4.2 Ionic Strength

Like pH, ionic strength also plays an important role in the physicochemical properties of chitosan solutions and can strongly influence their biological behavior.

Liu et al. studied the effect of ionic strength on adsorption capacity of Pb-imprinted dithiocarbamate modified chitosan beads (Pb-IDMCB) for Pb (II) adsorption. The effect of different concentrations of $NaNO_3$ on Pb (II) removal was studied. The adsorption capacity of Pb–IDMCB for Pb (II) decreased insignificantly with the increasing ionic strength; however, the adsorption capacity of NIDMCB (non imprinted dithiocarbamate modified chitosan beads) for Pb (II) decreased significantly. The observed decrease in Pb (II) sorption with ionic strength may be explained by the formation of outer-sphere complexes since sodium ions were presenting the background electrolyte. This could compete with the metal ions adsorbed on the outer-sphere sorption sites and reduce the adsorption capacity, but there are many specific cavities for Pb (II) in Pb–IDMCB, which could selectively adsorb Pb (II) from aqueous solution; meanwhile, Na^+ would not compete for the inner-sphere sites. This independence of sorption with background electrolyte concentration has been interpreted to indicate that the sorption process is primarily non-electrostatic in nature. Additionally, the presence of salts may compress the electric double layer surrounding negatively charged surfaces, which contributed to the release of adsorbed lead (Liu et al. 2013).

In another investigation, the effect of ionic strength on the stabilizing properties of chitosan in a model emulsion system containing whey protein isolate as emulsifier and canola oil was studied. Syneresis was favored by the increasing ionic strength to 0.3 M (Laplante et al. 2005).

1.4.3 Concentration

The biological properties of chitosan-based products are greatly influenced by its concentrations. The effects of concentration of chitosan-based products on the antimicrobial properties have been widely reported in literature. Ghaouth et al. studied the effect of chitosan concentration on the growth of *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Rhizopus stolonifer*. Chitosan markedly reduced the radial growth of all the fungi tested, with a greater effect at higher concentration (El Ghaouth et al. 1992).

In addition the adsorption efficiency of chitosan-based adsorbents is also governed by amount of adsorbent taken for adsorption of the targeted analyte. Rahangdale et al. reported that for the given analyte concentration, increase in concentration of chitosan-based adsorbent leads to the increase in adsorption capacity. The probable reason is that the increase in concentration of adsorbent provides more functional group and active sites, thus leading to the increase in adsorption capacity (Rahangdale et al. 2016, 2018; Rahangdale and Kumar 2018a, 2018c, 2019).

1.4.4 Molecular Weight

Chitosan can be categorized into three classes based on its molecular weight: low-molecular-weight chitosan, medium-molecular-weight chitosan, and high-molecular-weight chitosan (Sun et al. 2009). Some physicochemical and biological properties of chitosan and its solutions are affected by its molecular weight and thus molecular weight of chitosan plays a significant role in determination of its bioactivity.

Wang et al. tested chitosan with a molecular weight range between 3.5 and 15.8 kDa as a carrier for protein delivery (Wang et al. 2007). They reported that the loading of the targeted protein bovine serum albumin (BSA) increased from 8% to 48% for molecular weight from 3.5 to 6.3 kDa of chitosan, but only by a few percent for molecular weight 15.8 kDa. The BSA release rate decreased very quickly up to 6.3 kDa, but then only slightly to 15.8 kDa. These results confirmed that chitosan nanoparticles are suitable for delivering protein drugs (Wang et al. 2007; Zhang et al. 2010).

Jeon and Kim tested the antitumor activity of three kinds of chitosan (high molecular weight ranging from 6.5 to 12 kDa, medium molecular weight ranging from 1.5 to 5.5 kDa, and low molecular weight ranging from 0.5 to 1.4 kDa) against sarcoma 180 solid (S180) and uterine cervix carcinoma No. 14 (U14) (Jeon and Kim 2002). The efficiency of tumor growth inhibition for both types of tumor cells in mice was best in the case of medium-molecular-weight chitosan.

1.4.5 Degree of Deacetylation

Chitosan has been used in a wide range of biomedical applications including wound dressings, tissue engineering, implant coatings, and therapeutic agent delivery systems because of its excellent biological properties such as nontoxicity, biocompatibility, and biodegradability. The reactivity of chitosan is governed by factors like molecular weight, degree of deacetylation, pH, concentration crystallinity, etc. Degree of deacetylation (DDA) of chitosan has often been cited as an important parameter that determines many physiochemical and biological properties of chitosan such as crystallinity, hydrophilicity, degradation, and cell response (Prashanth et al. 2002; Prasitsilp et al. 2000; Hidaka et al. 1999). The degree of deacetylation in chitosan refers to the number of glucosamine units after deacetylation. DDA of chitosan is generally controlled by processing of the native polymer with alkali and with increasing time and temperature to obtain matrial with the highest DDA (>90) (Khor and Lim 2003; Kumar 2000; Freier et al. 2005).

Generally, the degree of deacetylation should be greater than 60% and it should solubilize in acidic media. Protonation of the amine group at the C-2 position leads to its complete solubility in an acidic solution below pH 5. Chitosan with a high DDA is suitable for the biomedical applications because DDA governs its biodegradability. Low DDA in chitosan induces an acute inflammatory response owing to its quick degradation, whereas high DDA causes minimal inflammation. However, higher the DDA, lower its affinity for the enzymes in vitro.

The molecular weight of chitosan influences its antibacterial activity, which tends to decrease with an increase in the molecular weight of chitosan. Freier et al. reported that chitosan with DDA 0% or 100% exhibits slower degradation rate and enhanced cell adhesion while chitosan with an intermediate DDA exhibits rapid degradation rates, but at the cost of limited cell adhesion (Freier et al. 2005). Hidaka et al. found that chitosan membranes synthesized with DDA between 65 and 80% exhibited marked inflammatory reactions that subsided in time with degradation of the films, granulation tissue formation, and osteogenesis while membranes made of chitosan with 94% DDA showed minimal degradation, mild inflammation, and minimal osteogenesis (Hidaka et al. 1999).

1.4.6 Temperature

Temperature affects the moisture content of chitosan-based materials. The mechanical strength and hardness of chitosan powder was reduced significantly at high temperature (40 °C) due to loss of moisture. Atmospheric temperature may affect the chitosan degradation ratio, especially in liquid and semisolid products. High temperature resulted into the faster degradation of chitosan chain in solution form and the rate of hydrolysis was found to follow first-order kinetics. However, no significant chain hydrolysis was noticed in the chitosan solution stored at 5 °C (Nguyen et al. 2008; Vårum et al. 2001).

1.5 Modification of Chitosan

Chitosan being basic in nature is insoluble in most of the aqueous solutions. As a result, restriction is imposed on the applications of chitosan. Thus in order to widen its area of applicability, it can be modified in terms of structure as well as chemical properties. Physical and chemical modifications are done to increase the solubility of chitosan in aqueous solutions and to improve its other important properties. Many times, modifications are done in order to increase its rigidity, porosity, etc. (Rahangdale et al. 2017; Rahangdale and Kumar 2018b).

The broad classification for the modification of chitosan is done as follows:

1. Physical modification
2. Chemical modification
3. Molecular imprinting

1.5.1 Physical Modification

The changes of the structural or tangible properties come under this classification. The formation of gels, membranes and beads of chitosan modifies them physically.

In one of the techniques proposed by Sandra Rivero et al., heat treatment was given to chitosan with tannic acid as well as without tannic acid to carry out the physical modification. After heat treatment at 180 °C, the moisture content as well as the water uptake capability of chitosan is reduced. Here the chitosan was modified in the form of films (Rivero et al. 2011).

A membrane of chitosan was synthesized by Mi et al., using immersion precipitation along with phase inversion method. The membrane was finally used for wound healing. The phase separation method can be efficiently modified using pre-evaporation method. This modified method can lead to an excellent control over porosity as well as thickness of skin surface. Chitosan when used for would healing purpose gives an all-round protection to the wound taking care of bacteria, fluid and gas penetration if the modifications suggested by Mi are practically implemented.

Chitosan can also be converted into various gels to enhance its properties (Ruel-Gariepy et al. 2000). Thermosensitive gels of chitosan were prepared for sustained drug delivery. The gels were quite stable and the viscosity remained unchanged for 3 months. It was specified that for gel formation of chitosan three types of interactions can be realized:

1. Hydrophobic interaction between two chitosan units
2. Hydrogen bonding
3. Electrostatic attraction

Another way of chitosan modification is formation of beads. Along with the wide use of modified chitosan in biomedical industry, it is also widely applicable for purification processes. Chitosan if modified properly has the property of absorbing

metal ions from aqueous solutions. Being a nontoxic biopolymer this can be used for water purification. Rorrer et al. fabricated magnetic and porous chitosan beads for the removal of Cu ions from water. Here three basic steps are involved in the formation of the beads. They are defined as bead casting, crosslinking, and drying. The formed beads were porous and had the capability for the uptake of Cu ions form the aqueous solutions (Rorrer et al. 1993).

Chitosan powders have also been synthesized as a part of its physical modification. Drug-loaded spray powder was synthesized by Learoyd et al., with high yield. The powder was amorphous in nature. Thus, a variety of physical modifications of chitosan are possible and each of the method gives some or the other application specific benefit.

1.5.2 Chemical Modification

Various methods can be employed for the chemical modification of chitosan.

1. Grafting of Chitosan

Grafting is a tailored method where a desired functional group is introduced on the backbone of the polymer whose grafting is to be done. Thus here a function group will be added onto chitosan in order to enhance its properties. The grafting can be of enzymatic, free radical, radiation, or cationic type. Enzymatic grafting is one of its kinds and has a lot of advantages. It eliminates the hazards associated with reactive agents and is specific in nature. Water solubility under basic conditions is obtained by phenolic grafting over chitosan (Jayakumar et al. 2005). Some of the examples of copolymer grafting are depicted in Table 1.2:

Enzymatic grafting of carboxylic group was done on chitosan to use it as cationic dye adsorbent by Chao et al. (2004). Here the four derivatives of phenol, namely, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenyl-acetic acid, and hydrocaffeic acid, were grafted onto chitosan. Here the yield of grafting was controlled by increasing the space between the benzene ring and carboxylic group. Thus grafting increased the adsorption capacity of the polymer chitosan.

Another innovative technique for grafting is through radiations. The various parameters controlling the grafting yield in this method were studied. The parameters such as radiation dose, monomer concentration, solvent composition, and time of exposure control the graft reaction as well as graft yield. Butyl acrylate was grafted on chitosan using gamma irradiations by Yu et al. (2003). This grafting had made chitosan hydrophobic and increased its impact strength.

Grafting can also be performed using free radical-initiated reactions. Liu and his coworkers performed the grafting of caffeic and ferulic acids on chitosan. The chitosan derivatives thus produced were soluble in water. However the thermal stability and crystallinity were found to decrease (Liu et al. 2014a, b).

Table 1.2 Different graft copolymers of chitosan

Sr. No.	Functionalized chitosan	Monomer grafted	Reference
1	Chitosan-graft-polyacrylonitrile	Acrylonitrile	Pourjavadi et al. (2003)
2.	Chitosan-*graft*-(*N*-isopropylacrylamide)	N-Isopropyl acrylamide	Kim et al. (2000)
3.	Chitosan-modified poly(vinyl acetate)	Vinyl acetate	Don et al. (2002)
4.	Graft copolymers of chitosan with Acrylic/methylacrylic acid	Acrylic, methylacrylic acid	Shantha et al. (1995)
5	Poly(*N*-vinylimidazole) grafted chitosan	N-Vinylimidazole	Caner et al. (2007)
6	Chitosan-*graft*-poly(triethylene glycol dimethacrylate)	Triethylene glycol dimethacrylate	Yilmaz et al. (2007)
7	Graft copolymer of acrylic acid (AA) and 2-hydroxyethyl methacrylate	Acrylic acid, 2-hydroxyethylmethacrylate	Dos Santos et al. (2006)
8	Polyacrylic acid grafted chitosan	Acrylic acid	Yazdani-Pedram et al. (2000)
9	Chitosan grafted poly(N,N-dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium)	N,N'-Dimethyl-N-methacryloxyethyl-N-(3-sulphopropyl) ammonium	Zhang et al. (2003)
10	Acrylamide grafted chitosan	Acrylamide	Rahangdale and Kumar (2018a)
11	Graft copolymers of maleoylchitosan and poly(acrylic acid)	Acrylic acid	Huang et al. (2006)
12	Chitosan-*graft*-poly(methyl methacrylate)	Methyl methacrylate	Singh et al. (2006)
13	Maleic acid grafted chitosan	Maleic acid	Hasipoglu et al. (2005)
14	Poly(2-acrylamido-2-methylpropanesulfonic acid) grafted chitosan	2-Acrylamide-methylpropanesulfonic acid	Najjar et al. (2000)
15	Vinyl pyrrolidone grafted chitosan	Vinyl pyrrolidone	Yazdani-Pedram and Retuert (1997)
16	Polyacrylamide grafted chitosan	Acrylamide	Yazdani-Pedram et al. (2002)
17	Graft copolymer of 2-hydroxyethyl acrylate and chitosan	2-Hydroxyethyl acrylate	Mun et al. (2008)
18	Poly(acrylic acid-co-acrylamide) grafted chitosan	Acrylic acid and acrylamide	Mahdavinia et al. (2004)
19	Graft copolymer of acrylonitrile/methyl methacrylate and chitosan	Acrylonitrile and methyl methacrylate	Prashanth and Tharanathan (2003)

Water solubility of chitosan can also be achieved by simple acetylation. This is a well-defined and convenient technique developed by Sashiwa and his coworkers (Sashiwa et al. 2002).

Chitosan can also be modified by crosslinking. In most of its applications, it was crosslinked to form oligomers. Dialdehydes are mostly used as crosslinking agents for chitosan. The crosslinking increases the elasticity as well as the strength of chitosan. Thus in order to use chitosan for any applications, crosslinking is one of the ways to improve its properties. Chemical, ion adsorption, mechanical, and physical properties of chitosan can be improved by crosslinking. Beppu et al. had crosslinked glutaraldehyde with chitosan to form the resulting polymer chain and various properties were studied. In these results, diffusivity also decreased.

Kumbar et al. crosslinked chitosan and increase in the crystallinity was observed with increase in crosslinking. The smallest particle size was obtained when glutaraldehyde was used as crosslinker.

Liu et al. crosslinked chitosan in situ using gamma glycidoxypropyltrimethoxysilane as a crosslinker. The result was formation of silica–chitosan hybrid membranes. Hydrophilicity of chitosan membrane was maintained in this process. The resultant membranes had increased thermal stability and increased water stability.

Acid chlorides and acid anhydrides were used by Tangpasuthadol et al. to do surface modification of chitosan. The surface hydrophobicity was increased and thus protein adsorption was enhanced when chloride derivatives were formed. Similarly when anhydride derivatives were formed, the behavior became selective adsorption type.

Amaral et al. (2005) performed phosphorylation of chitosan to form the chemical derivative. The phosphate functionalities have cationic exchange properties. Thus, the derivatives can be efficiently used for orthopedic applications. The chitosan thus formed would have amphoteric properties which would further widen its area of applications. Silva et al. (2008) performed plasma surface modification of chitosan. The chitosan membranes were treated with argon plasma, increasing its biocompatibility and potential in wound dressing. Functionality and etching processes occur when chitosan was exposed to plasma. However higher surface roughness was obtained which confirmed significant etching process. This also increased the fibroblast adhesion and also the chitosan membrane proliferation.

Chitosan was linked to cellulose (Darias and Villalonga 2001) where the pH and temperature of cellulose remained unchanged. The acidic stability of the modified chitosan was found to increase. The resultant polymer was biodegradable and nontoxic. The various glycol enzyme properties were also improved increasing the application of the resultant polymer.

Thus the chemical modification of chitosan can create wonders and unbelievable contrast properties can be achieved.

1.5.3 Molecular Imprinting of Chitosan

Molecularly imprinted polymers are memory-based polymers which can extract a specific template. Chitosan, due to its multifunctionality, can act as a functional polymer for forming the matrix of the MIP. However, in order to increase the efficiency of the chitosan-based MIPs, it should be suitably modified. The modification can be of any type like crosslinking, grafting, etc., or a combination of any of these methods. This modification would increase the strength, elasticity, and durability of chitosan (Rahangdale and Kumar 2018a, c).

Xia and his group used chitosan beads as functional matrix for the selective separation of quercetin. Here methacrylic acid was used as a separate function monomer. Chitosan porous beads were formed and the resultant beads were crosslinked to get the functional matrix. The adsorption capacity of the matrix was high and it had fast adsorption characteristics (Xia et al. 2006).

This technique is very impactful as the required characteristics of the monomer can be introduced into chitosan by modification and thus the MIPs formed will have nontoxic, biodegradable characteristics due to the use of chitosan. Guo et al. (2004) used chitosan for the preparation of the molecularly imprinted polymers for the separation of hemoglobin. Acrylamide was used as a functional monomer and chitosan beads as supporting matrix. The molecularly imprinted polymer had high adsorption capacity, high selectivity, and easy reproducibility. The use of such polymers can be further extended in the area of biosensors.

Rahangdale et al. prepared dual imprinted polymers for the simultaneous removal of salicylic acid and cadmium. Here chitosan was used as a functional polymer and epichlorohydrin was used as the crosslinker. The time, dose, binding capacity, and binding percentage were optimized in this study. The polymer had higher binding affinity for Cd and salicylic acid. Chitosan, being easily available and cost effective, makes the overall process simple and convenient as well as eco-friendly (Rahangdale et al. 2018).

Chitosan can also be used for selective adsorption of metal ions. Tianwei et al. prepared chitosan resins for selective removal of metal ions using epichlorohydrin and glutaraldehyde as crosslinking agents and a relative study was done. Adsorption capacity and selectivity of metal ions was increased. The pore diameter increased and the surface area of the resultant polymer decreased. For nickel ion, the stability and mechanical and chemical properties were found to be improved than the non-imprinted polymers. The adsorption behavior became 20 times than the non-imprinted polymer and the polymer was reusable (Tianwei et al. 2001).

Rahangdale et al. used chitosan grafted with acrylamide as a polymer and Cd was used as a template. The special application of this polymer was the recovery of Cd from Ni–Cd battery waste. In this case epichlorohydrin was used as a crosslinker. The detailed optimization of time, dose, and pH was done. The adsorption capacity was excellent and the recovery of Cd was 84% from the battery waste. Use of biopolymer chitosan makes this a green process and a very efficient way for the recovery of the valuable heavy metal. The polymer can be used repeatedly up

to multiple cycles with minimal loss in the efficiency (Rahangdale and Kumar 2018a).

Li et al. (2008) used chitosan–TiO_2 composite for simultaneous removal of organic and inorganic pollutant using molecular imprinting along with photodegradation. Here the polymer thus prepared was used for adsorption of heavy metal ions as well as for degradation of organic compounds up to 90%.

Perfluorooctane sulphonate was removed selectively by chitosan-based molecularly imprinted polymer from aqueous solution (Al Sagheer et al. 2009). Perfluorooctane sulphonate is a pollutant of global concern. The molecularly imprinted polymer prepared used epichlorohydrin as crosslinker. The reusability up to five times was achieved. The polymer thus formed was porous and specific for perfluorooctane sulphonate. The adsorption decreased with increased in pH. Polymer selectivity was also a function of molecular size and electrostatic attraction.

Thus, the modification of chitosan can create huge benefits and improve any desired characteristic if proper process is followed. Based on the application, the biopolymer chitosan can be modified physically and chemically or the process of molecular imprinting can be adopted.

1.6 Computational Modeling for Rational Designing of MIP and Chitosan-Based Material

The efficiency of imprinted polymer is governed by many factors such as the proper selection of the functional monomer, template or its structural analogue, porogenic agent, appropriate crosslinking agent, and the reaction initiator (Bastide et al. 2005). In order to obtain a précised imprinted polymer for a targeted analyte (template), one should also take into consideration the nature of the bonds to be formed between the functional monomer and the template/structural analogue as well as the solubility of the template/structural analogue in the polymerization mixture (Liu et al. 2017). Optimization of the above mentioned parameter may require significant quantities of solvents and reagents in order to achieve a satisfactory final outcome in the form of new adsorbent material (Pardeshi et al. 2012a). In addition, the adsorbent must be washed with the solvent after the completion of polymerization so as to extract the residue template or its structural analogue as well as to eliminate unreacted chemicals. Upon completion of the above steps, the obtained adsorbent may be further used for determination of its morphological and physiochemical properties and applications (Khan et al. 2016).

The exhaustive multistep processes mentioned above can be simplified using virtual designing of the imprinted polymers using a computer as a tool for the molecular modeling, which could significantly decrease the use of chemicals and reagents and can be considered as a greener and environmentally friendly approach. Molecular modeling for rational designing of MIP is based on the selection of the suitable method of calculation including ab initio Hartree–Fock (HF) or significantly more accurate second-order Moller–Plesset (MP2) which treats correlation energy

but is computationally expensive. The best choice in terms of accuracy and efficiency is density functional theory (DFT) (Azimi and Javanbakht 2014; Wang et al. 2015). The second important component of the molecular modeling is the choice of a suitable basis set. The flexible and saturated basis set allows the accurate modeling of small molecules and the use of small Pople-type basis sets (3-21G, 6-31G∗) is preferred for larger molecular systems or for fast and very approximate calculations. The B3LYP (Becke three-parameter Lee-Yang-Parr) hybrid functional with 6-31G (d) basis set is the most commonly used for the theoretical designing of the imprinted polymers for geometry optimization and frequency calculation of a large number of molecules and molecular complexes (Jun-Bo et al. 2015).

The B3LYP method with 6-31G (d) basis set allows the researchers to select an appropriate monomer–template combination on the basis of stability of prepolymerization complexes formed between template and monomer which is evaluated by calculating change in Gibbs free during complex formation as well as the type of interaction involved. All these factors govern the efficiency of imprinting process and thus help in successful synthesis of MIP.

Computational approaches, such as molecular modeling, have been widely reported for the rational designing of imprinted polymers specific toward the targeted analyte due to its advantages such as high accuracy level of information, reliability, and reasonable computational costs in comparison with other computational methods (Pardeshi et al. 2013).

It is considered as a rational, fast, and economic method which allows the rational choice of the most suitable monomer, crosslinker, and polymerization solvent among a set of chemicals traditionally used in imprinted polymer formulations for the molecular imprinting of a given template. It has been used to guide synthesis and performance of MIP for a specific template by the design of virtual libraries that screen the best possible functional monomers and also to study the nature of interaction between template–monomer complex (prepolymerization complex) (Pardeshi et al. 2012b). It also helps in understanding of various thermodynamic and spectroscopic properties of a system at molecular level.

It is documented that when a library of functional monomers is screened against a template using molecular modeling software, the monomers giving the highest value of Gibbs free energy change (ΔG) are more likely to form strong complexes with the template. Rahangdale et al. reported use of computational modeling for the selection of suitable grafting agent for the functionalization of chitosan in order to increase the interaction sites for the uptake of salicylic acid and cadmium. They have also used computational modeling to study the interaction present in prepolymerization complexes to support use of dummy template 4HBA during imprinting for salicylic acid recognition (Rahangdale and Kumar 2018a; Rahangdale and Kumar 2018c).

Computational approaches, such as molecular modeling, have also been widely reported for the rational designing of composites of chitosan due to its advantages such as high accuracy level of information, reliability, and reasonable computational costs in comparison with other computational methods (Pardeshi et al. 2013). Some of the examples are depicted below.

Hlavac et al. reported the use of molecular modeling for the selection of proper composition of chitosan/polyethylene oxide polymer blends for electrospinning. For this the initial models of chitosan and polyethylene oxide were prepared with varying lengths and compositions. Three variants of polyethylene oxide with three lengths of chains were prepared: 4 meters, 5 meters, and 15 meters. Two types of chitosan were studied for investigating the influence of deacetylation 80 % and 100 % deacetylated chitosan, both with the same length of 10 meters. Amino groups were protonated for simulation of acidic environment. On the basis of energy minimization it was revealed that deacetylation as well as deprotonation of chitosan amino groups plays a key role for stability of resulted polymer blend, and from the results of energy optimization it was observed that the strongest interaction exists between chitosan and polyethylene oxide in the case of more deacetylated chitosan (Hlaváč and Tokarský 2013).

The use of molecular modeling has also been reported to study the mechanical properties of chitosan/graphene composite by varying the graphene composition. For this the computational model of graphene/chitosan composite with varying amount of graphene (4, 7.67, 14.28 wt.%) was constructed. The elastic moduli constants for the pure chitosan and graphene/chitosan composite systems were calculated using molecular modeling and the result showed that a reinforcement of chitosan was observed with the addition of the graphene. The largest increase of ~ 23% was observed in the case of the composite with 7.67 wt.% graphene in their structure. Further increase in the graphene amount led to agglomeration and produced just a marginal effect (Pandele et al. 2014).

Lopez-Chavez and his coworkers utilized molecular modeling to construct an ionic conducting polymer–electrolyte system consisting of two polymeric chains of chitosan, each one with 12 amino group protonated chitosan monomeric units, one hydronium ion, one hydroxide ion, 200 water molecules, and 12 sulfate ions to study the ionic conductivity of both hydronium and hydroxide ions. The molecular modeling results were utilized to describe the ionic conductivity mechanism along the polymer matrix and compared with previously reported experimental data for chitosan membranes. To study the mechanism of the ionic conductivity in the system, three ionic species were used: hydronium, hydroxide, and sulfate ions. The results of molecular modeling showed that the hydronium and the hydroxide ionic species were responsible for the movement along the polymer matrix and the sulfates anchored on the amino groups of the membrane backbone leading to the mobility of the charge carrier ionic species (López-Chávez et al. 2005).

Molecular modeling based on quantum chemical calculations has been reported to understand the adsorption mechanism on the basis of estimation of activity coefficients of solute at the interface of solution adsorbent. The modeling results were compared with experimental data obtained for the adsorption of methylene blue onto lignin–chitosan blend. Modeling results revealed that methylene blue adsorption onto lignin–chitosan blend is favorable since the required desorption energy of methylene blue molecules from the lignin–chitosan blend surface is about eight times greater than the required desorption energy of water molecules (Rezakazemi et al. 2018).

Shen et al. reported the use of computational modeling to study the atomic interaction mechanism between chitosan and DNA as it is important in the design and application of chitosan-based drug and gene delivery systems. Molecular modeling results demonstrated that the functional groups of chitosan, the types of base, and length of polynucleotides regulate the interaction behavior between chitosan and polynucleotide. The results revealed that the aggregation effect in case of protonated chitosan could be partially eliminated due to the strong electrostatic interaction, especially the H-bond between $-NH_3^+$ groups on chitosan and phosphate groups on polynucleotide. The good dispersal capacity of polynucleotides may improve the encapsulation of polynucleotides by chitosan and hence increase the delivery and transfection efficiency of chitosan-based gene carrier (Shen et al. 2017).

In this way one can calculate the properties of various compounds and their interactions from the chemical structure of compound using molecular modeling approaches, and it is highly recommended as it provides an alternative approach to analyze the feasibility of a reaction prior to any experiment.

1.7 Application

1.7.1 Biomedical Application

1.7.1.1 Biosensor

Recently, enzyme biosensors are being reported as important tool in clinical, environmental, and food analysis over ordinary chemical sensors. The glucose electroenzymatic biosensors have been used in the food industry for quality control and most importantly as a clinical indicator of diabetes. Yang et al. fabricated a glucose biosensors for the efficient immobilization of enzymes on chemically modified biopolymer chitosan on the surface of a platinum electrode. The biosensor was effectively applied for the determination of glucose in beverage drinks through electrooxidation of H_2O_2 produced by oxidation of glucose at +0.6V by the glucose oxidase. The fabricated chitosan-based glucose biosensor has several advantages: a) easy and simple fabricating method which leads to the formation of interference-free film and b) cost effective, since very little enzyme was required during fabrication using the proposed protocol compared to electropolymerization method in which the immobilization method leads to the wastage of enzymes and some enzymes are extremely expensive (Yang et al. 2005).

1.7.1.2 Cancer Diagnosis

The quantum dots coordinated with heavy metals such as cadmium sulfide, cadmium selenide, and zinc selenide have been reported as a promising fluorescent probe for many biomedical applications. The quantum dots can replace the conventional organic fluorescent dyes in immunostaining and bioimaging of tissues and cancerous cells by appropriate bioconjugation. However, many of the quantum dots investigated for this purpose are cytotoxic owing to their heavy metal composition

(Derfus et al. 2004). To overcome this Manzoor et al. reported the synthesis of a heavy metal-free luminescent quantum dot (QD) based on doped zinc sulfide (ZnS), conjugated with a cancer-targeting ligand, folic acid (FA), by a simple aqueous method at room temperature. These quantum dots were found to be a promising biofriendly system for targeted cancer therapy (Manzoor et al. 2009).

Derfus et al. have shown that CdSe core quantum dots can induce cytotoxicity under certain conditions. The surface oxidation of CdSe core quantum dots led to the formation of reduced Cd on the surface of quantum dots and led to the release of free cadmium ions which finally led to cell death (Derfus et al. 2004).

Mathew et al. fabricated a novel multifunctional folic acid-conjugated carboxymethyl chitosan coordinated with manganese doped zinc sulfide quantum dot (FA–CMC–ZnS:Mn) nanoparticles for successful diagnosis and treatment of breast cancer. These nanoparticles can be used as targeted drug carrier and also for imaging of breast cancer cells by the fluorescence of ZnS:Mn attached to the system. These nanoparticles were synthesized by environmentally friendly simple aqueous route. The in vitro imaging of cancer cells with the nanoparticles was studied using fluorescent microscopy. The bright and stable luminescence of quantum dots can be used to image the drug carrier in cancer cells without affecting their metabolic activity and morphology. The anticancer drug selected in this study was 5-fluorouracil which can be used for the breast cancer treatment. The nontoxicity of FA-CMCS-ZnS:Mn nanoparticles was studied using L929 cells. Breast cancer cell line MCF-7 was used to study the imaging, specific targeting, and cytotoxicity of the drug-loaded nanoparticles. The targeted anticancer drug carrier with a biofriendly heavy metal-free quantum dot for tracking the path of the drug carrier is a great contribution to cancer therapy (Mathew et al. 2010).

1.7.1.3 Tissue Engineering

Tissue engineering is one of the most multidisciplinary research areas which involve the replacement of the body tissues and organs that are damaged far beyond recognition. The purpose of tissue engineering is to repair, replace, maintain, or enhance the function of a particular tissue or organ. Generally, autograft and allograft therapies are used clinically for tissue or organ replacement. However, these therapies exhibit several limitations such as limited availability, risk of disease transmission, pain at the graft site, lack of enough fusion, morbidity at the donor site, and cost. The alternative to these therapies is the replacement of tissue or organ with cells and biomaterials which shows better survival rates over autografts and allograft therapies. Various natural and synthetic polymers have been widely investigated for different facets of tissue engineering; among them chitin and chitosan have shown remarkable promise in the biomedical field. There are some properties that a polymer should bear for polymer scaffold designing such as high surface area, high porosity, nontoxic, biodegradability with the degradation rate matching the rate of neotissue formation, and structural integrity to prevent the pores of the scaffold from collapsing during neotissue formation.

Tissue engineering can be further divided into different segments based on the type of tissue/organ such as bone, ligament, cartilage, tendon, liver, neural and skin regeneration.

Shalumon et al. reported synthesis of water-soluble carboxymethyl chitin (CMC)/PVA blend fibrous membrane for tissue engineering applications. During the synthesis the concentration of carboxymethyl chitosan (7%) and PVA (8%) was optimized and electrospun to get nanofibers. Further the nanofibers were crosslinked with glutaraldehyde vapors followed by thermal treatment to get water-insoluble nanofibres. The prepared nanofibers were found to be bioactive and biocompatible. The cytotoxicity of the fibrous membrane was evaluated using human mesenchymal stem cells by the MTT assays. The results indicated that the nanofibrous CMC/PVA scaffold supports cell adhesion/attachment and proliferation and hence this scaffold will be a promising candidate for tissue engineering applications (Shalumon et al. 2009).

A novel biocomposite scaffold of chitosan and glass ceramic nanoparticles was prepared by Peter and his coworkers by blending glass ceramic nanoparticles with chitosan solution followed by lyophilization technique. The swelling, density, degradation, and in vitro biomineralization studies of the composite scaffolds were carried out and the results indicated that the degradation and swelling behavior of the nanocomposite scaffolds decreased, while protein adsorption increased with the addition of glass ceramic nanoparticles. Biomineralization studies showed higher amount of mineral deposits on the nanocomposite scaffold, which increased with increasing time of incubation. Cytocompatability of the composite scaffolds was assessed by MTT assay, direct contact test, and cell attachment studies. Results indicated that the nanocomposite scaffolds are better for cell attachment and spreading. The in vitro biomineralization studies confirmed the bioactive nature of the composite scaffolds. So, these nanocomposite scaffolds can be used effectively for alveolar bone regeneration (Peter et al. 2010).

Lee et al. synthesized chitosan–silica xerogel composite membranes using a sol–gel process. Silica xerogels, the inorganic constituent, have shown great promise in biomedical applications. Silica xerogels along with the chitosan, as the organic phase, allow the rigid silica xerogel to be handled in the form of a flexible membrane. The synthesized composite exhibits the properties of each constituent and thus has attracted considerable attention for novel bone substitute materials. The mechanical properties of chitosan were found to increase after composite membrane formation (Lee et al. 2009b).

Li et al. reported the synthesis and application of chitosan–alginate hybrid scaffolds for bone tissue engineering. A biodegradable scaffold acts as a temporary skeleton to accommodate and stimulate new tissue growth. The mechanical and biological properties of chitosan were increased after hybrid scaffold formation with alginate, which can be attributed to strong ionic interaction between chitosan and alginate. Bone-forming osteoblastic cells were found to attach easily to chitosan–alginate scaffold and grow actively and deposited calcified matrix. The in vivo studies showed that the hybrid scaffold had a high degree of tissue compatibility (Li et al. 2005a, b).

Jiang et al. fabricated novel three-dimensional chitosan/poly(lactic acid-glycolic acid) (PLAGA) composite porous scaffolds by sintering together chitosan/PLAGA microspheres for bone tissue engineering applications in contrast to the conventional freeze-drying technique which was believed to reduce the mechanical strength of the scaffold. The enhancement in mechanical strength of chitosan was observed after the addition of poly(lactic-co-glycolic) acid (PLAGA) which can be attributed to increase in sintering temperature and time; however the pore volume was decreased. The presence of chitosan on composite scaffold microsphere surface leads to increase in alkaline phosphatase activity of the cells cultivated on composite microsphere. It was also observed that the MC3T3-E1 osteoblast-like cells adhere well and grow actively as compared to PLAGA scaffolds. In addition, the presence of chitosan on microsphere surfaces increased the alkaline phosphatase activity of the cells cultured on the composite scaffolds (Jiang et al. 2006).

Further, Jiang et al. also fabricated a heparin-modified CS/PLAGA sintered scaffold to enhance the osteoblastic proliferation and differentiation, thereby stimulating rapid bone formation. The multifunctionality of chitosan facilitates the binding of the biomolecule heparin during scaffold synthesis (Jiang et al. 2010).

1.7.1.4 Wound Dressing

The incorporation of biopolymers such as gelatin, pectin, starch, cellulose, alginate, chitin, chitosan, collagen, polyamino acids, hyaluronates, and dextran into synthetic wound dressings has shown to enhance the healing process. The sugar and amino acid residues of these materials act as analogues of protein and growth factor present in human body required for stimulating the appropriate physiological responses required for cellular regeneration and tissue restructuring in wounds (Mi et al. 2001, 2003).

Mi et al. synthesized silver sulfadiazine-incorporated chitosan membrane with sustained antimicrobial capability by a dry/wet phase separation method to overcome current limitations in silver sulfadiazine cream for treating acute burn wounds. Silver sulfadiazine is an antibiotic which is applied to burn injuries in human (Fox 1975). However, it does not allow long-term protection of the wound from infection. Recently, some of the researchers have reported the use of antibiotic-incorporated wound dressing or artificial skin as a sustainable solution to the above problem, as it allows the delivery of the drugs in a controlled way. Thus, the wound can be effectively prevented from infection (Mi et al. 2003).

Chitosan–carboxymethyl chitosan–PVA composite membrane was fabricated by Pang and his coworkers and the results of the experiment conducted on animals demonstrated that the wound covered with composite membrane was hemostatic with fast healing and was histocompatible. It has been reported that chitosan had highest antimicrobial activity against E.coli. Whereas, carboxymethyl chitosan not only had capability to promote the growth of human skin fibroblast and inhibit the growth of keloid fibroblast, but also was biocompatibility with no cytotoxicity. Thus the composite membrane had found potential application to be a wound dressing material in biomedical application (Pang et al. 2008).

Chitin and chitosan have been reported to be effective biomaterials for wound dressing as it promotes the normal tissue regeneration and have bacteriostatic and fungistatic activities. Chitosan is a semicrystalline polymer and has lower gas permeability (Muzzarelli et al. 1974). However, wound dressing must have gas exchange through it, because high CO_2 pressure increases the acidity and slows down the healing process, and in addition a low oxygen concentration decreases the regeneration of tissue cell or makes possible the proliferation of anaerobic bacteria. In this regard modification of chitosan is necessary in order to achieve higher gas and water transport (Shigemasa et al. 1992; Urgen Kaessmann and Haak 1997).

1.7.1.5 Drug Carrier

The discovery and development of synthetic drugs is highly challenging, laborious, and expensive processes. However in spite of the successful trial in the clinical phase, most of the drugs fail to achieve favorable clinical outcomes because they do not have the ability to reach the target site of action. Some quantity of administrated drug is distributed over the normal tissues or organs leading to severe side effects. To overcome this, an effective approach is the synthesis of targeted drug that releases the drugs or bioactive agents at the desired site of action. Chitosan has been widely utilized as drug delivery systems for low molecular drugs, peptides, and genes.

Tripolyphosphate-crosslinked chitosan nanoparticles have been employed as carriers for the anthracycline drug, doxorubicin, to deliver it into the cells in its active form. Janes et al. effectively entrapped doxorubicin into the chitosan nanoparticles during ionotropic gelation of the chitosan with tripolyphosphate. The doxorubicin-loaded nanoparticles showed significant cytostatic activity against human melanoma A375 cells and C26 murine colorectal carcinoma cells relative to free doxorubicin and thus found to be more efficient. Further the confocal microscopy studies demonstrated that doxorubicin-loaded chitosan nanoparticles enter the cells via an endocytic mechanism and degrade intracellularly to release the doxorubicin (Janes et al. 2001).

The poly(L-lactic acid) (PLLA)–chitosan hybrid scaffolds were synthesized by Prabaharan by using PLLA with different concentrations of chitosan and glutaraldehyde in order to be used as a drug carrier. The incorporation of chitosan into the PLLA porous structure allows for producing chitosan-based scaffold devices with interesting damping and stiffness aimed at being used in tissue engineering of bone or cartilage. The porosity of hybrid scaffolds was governed by the concentration of the chitosan incorporated into the PLLA scaffold. At lower concentrations, chitosan was mainly adsorbed onto the surface of PLLA, whereas at higher concentration chitosan formed microfibrillar structures within the pore walls of the PLLA foam that may act as additional soft anchorage sites for cells. An anti-inflammatory drug, ketoprofen, was loaded within the chitosan component of the hybrid scaffolds by immersing the scaffolds in a drug–ethanol solution. The drug release rate can be controlled by the chitosan content and crosslink densities, suggesting the effectiveness of the hybrid scaffold as a drug delivery system (Prabaharan et al. 2007).

A novel biodegradable chitosan–β-cyclodextrin composite scaffold was fabricated by Prabaharan and his coworkers using freeze-drying method and has

been applied as synthetic extracellular matrices to fill the gap during the healing process. It can also be used as a matrix for drug loading and controlled release due to the presence of β-cyclodextrin. The morphology, swelling, and drug release properties of the scaffolds were governed by the extent of crosslinker glutaraldehyde used during scaffold synthesis. The cytotoxicity study revealed that there is no obvious cytotoxicity of chitosan–β-cyclodextrin composite scaffolds crosslinked with 0.01 M glutaraldehyde against the fibroblast (L929) cells. These results indicated that chitosan–β-cyclodextrin composite scaffolds may become a potential biodegradable active filling material with controlled drug release capability, which provide a healthy environment and enhance the surrounding tissue regeneration (Prabaharan and Jayakumar 2009).

1.7.1.6 Antimicrobial Activity

Chitosan has been investigated as an antimicrobial agent against a wide range of microorganisms like algae, bacteria, yeasts, and fungi because of its interesting biological properties.

The antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan deacetylation, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions (e.g., substrate water activity or moisture or both). Chitosan with different degrees of deacetylation has been tested against fungi (*Aspergillus fumigatus*, *Aspergillus parasiticus*, *Fusarium oxysporum*, *Candida albicans*), Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Bacillus cereus*, *Listeria monocytogenes*), and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Aeromonas hydrophila*, *Shigella dysenteriae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*) in order to see the effect of degree of deacetylation on antimicrobial activity of chitosan. It has been observed that the antimicrobial activity of chitosan increased with increase in degree of deacetylation due to the increasing number of ionizable amino groups. Antibacterial activity also increases with increasing molecular weight of chitosan, though too high molecular weight or concentration is counterproductive (Andres et al. 2007; Tipparat and Riyaphan 2008; Tsai et al. 2002).

Park et al. studied the effect of DDA of chitosan on its antimicrobial activity. For this the chitosan with different degrees of deacetylation was tested against three Gram-negative bacteria and five Gram-positive bacteria. The results demonstrated that the chitosan with DDA 75% exhibited more antimicrobial activity than chitosan with 50% DDA (Park et al. 2004).

However, some researchers have mentioned that there is no exact relationship between DDA and antimicrobial activity. They suggested that the antimicrobial activity of chitosan is dependent on both the chitosan and the microorganism used (Chien and Chou 2006; Oh et al. 2001).

The different mechanisms have been proposed to show the antimicrobial activity of chitosan. The first mechanism deals with the ionic interaction between chitosan and surface of cell wall of bacteria resulting into microbial death. In this, they proposed that chitosan, a positively charged molecule, interacts with the negatively charged cell wall of bacteria, thereby disrupting the normal functions of the

membrane, via promoting the leakage of intracellular components and inhibiting the transport of nutrients into the cells. The antimicrobial activity of chitosan against a variety of bacteria and fungi is due to its polycationic nature.

The second mechanism proposed deals with the inhibition of m-RNA and protein synthesis through the penetration of chitosan within the nuclei of microorganisms and interfering with the synthesis of mRNA and proteins is responsible for the inhibition of the bacterial growth.

The third proposed mechanism deals with the formation of an external barrier chelating metals which initiate the suppression of essential nutrients required for microbial growth. All these events are supposed to occur simultaneously but at different intensities resulting into microbial death (Goy et al. 2009; Helander et al. 2001; Fei Liu et al. 2001; Roller and Covill 1999).

Furthermore, biopolymer chitosan has been widely reported for effective delivery of many pharmaceuticals. Thus, the chitosan may be used for the incorporation of other antipyretic agents for the synthesis of long-acting antibacterial wound dressing.

Although both native chitosan and its derivatives are effective as antimicrobial agents, there is a clear difference between them. Their different antimicrobial effect is mainly exhibited in live host plants. In addition, oligomeric chitosans (pentamer and heptamer) have a better antifungal effect than larger units. The antimicrobial activity of chitosan is more immediate on fungi and algae than on bacteria. Chitosan has been shown to be fungicidal against several fungi. It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation (Fei Liu et al. 2001; Savard et al. 2002).

In general, such mechanism is more efficient at higher pH, where positive ions are bounded to chitosan, since the amine groups are unprotonated and the electron pair on the amine nitrogen is available for donation to metal ions. A model based on the system chitosan–Cu was proposed to relate the pH dependence on the proportion of available sites for interacting in polysaccharide backbone. At $pH < 6$ the complexation involves only one NH_2 group and three hydroxyls or H_2O molecules, while at $pH > 6.7$ it is likely to have two NH_2 involved in the complex formation. For higher pHs, i.e., 7–9, the deprotonation of hydroxyl groups is considered to occur and the predominant complexation is ruled by two $–NH_2$ and two hydroxyl groups dissociated (Wang et al. 2005).

Helander et al. studied the effects of chitosan treatment on the cell membranes of Gram-negative bacteria and found evidence for extensive cell surface alterations, marked by thickening and formation of vesicular structures on the outer membranes of both *Escherichia coli* and *Salmonella typhimurium*. They reasoned that chitosan binds to the outer membrane of Gram-negative bacteria, thereby affecting its barrier properties, probably through complex formation with various lipopolysaccharides. Highly cationic mutants of *S. typhimurium* were also found to be more resistant to chitosan than the parent strains. Morimoto et al. reported the specific binding of a chitosan derivative with a receptor on the cell surface of *Pseudomonas aeruginosa* (Helander et al. 2001).

More interestingly, Chung et al. proposed that the inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from the cell membrane, followed by destruction of the cell membrane. They came to this conclusion based on similarities between the antibacterial pattern of chitosan and those of polymyxin and EDTA. Electron microscopical examinations of various chitosan-treated microorganisms suggest that its site of action is at the microbial cell surface (Chung and Chen 2008).

1.7.2 Industrial Applications

Chitosan is being used in wide range of applications such as cosmetics, paper, pharmaceuticals, water treatment, etc., because of its physicochemical properties. Different properties of chitosan are involved in its different application. These properties of chitosan are greatly influenced by its degree of deacetylation and molecular weight.

1.7.2.1 Cosmetics

Chitosan, a biologically active material, is fungicidal and fungistatic in nature. It also shows good compatibility with lots of biological compounds that are the main constituents of many cosmetic products. Chitosan comes under the category of hydrocolloids and is the only natural aminopolysachharide which becomes viscous when neutralized under acidic environment because of its polycationic nature. Its viscous nature under acidic environment facilitates its interaction with skin and hair. The researchers reported the use of chitosan for hair treatment because of their complementary nature. The clear solution of chitosan forms a thin layer on hair, thereby increasing its softness, mechanical strength, and smoothness. The alcoholic solution of chitosan can also be used in hair treatment as it forms gel when added to mixture of water and alcohol. Researchers reported use of chitosan in hair sprays, shampoos, hair colorant, and hair tonics. Some derivatives of chitosan can be used in shampoos as it can form foam and create emulsifying action (www.genocite.com).

Chitosan because of its cationic nature and high molecular weight has been widely used in many skin care products. Most of the chitosan derivatives used in skin care products have high molecular weight and therefore cannot penetrate the skin. In addition it can also be used as a moisturizer instead of hyaluronic acid because of its low cost and availability (www.Meronbiopolymer.com).

1.7.2.2 Paper Industry

Chitosan can be used to increase the strength of recycled paper and is found to be an environmentally friendly option for packaging because of its biodegradable nature. Chitosan has been involved in synthesis of paper because of its structural similarity with cellulose (a main constitute of plant cell walls from which paper is

manufactured). The use of chitosan in paper making is prevalent as the paper produced is resistant to moisture and has smooth surface. Chitosan has been widely used in manufacturing of wrapping paper, toilet paper, cardboard, and food packaging material (Dutta et al. 2002).

1.7.2.3 Textile Industry

Researchers reported the grafting of chitosan on nylon/silk fiber to increase the hydrophilic and antibacterial properties of grafted fiber (Tseng et al. 2009). Wool fiber undergoes shrinkage during aqueous treatment. To overcome this the wool fiber was grafted with chitosan, as grafting results in increase in the number of hydroxyl and amino groups that can form a hydrogen bond with water molecule and decreases the hydrophilicity of the grafted wool fiber, thus improving its antifelting properties (Vilchez et al. 2008).

1.7.2.4 Solid-State Batteries

Chitosan powder as such cannot be used in the manufacturing of proton-conducting solid-state batteries. Thus acidic solution of chitosan is used to induce ionic conductivity which is mainly due to the presence of proton in acidic solution of chitosan. The piezoelectric study of chitosan solution indicated that it has small value of dielectric constant, thus confirming the presence of more number of microvoids in chitosan polymer which are responsible for the transport of protons. Thus chitosan solution with suitable electrode may result into a better battery system (Ravikumar and Dutta 1998).

1.7.2.5 Agriculture

Recently, environmentally friendly treatments are being employed in agriculture for controlling crop diseases as alternative to chemical pesticides, including the use of natural compounds such as chitosan. “Seed treatments” are the biological, physical, or chemical agents and techniques applied to seed to provide protection and improve the establishment of healthy crops [International Seed Federation (ISF)]. This technique involves the application of thin layer of the active product, such as pesticides, fertilizers, or growth promoters, often in combination with other additives on the seed surface to promote healthy plant growth (Rhee et al. 1998).

Chitosan can be easily modified into different polymorphic forms and thus can be used as film, forming physical barriers (film) around the seeds preventing the pathogen infection (Rhee et al. 1998; Ravikumar and Dutta 1998).

Chitosan has great potential as protector against diseases because of its antibacterial property and thus can be used against a wide variety of microorganisms such as bacteria, yeast, and fungi (Domard and Domard 2001) to induce plant resistance.

Another important application of chitosan in agriculture is the use of chitosan as film coating as a delivery system for fertilizers, plant protection products, and micronutrients for crop growth promotion (Kurit et al. 1993).

1.7.2.6 Food Processing

Chitosan has been widely used in food processing industry because of its nontoxic and antibacterial nature. Chitosan because of its biodegradability, nontoxicity, and intrinsic antibacterial effects has been widely used as an antimicrobial agent to improve food quality and extend shelf life. The desired antimicrobial effects may be achieved by using chitosan and its derivatives either alone or blended with other ingredients. For example, chitosan combined with biocontrol yeast and/or calcium chloride has been used to control blue mold in pears (Madihally and Matthew 1999). The combined effect of chitosan, biocontrol yeast, and calcium chloride produced a more effective and stable reduction in fungal decay than treatment compared to either chitosan or biocontrol yeast alone. In addition to bacterial and antifungal activity, chitosan has been recently tested for its efficacy against foodborne viruses, including human norovirus and enteric virus surrogates (Zhang and Zhang 2001). In some cases, chitosan has been modified to improve its antimicrobial efficacy in specific food systems. For example, a chitosan–glucose complex coating was found to be the most effective treatment for protecting mushrooms from microbial degradation and improving postharvest quality, compared to treatment with a chitosan or glucose coating alone (Suh and Matthew 2000). The chitosan–glucose coating maintained mushroom tissue firmness, inhibited an increase in the respiration rate, reduced microorganism counts (tested pseudomonads, yeasts, and molds), and delayed changes in ascorbic acid concentrations while maintaining overall sensory quality. The antimicrobial activities of water-soluble chitosan have also been well characterized (www.bae.ncsu.edu; www.vanson.com). A recent study developed chitosan and carboxymethyl chitosan–zinc complexes to compare their antimicrobial activities (Mucha 1997). The study revealed that the carboxymethyl chitosan–zinc complex exhibited much greater antimicrobial activity against both Gram-positive and Gram-negative bacteria than the chitosan–zinc complex. The authors deduced that the carboxymethyl groups greatly improved the water solubility of chitosan enhancing its diffusivity, thus making higher concentrations available at the site of action.

1.7.3 Environmental Applications

Chitosan has great potential for certain environmental applications, such as remediation of organic and inorganic contaminants, including toxic metals and dyes in soil, sediment, and water, and development of contaminant sensors. Some of the examples are depicted in Table 1.3.

1.7.3.1 Flocculating Agent

The rapid industrialization and urbanization world over has been accompanied by the production of wastewaters containing different types of dissolved and undissolved contaminants causing water pollution (Schwarzenbach et al. 2006; Shannon et al. 2008). The various techniques, adsorption, coagulation/flocculation (Dao et al. 2016; Jiang 2015), oxidation/reduction (Arena et al. 2015; Chen 2004), membrane

Table 1.3 Modified chitosan derivatives for environmental application

Sr. No.	Chitosan derivative	Targeted pollutants	Adsorption capacity (mg/g)/% removal efficiency	Reference
1	Carboxymethyl chitosan	Cu(II)	155.49	Sun and Wang (2006)
2	Crosslinked chitosan	Cu(II), Zn(II), Pb(II)	35.46, 10.21, 34.13	Chen et al. (2008)
3	Glycine-modified crosslinked chitosan resin	Au(III), Pt(IV), Pd(IV)	169.98, 122.47, 120.39	Ramesh et al. (2008)
4	Crosslinked chitosan	Cu(II), Zn(II), NI(II), Pb (II)	33.00, 15.08, 37.88, 105.26	Chen et al. (2009)
5	Chitosan magnetite composite	Pb (II), Ni(II)	63.33, 52.55	Tran et al. (2010)
7	Magnetic thiourea chitosan	Ag(I)	531.454	Fan et al. (2011)
8	CTS/*Sargassum* sp. composite	Cu(II)	68.63	Liu et al. 2011a, b)
9	Zero valent iron/ chitosan composite	Cr(VI)	Not reported	Liu et al. (2012)
10	Magnetic chitosan	Sr (II)	11.58	Chen and Wang (2012)
11	Chitosan/activated carbon composite	Cd (II)	52.63	Hydari et al. (2012)
12	Magnetic chitosan resin	U(VI)	187.26	Zhou et al. (2012)
13	Ion-imprinted chitosan	Co(II)	92.2	Nishad et al. (2012)
14	Chitosan/TEA composite	Ag(I)	510	Zhang et al. (2012)
15	Chitosan nanofiber mat	Pb(II)	359	Li et al. (2013)
16	Chitosan/activated clay	Methylene blue, reactive dye (RR222)	330, 1912	Chang and Juang (2004)
17	Chitosan/activated clay	Tannic acid, humic acid	1490, 243	Chang and Juang (2004)
18	Acrylic acid grafted chitosan microsphere	Basic Blue 3G	384.37	Lazaridis et al. (2007)
19	Chitosan/ montmorillonite nanocomposite	Congo red	54.52	Wang and Wang et al. (2007)
20	Chitosan/oil palm	Reactive Blue 19	909.1	Hasan et al. (2008)

(continued)

Table 1.3 (continued)

Sr. No.	Chitosan derivative	Targeted pollutants	Adsorption capacity (mg/g)/% removal efficiency	Reference
21	Chitosan/polyurethane	Acid violet 48	30	Lee et al. (2009a)
22	Chitosan/bentonite	Tartrazine	294.1	Wan Ngah et al. (2010a)
23	Chitosan/bentonite	Malachite green	435	Wan Ngah et al. (2010b)
24	Chitosan/ kaoline/ã-Fe2O3	Methyl orange	71 %	Zhu et al. (2010)
25	Chitosan/γ-Fe2O3/fly ash cenosphere	Bisphenol A, 2,4,6-Trichlorophenol	17.61, 60.98	Pan et al. (2011)
26	Magnetic chitosan composite	Sulfamethazine	3.23	Xu et al. (2012)
27	Magnetic chitosan composite	Alizarin red	40.12	Fan et al. (2012)
28	Chitosan/graphene oxide composite	Sulfamethoxazole	0.52	Huamin et al. (2013)
29	β-Cyclodextrin derivatized chitosan	Remazol Blue RN Remazol Red 3BS Remazol Yellow	Not reported	(Kyzas et al. 2013)
30	Chitosan–Fe_3O_4 composite	Carbamazepine	Not reported	Zhang et al. (2013)
31	Chitosan MIP	Methandrostenolone	69.13	Wang et al. (2014)
32	Chitosan/TiO_2 composite	Ni(II)/methyl orange	10.97 mg/g (Ni) 90% degradation	Li et al. (2008)
33	Chitosan/TiO_2 composite	Cd (II) 2,4-Dichlorophenol (2,4 DCP)	256.41 mg/g (Cd) 98 % degradation of 2,4 DCP	Chen et al. (2012)
34	Chitosan stabilized bimetallic Fe–Ni composite	Cd(II) Amoxicillin	81.3 % (Cd) 68.9 % (amoxicillin)	Weng et al. (2013)
35	Succinyl grafted chitosan	Zn(II) Remacryl Red TGL (CR)	290 (Zn) 1404 (CR)	Kyzas et al. (2015)
36	Chitosan-based dual imprinted polymer	Cd/salicylic acid	38.46 / 23.81	Rahangdale et al. (2018)
37	Acrylamide grafted chitosan-based dual imprinted polymer	Cd/salicylic acid	53.42 /45.77	Rahangdale and Kumar (2018c)

filtration (Fane et al. 2015; Hillis 2007), and biotechnology (Imran et al. 2015; Delgado Vela et al. 2015), are employed for the water treatment. Among these techniques, coagulation/flocculation is one of the most commonly used to achieve solid-liquid separation, based on its cost effectiveness and ease of operation (Dao et al. 2016; Jiang 2015; Lee et al. 2014).

In this process the small colloidal particles suspended in wastewater are destabilized by addition of coagulant. Further the coagulation is followed by flocculation, during which the destabilized particles are aggregated to form larger flocs which can be removed by sedimentation. The selection of appropriate flocculent is very necessary as it decides the efficiency of flocculation process. Flocculants are broadly classified into two classes: (1) inorganic coagulants (aluminum sulfate ($Al_2(SO_4)_3$), poly-aluminum chloride (PAC), and polymerized ferrous sulfate (PFS)) and (2) organic polymeric flocculants including synthetic and natural ones. The use of inorganic salts as a flocculant has been widely reported because of their low cost. They have also been used in coagulation/flocculation process as they get readily adsorbed on negatively charged colloidal particles, resulting in the simultaneous surface charge reduction and formation of microflocs (Li et al. 2005a, b; Li et al. 2006). However, for efficient flocculation a large quantity of inorganic flocculant is required which lead to the formation of huge volumes of metal hydroxide sludge and subsequent disposal problem. Other drawbacks associated with inorganic coagulants are that they are highly sensitive to pH, inefficient toward very fine particles, and applicable only to a few disperse systems (Bratby 2006; Sharma et al. 2006). Moreover, an increase in metal concentration in treated water may have serious human health concerns (Walton 2013; Ward et al. 2006).

Organic flocculants are highly efficient even at very low concentration and thus generate smaller sludge volume without consumption of alkalinity unlike inorganic coagulants. They have different molecular weight, structure, ionic nature, charge density, chemical composition, and degree of substitution of the various functional groups (Bolto and Gregory 2007). The flocs generated during flocculation are easily settled down as they are bigger in size (Bolto and Gregory 2007; Razali et al. 2011). One more advantage associated with cationic polymeric flocculent is that they possess dual functions of coagulation and flocculation by neutralizing the negative charges and bridging the aggregated destabilized particles (Chong 2012; Lee et al. 2014). However, synthetic organic flocculent such as polyacrylamide (PAM) and its derivatives and ethyleneimine are extremely toxic causing severe neurotoxic effects (Bolto and Gregory 2007; Bratby 2006; Dao et al. 2016). Therefore, the development of natural polymeric flocculants, such as chitosan (Guibal et al. 2006; Renault et al. 2009), starch (Singh et al. 2000), and cellulose (Liu et al. 2014a, b) has been promoted because of their biodegradability, widespread availability, low cost, and nontoxicity.

1.7.3.2 Chelating Agent and Heave Metal Ion Trapper

Chitosan can act as a chelating agent for binding of different toxic heavy metal ions such as uranium, lead, cadmium, mercury, etc. The amino group present on chitosan is responsible for the adsorption of metal cation through coordinate interaction at

neutral pH. The adsorption of metal anion onto chitosan takes place through electrostatic attraction between metal anion and protonated amine groups in acidic conditions.

The modification of chitosan through crosslinking and imprinting enhances its absorption efficiency and specificity toward targeted analyte. For example, Chen et al. synthesized the chemically crosslinked metal-complexed chitosan using the ion-imprinting method with four metals (Cu(II), Zn(II), Ni(II), and Pb(II)) as templates and glutaraldehyde and epichlorohydrin as a crosslinker. The results of adsorption studies demonstrated that the crosslinking and imprinting of chitosan leads to higher adsorption capacity toward metals in an aqueous medium (Chen et al. 2008, 2009).

The functionalization of chitosan with different grafting agent containing reactive functional groups improves its specificity and efficiency for some metal ions. The adsorption efficiency of chitosan toward heavy metal ion is good; however, its low stability has prompted many researchers to modify them. Different modifications such as grafting of acrylamide, acrylic acid, dithiocarbamate, thiourea, and carboxymethyl were studied and employed to improve the adsorption efficiency as well as the mechanical and physical properties for metal ion removal.

Ag (I)-imprinted polymer crosslinked with glutaraldehyde was synthesized by Fan and his coworkers using thiourea-modified magnetite chitosan to remove Ag^+ ion. The high binding efficiency of the modified chitosan-based adsorbent resulted from chelation of the Ag^+ ion with the lone pair of electrons on the sulfur atom of the thiourea and amine groups of chitosan (Fan et al. 2011). Liu and coworkers successfully synthesized an innovative chitosan biopolymer impregnated with α-Fe_2O_3 for arsenic metal decontamination from aqueous matrix using ion-imprinting technique (Liu et al. 2011a, b).

1.7.3.3 Removal of Organic Pollutants

Chitosan is regarded as one of the most efficient adsorbents for adsorption of various organic pollutants such as dyes, pharmaceuticals, detergents, pesticides, etc., in water treatment systems. The amino and hydroxyl groups present on backbone of chitosan allow its adsorption interactions with various pollutants. Organic pollutants, including phenolic compounds, polycyclic aromatic hydrocarbons, organic pesticides, and herbicides, cause health and environmental problems due to their toxic effects coupled with poor biodegradability. Chitosan adsorption for organic pollutants offers high adsorption capacities, insensitivity to toxic substances, good modifiability, as well as recoverability. Chang and Jiang have reported the use of chitosan/activated clay composite beads for the adsorption of two organic acids (tannic acid, humic acid) and two dyes (methylene blue, reactive dye RR222).

Wang et al. prepared methandrostenolone-imprinted chitosan-based polymer using chitosan as functional polymer and epichlorohydrin as crosslinker and applied it for the extraction of methandrostenolone from real samples using the MIPs as SPE adsorbent (Wang et al. 2014). Meng et al. used highly selective cellulose acetate blend imprinted membranes for SA removal using chitosan as a functional polymer and chitosan-wrapped multi-walled carbon nanotubes as the additives (Meng et al.

2014). Some researchers have reported the use of metal ion-imprinted chitosan-based polymer for the selective removal of the metal ion and its further use for the degradation of an organic pollutant (Chen et al. 2012; Li et al. 2008; Kyzas et al. 2015; Weng et al. 2013).

Huamin et al. fabricated a novel chemosensor for the determination of sulfamethoxazole (SMZ) using chitosan/graphene oxide-molecularly imprinted polymers as recognition element. It was synthesized by using acrylamide as functional monomer, ethylene glycol dimethacrylate as crosslinker and 2,2-azobisisobutyronitrile as initiator, acetone as solvent, and chitosan/graphene oxide for support. The chitosan/graphene oxide-based MIP showed satisfactory recognition capacity for the sulfamethoxazole (Huamin et al. 2013).

Zhang et al. reported the synthesis of chitosan–Fe_3O_4-based magnetic molecularly imprinted polymer (MMIP) for efficient selective removal of carbamazepine (CBZ) from aqueous matrix. The chitosan–Fe_3O_4 not only acted as a support but also as a functional polymer during imprinting process. The adsorption capacity of synthesized polymer was found to be nearly same in various aqueous matrixes. The feasibility of selective sorption of CBZ from real water by the MMIP was analyzed by using spiked real water samples. The result showed that the sorption capacity of MMIP has no obvious decrease in different water samples whereas there was obvious decline in the sorption amount of the MNIP (magnetic non-imprinted polymer) (Zhang et al. 2013).

1.8 Conclusion

Chitosan, a green polymer, is derived from a highly basic polysaccharide, chitin, which can be obtained from various sources like shrimp waste shells, shell fish waste, blue swimming crab, cuttlefish, etc. The extraction of chitin from raw material includes a specific algorithm. However, depending upon the extent of processing needed, some steps may be neglected. The industrial use of chitosan, the derivative of chitin, is increasing with great pace due to its extraordinary advantages and multiple uses. The various factors affecting the physical as well as the chemical properties of chitosan include pH value, ionic strength, concentration, molecular weight, degree of deacetylation, temperature, etc. Using chitosan in its raw form makes it impossible to take the advantages of its special properties due to low elasticity and durability. Thus, it is modified physically as well as chemically to make it suitable for specific applications. The physical modifications of chitosan include formation of membranes, beads, etc. Its chemical modifications can be a topic of unending research depending upon the need of improvement of the properties by a specific application. There exist some biomedical applications of chitosan which are a strong demand of today's scenario such as cancer diagnosis, tissue engineering, wound dressing, etc. Chitosan with required modifications can also be used as a drug carrier. The molecularly imprinted polymers designed using computational modeling can be synthesized using the modified versions of chitosan, which can be used for specific removal of pollutants from the environment, with no

harmful effect on the ecosystem. All the above aspects suggest that the use of chitosan in various industries is varied. Thus, any field that one can think of can have use of this material in it in future. The study and research of chitin should be done which is directly proportional to its use in various industries. The progressive use of chitosan as a functional polymer in imprinting technique is very appreciable.

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Application of Chitosan in Oral Drug Delivery

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Abstract

Oral drug delivery is counted as the preferable route of drug administration due to its convenience, safety, and cost-effectiveness. However, many drugs are not good candidates for oral application mainly because of drug degradation within the gastrointestinal system. Overcoming the obstacles for effective oral delivery of drugs is currently one of the chief goals driving drug delivery research. Recently, remarkable advances in drug delivery technology have led to the increase in the use of various carriers for oral drug delivery. Polymers, as one of the most widely utilized tools, have demonstrated a considerable number of benefits of which stable physicochemical properties and cost-effectiveness are the prominent ones. Along with the mentioned features, an ideal polymeric delivery vehicle should be biocompatible and protect the incorporated drug from enzymatic degradation in the gastrointestinal tract. Chitosan has been extensively studied by many researchers, and a massive data is now available upon its distinctive benefits and restrictions as well as its unique characteristics appreciable for oral drug delivery. It is safe, biocompatible, low cost, and readily available. In addition, intrinsic mucoadhesion ability of chitosan urges its use as an oral drug delivery vehicle. The goal of this chapter is to focus on the application of chitosan as an oral delivery carrier for therapeutic molecules and drugs. Current conventional formulations of chitosan are first reviewed, and the

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_2

related limitations are investigated to lead readers to the next sections in which novel approaches for improved delivery system are explained as fully as possible. Application of chitosan in oral gene and peptide delivery is explained as separate sections since these two areas have been attracting much attention in recent years due to the intrinsic properties of chitosan making it a promising candidate in the areas. Different strategies employed to improve chitosan polymers regarding physicochemical and targeting properties are covered throughout the script. Diverse modification approaches as well as their limitations are explained, exemplified, and illustrated within the body of the chapter. In the end, the future concept of chitosan oral drug delivery is argued followed by a concise summary.

Keywords
Chitosan · Oral delivery · Chitosan tablets · Chitosan capsules · Chitosan beads · Chitosan granules · Chitosan nanoparticles · Oral gene delivery · Oral peptides and proteins delivery · Chitosan hydrogels

2.1 Introduction

It goes without saying, the benefits of oral drug delivery are adequate enough to consider it as the first choice and convenient route of drug administration. Patient compliance, cost-effectiveness, and ease of high-scale manufacturing as well as no requirement of costly sterility processes are the prominent advantages of oral route of drug administration. Researchers are increasingly seeking ways to design novel oral drug delivery systems and to substitute the current parenteral drug for oral ones, especially in cases where the overall treatment outcomes are the same. Various technologies in oral formulations, even tiny enhancements in drug delivery systems, can make primitive differences in increasing patient compliance. Insulin oral delivery, as a quintessential instant, is a solution many scientists are working on for it can greatly facilitate treatment of diabetes. Chitosan is one of the well-studied polymers with suitable properties in oral drug delivery systems. Numerous works have been done up to this day in order to orally administer drugs and genes with the aid of chitosan polymers. This chapter focuses on chitosan as a carrier in oral drug and gene delivery systems. At the beginning, the general concept of oral application of drugs and its pros and cons are provided, and as the chapter goes, various strategies in chitosan oral delivery systems are explained with examples.

2.2 Oral Drug Delivery

Oral route is composed of several sites in the gastrointestinal system. The intrinsic features of gastrointestinal route provide diverse environments with distinct properties which can be counted as both opportunities and challenges (Hadisoewignyo et al. 2018). PH values, available absorption site, presence of

Table 2.1 Characteristic features of gastrointestinal tract affecting drug absorption

Site	PH values	Special properties
Mouth saliva/ buccal cavity	Approx. 6.8	Lipophilic, neutral, and basic drugs absorbed directly into the blood and circularly system. However, small surface area and little retention time decrease the chance of drug absorption (Montero-Padilla et al. 2017; Zeng et al. 2017)
Stomach	Before meal: 1–3 After meal: 4–5	Medium surface area, suitable site for acidic drug absorption than neutral and lipophilic drugs (Sutton et al. 2017)
Small intestine	5–7.5	A vast surface area that is the major site for absorption of all types of drugs (neutral, acidic, or basic) (Huang et al. 2015)
Large intestine (colon)	7.9–8	A small amount of free water at this site provides a little chance for all kinds of drugs to be absorbed into the bloodstream (Teruel et al. 2018)

digestive enzymes, and amount of free water play critical role in the fate of administrated drug (Spinks et al. 2017). A quick glance of explicit characteristics of different parts of oral route is provided in Table 2.1.

These diversities among different sites of absorption in the oral route are one of the hurdles to overcome. Along with this, other challenges also must be taken heed of such as:

1. The presence of digestive enzymes within the gastrointestinal tract increases the chance of instability of the administrated drug. This issue is bolded in case of application of large molecules as proteins and nucleic acids.
2. Macromolecules have slight chance to be absorbed by gastrointestinal cells.
3. Since stomach emptying time is highly dependable on volume of the taken food, the absorption of the intended drug may be affected by nourishment.
4. Slow onset of action compared with parenteral administration route.
5. Control over release of the drug can be a challenging issue since most of the times there is no specific delivery site.
6. Though it is one of the most preferred routes of drug administration, for some patients the parenteral or rectal route is still the route of choice.

Thus, a favorable oral delivery system must possess enough stability against degradation, controlled drug release profile, as well as targeted site of absorption/ action in case of necessity.

2.3 Chitosan as an Ideal Carrier for Oral Drug Delivery

Chitosan, a linear polysaccharide, has structural features similar to glycosaminoglycans composed of arbitrarily distributed β-(1 → 4)-linked D-glucosamine and N-acetyl-D-glucosamine. This cationic natural polymer is

produced commercially by deacetylation of chitin, widely found in cell walls in fungi, the exoskeletons of crustaceans (e.g., crabs and shrimps) and insects. Chitosan possesses biological and physicochemical properties which made it a promising carrier for oral drug delivery. Primarily, chitosan is safe. It is approved for use outdoors and indoors on many plants grown commercially and by consumers. Its use as wound dressing component was approved by the Food and Drug Administration (FDA) in 2002 (Wedmore et al. 2006). Chitosan is also approved for use as bandages and hemostatic agents in Europe. Japan and Korea had proposed the use of chitosan in potable water (Kumar et al. 2017). No adverse effects were reported in male (4.5 g chitosan per day) or female (2.5 g per day) volunteers following oral chitosan administration for 12 days (Gades and Stern 2005). In the second place, the presence of abundant NH2 functional groups on the chitosan chains provides a cationic nature for the polymer, enabling chitosan to easily interact with negatively charged cell membrane which can promote cellular uptake. Furthermore, it is also able to interact with negatively charged polymers, macromolecules, and polyanions in contact in an aqueous environment. While many polymers are insoluble in water, chitosan can be partially water soluble and form a hydrogel in an aqueous environment. It has the particular quality of adhering to mucosal surfaces, indicating it can be an ideal option in terms of oral drug delivery. Moreover, some chitosan derivatives possess unique features making them favorable for drug delivery applications. As an instance, it is reported that glycol chitosan solution or glycol chitosan-based nanoparticles can block the P-glycoprotein efflux pump (Mandracchia et al. 2017). In the following pages, firstly, conventional applications of chitosan are presented along with highlighting the ideal properties with which chitosan serves the work, and the next sections focus on the novel usage of this natural polysaccharide and its high potentials in oral drug delivery.

2.4 Chitosan Tablets

To begin with, addition of chitosan as an expedient to tablet dosage forms or films results in a more controlled drug release profile. Ample studies support this benefit of chitosan polymers (Kawashima et al. 1985; Park et al. 2008; Mi et al. 1997). Chitosan was also employed to prevent the release of drug within the gastric acidic environment. Directly compressible tablets were prepared from admixtures of distinct amount of diclofenac and chitosan with various degrees of N-deacetylation. The obtained tablets were able to selectively inhibit the release of diclofenac within an acidic environment. The demonstrated benefit might be due to gel formation by chitosan in an acid medium which can limit the rate of diffusion and formation of an ionic complex between negatively charged diclofenac sodium and the cationic polymer when exposed to the solvent in gastric environment (Sabnis et al. 1997). As pH lowers in gastric environment, amine fictional groups of chitosan become positively charged which enable the polymer to form electrostatic interaction with negatively charged molecules, via the well-known process “ion gelation” which will be further investigated in this very section; the formed structure can preserve any

positively charged molecule within itself (in this case, diclofenac). Similar results were gained in the case of complexation between chitosan and anionic polyelectrolytes of Eudragit L100, xanthan gum, and Carbopol 934 in sustained release of bupropion HCl (Varshosaz et al. 2015). A more detailed explanation of interaction between cationic groups of chitosan and anionic molecules is provided further in a separate section titled "chitosan hydrogels and films."

As for oral delivery, the ability of chitosan to adhere to intestinal wall of digestive system has been a subject of interest for scientists. In fact, chitosan has been demonstrated to enhance penetration through mucosal tissues by opening tight junctions. Chitosan is rich in possessing high positive surface charges due to the presence of free NH2 groups, and this leads to intense electrostatic interaction of chitosan chains and negatively charged mucins. Hydrogen and hydrophobic bonding also play contributing roles in this phenomenon. Hence, formulation with chitosan has higher chances of effective interactions with mucosal layer. Another gain for this is that dosage form will have increased residual time within intestinal tract, improving drug bioavailability. The high content of positive charges of chitosan can benefit oral delivery of some conventional drugs, especially those with poor solubility.

Oral delivery of some conventional drugs can also greatly benefit from the high content of positive charges of chitosan, especially those with poor solubility. One of the contributions to the poor solubility of some drugs comes from their crystallinity. Chitosan as a positively charged polyelectrolyte can interact with negative constitutes of drugs and disrupt their crystal structure leading to improved solubility profile in water physiological medium. Aceclofenac is a typical example in this term, in a study done by Srinivas Mutalik et al. Chitosan was precipitated on aceclofenac crystals with the aid of sodium citrate as the salting-out agent. Various formulations with different concentrations of chitosan were prepared and examined in terms of their solubility. All the formulations with chitosan showed considerably improved solubility profile in water compared to free aceclofenac. The XRD patterns of the pure drug and drug-chitosan crystals also agreed with the abovementioned statement; in fact, the XRD patterns exhibited a significant decrease in crystallinity, supporting the fact that chitosan can increase solubility by crystal structure disruption (Mutalik et al. 2008). Alteration in surface morphology of drug-chitosan formulations was considered as another contributing factor in the increase of solubility. The result is also in agreement with other studies done on acelofenac (Gavhane and Yadav 2013; Rao et al. 2010). The same result was also observed in case of ibuprofen-chitosan tablets (Sogias et al. 2012).

2.5 Chitosan Capsules

Chitosan as a capsule body has also been investigated by researches for oral drug delivery. Again, the idea of this strategy stems from the fact that chitosan is a safe, biocompatible, biodegradable, and natural polymer, and last but not least, it provides dosage form sustainability in terms of drug release.

In a study done by Tozaki et al., chitosan capsules containing several agents, including insulin, were prepared and coated with hydroxypropyl methylcellulose phthalate (HPMCP) as an enteric coating material. Insulin had been absorbed significantly greater in chitosan capsule formulations rather than free insulin. In fact, insulin is poorly stable within the gastrointestinal tract and is drastically degraded by enzymes; however, chitosan capsules were able to protect insulin from degradation as well as increase drug adsorption (Tozaki et al. 1997). Similar study by this group also showed the same better therapeutic responses with chitosan capsules containing 5-aminosalicylic acid (5-ASA) compared to carboxymethyl cellulose (CMC) suspension comprising the same drug in the treatment of TNBS-induced colitis in rats (Tozaki et al. 2002). The colon-targeting features of chitosan stems from the fact that while chitosan can preserve its structure within small intestine, it is fully degraded in the colon by the enzymatic digestion of the colonic microflora. Other studies also support the abovementioned property of chitosan capsules (Fetih et al. 2005; Shimono et al. 2002; TOZAKI et al. 1999). According to the literature, the interest in which chitosan capsules serve is in the colonic delivery of chitosan capsules mostly for the treatment of inflammatory diseases. However, a few other applications have been reported in studies employing chitosan capsules in oral delivery of drugs.

2.6 Chitosan Beads and Granules

Chitosan beads were also investigated in case of sustained drug delivery. In a study done by Chandy and P. Sharma, chitosan beads and microgranules were prepared and loaded with nifedipine. As for chitosan beads, chitosan solution in 2% acetic acid was blown into NaOH-methanol solution by high-pressure air through nozzles, and then the obtained porous beads were washed by hot then cold water consecutively. From the nozzle (0.15 mm diameter), bead preparations were produced. In vitro analysis showed that chitosan beads have zero-order drug release kinetic. The demonstrated uniform drug release stems from the fact that the outer core swells first, followed by diffusion of drug, and then swelling continues to the inner core of the beads. This results in homogeneous drug release rates (Chandy and Sharma 1992). Chitosan granules also showed the same results in studies. Sustained release of indomethacin by chitosan granules was observed in vivo. Chitosan granules, containing 25 mg of indomethacin, were encapsulated in hard gelatin capsules and administered orally to the male rabbits. Blood samples were taken from ear vein and assayed for indomethacin concentrations. The results indicated a sustained-release profile for indomethacin-loaded chitosan granules (MIYAZAKI et al. 1988a). Most of the works done on chitosan granules and beads date back to the late 1990s and the starting years of the twentieth century. This mainly stems from the great advantages chitosan delivery systems offered such as chitosan micro- and nanoparticles which

will be explained further in this chapter. Nonetheless, a quick glance on some of the works done on the subject is summarized in Table 2.2.

In one of the most recent works, chitosan beads were employed for colonic delivery of azathioprine for treatment of inflammatory bowel disease. The ideal feature of chitosan that made it a suitable drug vehicle in this work is the fact that chitosan is poorly soluble at pH higher than 7, while protonation of its amine groups at acidic pH facilitates its solution. Thus, limited drug release from the chitosan beads is expected in the small intestine, while drug release could be enhanced at the colonic inflamed tissues in IBD that have low pHs. For avoidance of gastric degradation of the beads, enteric coated capsules were used to fill the prepared formulations (Helmy et al. 2017). Chitosan beads and granules have been investigated in many studies for their potentials as oral drug delivery vehicle. However, chitosan granules seem to be replaced with more novel and enhanced chitosan-based delivery system; they both have been observed to possess sustained drug release profile along with biocompatibility which is an inherent feature of chitosan itself making them promising tools in oral drug delivery. Nonetheless, the advent of nanotechnology has brought out notable advantages facilitating the field of oral drug delivery (which will be explained in more details in the next parts) and might stand for the decrease in the number of researches done on chitosan granules and beads in the case of oral drug delivery.

Table 2.2 Examples of research works done on the application of chitosan beads and granules in oral drug delivery

Chitosan delivery system	Drug	Ref.
Chitosan granules	Indomethacin	HOU et al. (1985) and Miyazaki et al. (1988b)
Chitosan granules	Diclofenac	Gupta and Kumar (2000)
Chitosan beads	Riboflavin	Shu and Zhu (2002)
Chitosan beads	Calcium sulfate	Cho et al. (2005)
Alginate-konjac glucomannan-chitosan beads	Bovine serum albumin (BSA) and insulin	Wang and He (2002)
Chitosan/calcium-alginate beads	Insulin	Hari et al. (1996a)
Chitosan-alginate beads	BSA	Xu et al. (2007) and Takka and Gürel (2010)
Chitosan-calcium alginate beads	Verapamil	Pasparakis and Bouropoulos (2006)
Chitosan-alginate multilayer beads	Ampicillin	Anal and Stevens (2005)
pH-responsive carboxymethyl chitosan-alginate beads	Insulin	Mukhopadhyay et al. (2013)
Alginate-chitosan beads	Metformin	Mokhtare et al. (2017)
Chitosan-xanthan beads	Glipizide	Kulkarni et al. (2015)

2.7 Chitosan Oral Gene Delivery

Gene delivery, as one of the most emerging terms in novel therapy approaches, is attracting much attention, either in the form of delivery of genetic molecules as therapeutic agents against cancer or components of a vaccine. As expected, major challenges facing oral drug delivery are similar in the case of oral gene delivery. Hence, protection of acid labile genetic molecules in the stomach, their safe transition through intestines, and, finally, effective cellular transport across intestinal epithelial cells are all of the most important issues in the field. Instinctive properties of chitosan make the polymer a suitable candidate for oral gene delivery. In this section, application of chitosan as a carrier of genetic molecules is divided into two subsections: delivery of DNAs and delivery of siRNAs/miRNAs.

To begin with the first category, it is worth remembering the utmost importance of DNAs and their functions within the cells. As compellingly explained in many studies, DNAs as therapeutic options in cancer therapy are able to express the protein of interest; thus, potentially they can regulate again the altered regulation pathways within the cancer cells. In the case of oral delivery of DNAs with chitosan nanoparticles, the section can be divided into two main areas: DNA vaccination and therapeutic DNAs.

Needless to mention, perhaps the most crucial gain of oral vaccination is better acceptance of this route of immunization by patient. Moreover, oral vaccine provokes immune responses at mucosal surfaces, the place where many pathogens generally invade (Clark et al. 2001). The most important role in oral uptake of particulate vaccines is on the shoulder of antigen-sampling membranous (M) cells presenting in intestinal Peyer's patches with perhaps a minor role for intestinal enterocyte as observed in some studies (Roy et al. 1999; Desai et al. 1997; Desai et al. 1996). It is reported that particles with a maximum size of 10 micrometers can penetrate into Peyer's patches (Eldridge et al. 1990); thus, it is of great importance to form chitosan micro- and nanoparticles with the suitable size for enhanced cellular transfection in intestinal tract.

The feasibility of chitosan in oral DNA vaccination has been explored by a number of studies all suggesting the practical applicability of chitosan nanoparticles. Before providing more details on the issue, it should be noted that chitosan might have properties that affect immune responses, highlighting its role in vaccination. Intranasal co-administration of chitosan glutamate solution and purified influenza surface antigens results in elevated levels of IgG and IgA compared with free surface antigens(Bacon et al. 2000). An increase in the level of IL-4 and TGF-beta mRNA was reported in oral feeding of chitosan solution to rats (Chellat et al. 2005). As per similar studies, chitosan may not only act as an adjuvant in vaccine immunization, but also it can increase the antigen size and activate the immune system (Danesh-Bahreini et al. 2011). Chitosan nanoparticles containing vaccine against nervous necrosis virus (NNV) were orally administrated in European sea bass juveniles to assess the immunization of the vaccinated animals against the virus. Increase in levels of transcription of genes related to cell-mediated cytotoxicity and the interferon pathway in the posterior gut of the vaccinated group was observed along with

the significant enhancement in the survival rate against NNV challenge 90 days after the start of vaccination (Valero et al. 2016). In a similar study, a DNA vaccine encoding the VP2 gene of infectious pancreatic necrosis virus (IPNV) was encapsulated into chitosan-tripolyphosphate (Chi-TPP) nanoparticles and orally administrated in rainbow trout (*Oncorhynchus mykiss*). An increase in transcript levels of CD4 in vaccinated fish with 25 mg pcDNA-VP2 vaccines encapsulated in Chi-TPP nanoparticles suggests the involvement of helper T cells in group fed with vaccine. The levels of anti-IPNV antibodies were drastically increased 45–60 days after vaccination, being overall significantly higher in the vaccinated groups compared with the control group (Ahmadivand et al. 2017). Table 2.3 casts a concise glance at some of the most recent works done on the issue of oral vaccination with chitosan nano- and microparticles.

Table 2.3 Examples of research works done on the application of chitosan nano- and microparticles in oral vaccination

Vaccination against	Sample species	Carrier	Ref.
Turbot reddish body iridovirus	Turbots (*Scophthalmus maximus*)	Chitosan nanoparticles	Zheng et al. (2016)
Measles virus	BALB/c mice	Alginate-coated chitosan nanoparticles	Biswas et al. (2015)
Streptococcus iniae	Channel catfish (*Ictalurus punctatus*)	Alginate/chitosan microspheres	Wang et al. (2018)
A. hydrophila	European carp (*Cyprinus carpio*)	Nano-polyplexes consisting of oleoyl-carboxymethy-chitosan/ hyaluronic acid	Liu et al. (2016)
Trueperella pyogenes	Female Kunming mice	Chitosan nanoparticles	Huang et al. (2018)
Salmonella	Laying chickens	Chitosan nanoparticles	Renu et al. (2018)
Avian avulavirus (cause of Newcastle disease)	Specific pathogen-free (SPF) chickens	Chitosan-coated poly(lactic-co-glycolic) acid nanoparticles	Zhao et al. (2014)
Brucella melitensis	BALB/c mice		Abkar et al. (2017)
Hepatitis B virus	C57BL/6 mice	Alginate-coated chitosan particles	Soares et al. (2018)
Vibrio anguillarum	Turbot (*Scophthalmus maximus*)	Carboxymethyl chitosan/chitosan nanoparticles	Gao et al. (2016)

Chitosan was also deployed in order to orally deliver therapeutic DNAs. Regarding this application of chitosan nano- and microparticles, a number of benefits have been reported in studies mainly including patient compliance, safety, and reduction of the administrated dose. With regard to safety, however, a clearer interpretation might be necessary to regard it as a benefit. In other words, in the case of targeting short-lived intestinal epithelial cells, oral administration of DNA molecules can be considered safe compared to parenteral route, since lack (or very low in some cases) of systematic effect can be detected with oral route administration. Functional coagulation factor IX (FIX) is one of the main players in the blood coagulation cascade, as its deficiency causes hemophilia B, a current challenge in health care. In a study done by Quade-Lyss et al., bioengineered FIX variants possessing high clotting activity were encapsulated within chitosan nanoparticles for oral administration in FIX-deficient mice. Following oral administration of nanoparticles, GFP (green fluorescent protein) expression in the small intestine was successfully stained, while no detection was made in the liver, spleen, or colon. Seven consecutive daily doses of nanoparticles containing hyperfunctional FIX variants made clotting activities in treated control lasting for at least 3 weeks. Moreover, chitosan nanoparticles were not affected by immunotolerance mechanisms indicating their promising role in the oral delivery of FIX variants (Quade-Lyssy et al. 2014). Thiolated chitosan as one of the main derivatives has attracted attention since modification of chitosan chains with thiol groups provides obtained nanoparticles more stability mainly due to formed inter- and intramolecular disulfide bonds within the thiomer itself. What is more is that interaction of negatively charged DNAs with chitosan chains would be strengthened by addition of thiol groups. Ronny Martien et al. investigated thiolated chitosan in delivery of pDNA on Caco-2 cells as a model of enterocytes lining the small intestine. The obtained nanoparticles showed better DNA protection against DNase I compared with unmodified chitosan-DNA nanoparticles indicating the feasibility of these nanoparticles to be used as oral carrier for genes as nucleases are found abundantly in the small intestinal fluid. Nanoparticles carrying plasmid DNA, expressing a GFP, have successfully entered the cells after 4 h, and the expression was detected after 36 h of transfection (Martien et al. 2007). In another study conducted by HaoZheng et al., survivin short hairpin RNA (shRNA)-expressing plasmid DNA (shSur-pDNA) was encapsulated within amino acid-modified chitosan nanoparticles to investigate the effect of nanocomplexes in hepatoma. The idea of amino acid modification stems in the fact that modified chitosan nanoparticles present enhanced quality in terms of their stability in simulated environment of GI tract, cellular uptake, endosomal escape, release behavior, pDNA permeation across intestinal cells, proliferation inhibition of tumor cells, and the overall antitumor efficacy. In comparison with unmodified chitosan-pDNA nanoparticles (CS-NPs), chitosan-histidine-cysteine nanoparticles (CHC-NPs) were able to preserve their physicochemical properties when exposed to dilution and ion challenges as well as experienced smaller changes in acidic conditions as a model for stomach environment. This indicates that modified chitosan nanoparticle might preserve its features in the gastrointestinal tract. Intracellular uptake of nanoparticles also suggested that CHC-NPs have a significantly

higher transfection than free CS-NPs with further studies suggesting clathrin- and caveolae-dependent pathways were responsible for the internalization process. In vitro and in vivo antitumor analysis also stands out for the better and higher antitumor efficacy for CHC-NPs. In fact, CHC-NPs could decrease tumor volume significantly after 16 days of tumor size measurement (Zheng et al. 2015).

Chitosan is a suitable carrier for delivery of microRNAs and siRNAs. The great importance of miRNAs and siRNAs has urged scientists in recent decades to put more effort into the practical applicability of these therapeutic molecules in treatment of terminating diseases including cancer. microRNAs and siRNAs are double-stranded RNAs consisting of 21–23 nucleotides potentially able to knock down any gene of interest; thus, deploying the RNA interference system might be a promising solution to a number of current challenging problems. As parenteral route of administration of these genetic molecules is the first choice in many studies, oral route is the best choice when it comes to patient compliance. In one study, mannose-modified trimethyl chitosan-cysteine (MTC) were employed to form nanoparticles containing tumor necrosis factor-a (TNF-a) siRNA to investigate their effects against acute hepatic injury in rats. Nanoparticle formation was achieved in three methods. In the first and well-known one, nanoparticles were formed according to the process "ion gelation" in which siRNA is premixed with tripolyphosphate (TPP) solution and added dropwise into ionic MTC solution (en-MTC NPs). The second method was a simple direct self-assembly between MTC conjugates and siRNA (sa-MTC NPs), and the last one was siRNA adsorption after the process of TPP-mediated ionic gelation of MTC conjugates (ad-MTC NPs). Gel retardation assay revealed that while sa-MTC NPs and ad-MTC NPs cannot retain the negatively charged siRNA molecules (with the sa-MTC NPs as the weakest interaction with siRNAs), en-MTC NPs are able to retain and protect entrapped siRNAs from degradation. With the aid of FAM-labeled TNF-a siRNA, intestinal transfection of nanoparticles was evaluated which demonstrated that mannose-modified trimethyl chitosan-cysteine had significantly higher cellular transfection compared with unmodified trimethyl chitosan-cysteine, a fact which highlights the crucial role of mannose in the uptake process of nanoparticles since normal enterocytes and M cells that express mannose receptor can actively uptake the modified nanoparticles. In vivo studies showed that orally administrated en-MTC NPs were able to decrease TNF-a levels in LPS/D-GalN-induced acute hepatic injury rat models which surpassed Lipofectamine/siRNA complexes and significantly higher than unmodified trimethyl chitosan-cysteine siRNA nanoparticles (He et al. 2015).

Considering different aspects of delivery of genetic molecules into intended cells, chitosan polymer seems to serve not only as a good carrier, but also it might be of first choice when it comes to orally administrated formulations. It is worth mentioning again that chitosan is a natural polymer and its oral administration of large amounts is proven to be safe in humans. Besides its mucoadhesive features, it also is a cationic polymer and thus is able to form stable complexes with negatively charged molecules including DNAs and RNAs.

2.8 Chitosan Oral Peptide/Protein Delivery

Therapeutic peptides have a rich history of use, and a massive data on their physicochemical properties and challenges are available in the literature. In fact, the first attempt of peptide therapy dates back to the 1920s when the isolation and first therapeutic use of insulin in diabetics did not produce sufficient quantities of the hormone. Since that very day, numerous attempts have been devoted to the safe and efficient application of therapeutic peptides and proteins (Lau and Dunn 2018). To begin with, an understanding of the challenges of oral peptide delivery can be useful; therefore the main problems limiting the practical applicability of oral peptide delivery systems are briefly explained here. First, the presence of abundant digestive enzymes in the intestinal tract (namely, proteolysis of peptide bonds by pepsin in the stomach, degradation by pancreatic enzymes or membrane-bound proteolytic enzymes in the small intestine, and degradation by chymotrypsin and peptidases in the jejunum) is a crucial challenge since they are designed to break down amide bonds, which are one of the main bonds existing in proteins; thus almost any protein is susceptible to degradation by digestive enzymes. Second, many peptides suffer from poor intestinal permeability due to high polarity and molecular weight. Third, many peptides are acid labile and poorly stable in extreme pH conditions causing reduction of disulfide bonds and hydrolysis (Richard 2017). Therefore, a favorable peptide/protein delivery system has to protect the entrapped peptide against gastrointestinal environment and enzymatic degradation as well as increasing adsorption by intestinal cells. Application of chitosan in oral delivery of peptides has attracted scientists' interest because of the intrinsic properties of this natural polymer. Insulin, as one of the well-studied peptides in this field, was encapsulated in trimethyl chitosan nanoparticles formed via ionotropic pregelation method with the aid of alginate and cationic β-cyclodextrin polymers. The advantage of trimethyl chitosan to unmodified chitosan is its high solubility up to a pH value of 9.0 due to its permanent cationic charge (more details on trimethyl chitosan derivative are provided in a different section further in this chapter). The idea of using cyclodextrins (CDs) in oral peptide delivery systems stems form reports indicating the ability of the polymer to increase absorption by modifying the mucosal membrane fluidity and protection of the trapped therapeutic peptide against denaturation and degredation. In vitro release analysis showed that nanoparticles prevented insulin release in simulated gastric fluid, while a burst release was observed in basic pH amounts as a simulation of intestinal fluid. Nanoparticles were capable of transporting insulin across Caco-2 cell monolayer significantly compared to free insulin with the superiority of trimethyl chitosan nanoparticles to unmodified chitosan nanoparticles indicating trimethyl chitosan as a promising candidate for oral delivery of insulin (Mansourpour et al. 2015).

2.9 Chitosan Nanoparticles

Polymeric nanoparticles have captivated attention inasmuch as their ability to distribute drugs in the whole body for an extended period of time (Sithole et al. 2017). Over the past few years, in order to develop adequate oral drug delivery systems, the area of focus has shifted from chitosan to chitosan derivatives. By means of this approach, it was reported that modifying chitosan brings about notable improved properties, such as increased drug retention capacity, mucoadhesion, as well as sustained release of therapeutic agents (Chaudhury and Das 2011). Chitosan has been used both in gene and protein delivery, especially by the role of oral absorption enhancer (Khan et al. 2002). Chitosan derivatives enable us to protect the proteins from degradation in the acidic environment of gastrointestinal tract and to achieve a sustained drug delivery (George and Abraham 2006a).

To begin with, a deeper knowledge of formation of chitosan micro- and nanoparticles containing therapeutic molecules would help to digest the issue; thus, in the following paragraphs, the main principals of chitosan nanoparticle formation are explained with the aid of some recent related works.

According to the literature, the well-established method for chitosan nanoparticle formation is the process called "ioinic gelation." The idea of the method derives from the intrinsic property of chitosan resulting from the presence of abundant free NH2 groups in the polymer chains. The NH2 functional group in chitosan has a pKa value of approximately 6.4; therefore, in acidic environment, NH2 groups tend to become cationic NH3+ which in turn gives the whole chitosan chain cationic charge.

In order to alter some of the conspicuous characteristics of chitosan such as solubility, mucoadhesion, and stability, both the -NH2 and -OH groups in the backbone of chitosan enable it to be modified easily (Werle et al. 2009). By chemical modification of chitosan, different derivatives such as quaternized chitosan, thiolated chitosan, carboxylated chitosan, amphiphilic chitosan, chitosan with chelating agents, PEGylated chitosan and lactose-modified chitosan can be achieved (Sutton et al. 2017). Several therapeutic agents have been orally delivered using NPs prepared from various chitosan derivatives as mentioned below.

2.9.1 Quaternized Derivatives of Chitosan

One of the challenges associated with the use of chitosan is its low solubility at neutral to basic pH. This can be triumph over by methylating amino groups of chitosan to produce N-trimethyl chitosan (TMC), a quaternized derivate (Thanou et al. 2000), which is water soluble in a broad range of pH (pH =1–9) (Biswas et al. 2014). With the aim of delivering peptides and proteins, various quaternized derivatives of chitosan have been synthetized such as diethylmethyl chitosan (DEMC), TMC and triethyl chitosan (TEC).

Studies reveal that quaternization of chitosan facilitates the opening of tight junctions and improves the permeability of insulin across Caco-2 cells (Sadeghi et al. 2008a).

Sadeghi et al. developed both diethylmethyl chitosan and trimethyl chitosan using ionotropic gelation and polyelectrolyte complexation methods in order to improve insulin drug delivery. The obtained results demonstrated that nanoparticles prepared by the polyelectrolyte complexation method had higher insulin loading efficiency and zeta potential than those made by the ionotropic gelation method and may subsequently be used for further studies (Sadeghi et al. 2008b).

Bayat et al. established another insulin nanoparticle using TEC and DMEC by polyelectrolyte complexation method. Release studies revealed a small burst release at the beginning and then a sustained release for 5 h. Quaternized derivatives showed better insulin transport across the colon membrane of rats in comparison with chitosan (Bayat et al. 2008).

In a research by Lin et al. N,O-carboxymethyl chitosan nanoparticles were prepared as insulin carrier by ionic gelation method with TPP. These nanoparticles showed pH-sensitive characteristics and could be used for controlled release of insulin via oral route (Lin and Lin 2009).

In another study conducted by Cui et al. a pH-sensitive carboxylated chitosan-grafted poly(methyl methacrylate) nanoparticles were established for oral delivery of insulin. These nanoparticles displayed minimal release rate at pH of 2.0 and an instant release rate at pH of 6.8 and 7.4 (Cui et al. 2009).

2.9.2 Thiolated Chitosan

Various mucoadhesive polymers are currently used in oral drug delivery, among which thiolated chitosans were found to be highly promising owing to their mucoadhesive profile. The free thiol groups of thiolated chitosans form disulfide bridges with cysteine-rich subdomains of the mucus. The resulting covalent bond between the polymer and mucus is the reason for improved mucoadhesive profile (Werle and Bernkop-Schnürch 2008). It could be concluded that thiol-functionalized carriers could be beneficial for mucosal drug delivery.

There are three main reasons for the wide usage of thiolated chitosan in oral drug delivery:

1. During tensile studies, it was manifested that there is a positive correlation coefficient among the amount of free immobilized thiol groups and the mucoadhesive attributes of the polymer (Kast and Bernkop-Schnürch 2001).
2. Thiolated chitosan demonstrated improved permeation enhancing effect owing to the reversible opening of tight junctions (Werle and Bernkop-Schnürch 2008).
3. Recently, it has been proved that thiolated chitosans can inhibit efflux pumps such as P-glycoproteins. Therefore, by using thiolated chitosan, the oral delivery of efflux pump substrates can be improved (Werle and Hoffer 2006).
4. The cross linkage of the thiolated chitosan chains results in a stable polymeric matrix that allows a controlled, continuous drug release (Bernkop-Schnürch et al. 2003).

Various thiolated chitosan derivatives are presently in use including chitosan-thioglycolic acid (chitosan-TGA) conjugates (Kast and Bernkop-Schnürch 2001), chitosan-cysteine (chitosan-Cys) conjugates (Bernkop-Schnürch et al. 1999), chitosan-glutathione conjugates (chitosan-GSH) (Kafedjiiski et al. 2005a), chitosan-thioethylamidine conjugates (chitosan-TEA) (Kafedjiiski et al. 2005b), chitosan-6-mercaptonicotinic acid conjugates (Millotti et al. 2014), and chitosan-4-thio-butyl-amidine conjugates (chitosan-TBA) (Andreas et al. 2003). By coupling the primary amino groups of chitosan with the thiol-bearing functional groups, thiolated chitosan is designed (Bernkop-Schnürch et al. 2004).

In a study conducted by Millotti G (Millotti et al. 2014), chitosan-6-mercaptonicotinic acid was obtained as a thiolated chitosan carrier for in vivo evaluation of oral drug delivery of insulin. Thiolated chitosan formulations were about 80-fold more mucoadhesive in comparison with unmodified chitosan. In vivo results demonstrated that absorption of insulin by rats was highly enhanced by thiolation. The areas under the concentration-time curves (AUC) of chitosan-6-mercaptonicotinic acid were up to 6.8-fold improved, compared with unmodified chitosan. So, it could be concluded that chitosan-6-mercaptonicotinic acid holds promise for oral delivery of insulin.

Thiolated chitosan nanoparticles were also used for the oral delivery of low molecular weight heparin (LMWH). A pH-responsive system was developed, using thiolated chitosan (TCS) and HPMCP, a pH-sensitive polymer. The developed nanoparticles showed a significant improvement in mucoadhesion on rat intestinal mucosa. After oral delivery of LMWH-loaded TCS/HPMCP nanoparticles in rats, an increase in the oral bioavailability of LMWH was reported. Furthermore, the activated partial thromboplastin time (APTT) was significantly extended, which indicated enhanced anticoagulant effects. In conclusion, pH-responsive TCS/HPMCP nanoparticles represent a promising vehicle for oral delivery of LMWH (Fan et al. 2016).

A chitosan-cysteine conjugate/polymalic acid (PMLA) was synthesized as a drug delivery system for treatment of *Helicobacter pylori*. The resulting nano-system was encapsulated with amoxicillin. The results showed that amoxicillin-cysteine-chitosan/PMLA nanoparticles exhibit favorable pH-sensitive properties that could prolong the release of amoxicillin at gastric acid. Inhibition of *Helicobacter pylori* growth was compared using unmodified amoxicillin-chitosan/PMLA nanoparticles and amoxicillin-cysteine-chitosan/PMLA nanoparticles. More specific and effective inhibition profile was observed using amoxicillin-cysteine-chitosan/PMLA nanoparticles. This research sheds a light on the design of a new generation of pH-sensitive cysteine-conjugated nanocarriers that can be used as new promising oral drug delivery systems (Arif et al. 2018).

2.9.3 PEGylated Chitosan

A variety of strategies have been used for further improvement of the surface properties of chitosan nanoparticles. The usage of poly(ethylene glycol) (PEG) in this context has been popular due to two main reasons: First, biocompatibility of chitosan can be improved by chemical modification of chitosan with PEG (Zhang et al. 2002a). Second, the stability of nanoparticles is enhanced by coating with PEG, which promotes further transport of target molecules through intestinal and also nasal epithelia (Tobıo et al. 2000; Tobio et al. 1998).

Human parathyroid hormone 1–34 (PTH 1–34) was loaded in chitosan nanoparticles and then PEGylated (PEG-CS-PTH NPs). The resulting nanoparticles were subjected to an in vitro release in simulated rat body fluids. The anabolic effects of PTH peptide were remarkable in comparison with bare PTH 1–34 and CS-PTH NPs (Narayanan et al. 2013).

2.10 Chitosan Films in Oral Drug Delivery Systems

Several polymers have been used as carrier films in oral drug delivery systems. Among them polymers such as chitosan that has achieved some features such as compatibility with the gastric environment, mucoadhesive property, stability during the drug release time, adequate mechanical properties, ease of preparation, flexible and elastic feature resulting in patient compliance, biocompatibility and biodegradability, prolonged residence time of the formulation leading to controlled delivery system, more accurate dosing of the drug compared to gels and ointments, and favoring the drug localization to improve the bioavailability of drug could be a useful choice in drug delivery systems (Tang et al. 2014). Suitable flexibility and drug diffusion could be developed by using plasticizers and cross linkers along with the base polymer in forming the carrier films. Increasing of deacetylation degree in chitosan has an increasing effect on tensile strength of the water-swollen films and possesses less rapidly degradation of the films (Kumar et al. 2004). Chitosan has mucoadhesive property by its positively charged amino groups' interaction with negatively charged sialic acid residues in mucus that makes chitosan an appropriate choice in oral drug delivery systems. Chitosan could also increase the drug absorption by opening the tight junctions (Tang et al. 2014).

Chitosan films could be prepared by chemical cross-linking agents or electrostatic interaction in a casting solvent evaporation method in which the second method is safer than chemical cross-linking process. Polyanions form electrostatic interaction with chitosan leading to polyelectrolyte complex films. Chitosan could also be developed by dipping the polymer film into a cross-linking ion solution by using low molecular weight ions such as TPP and other polyphosphate molecules (Hadisoewignyo et al. 2018). Properties of ionically cross-linked chitosan films with multivalent phosphates can be significantly influenced by ionic strength and media pH. These films release the drug quickly in acidic conditions; however, the release is slow in neutral conditions such as simulated intestinal fluid. They play a

vital role in site-specific drug delivery systems in the stomach (Teruel et al. 2018). Low activity of proteolytic enzymes in the colon compared with the intestine can increase absorption of the protein drugs (Hejazi and Amiji 2003). Therefore, bacterial degradable polymers can be used as a site-specific candidate for colon-specific drug delivery. By degradation of these polymers with enzymes or microorganisms, their molecular weight and mechanical strength are reduced, and they would be able to release the drug in the desired site (Park et al. 2011). Chitosan films could be a proper choice in colon drug delivery systems as chitosan is susceptible to lipase, pectinases, and amylases (Roy et al. 2003). An enteric coating of chitosan can protect it from the acidity of the stomach resulting in dissolving of enteric layer in the intestine and exposing drug-bearing chitosan core to the bacterial enzymes in the colon leading to drug release (Zhang et al. 2002b).

Drug release from oral gel systems can be prolonged by using chitosan polymer due to its bioadhesion property that makes chitosan as a proper choice for periodontal controlled drug delivery systems. Chitosan also has the ability to inhibit the adhesion of *Candida albicans* to human buccal cells leading to antifungal activity. In a study, chitosan films containing chlorhexidine (Chx) drug were prepared by using glycerin as a plasticizer and tripolyphosphate pentasodium salt as a cross-linking agent. Chx release from cross-linked chitosan films was lower than free chitosan films (without cross-linking agent) up to 4 h due to a change in the porosity of the film after cross linking. The water uptake capacities of films were reported $180 \pm 10\%$ that indicates a significant bioadhesion property due to the direct relationship between the rate of hydration and bioadhesion (Şenel et al. 2000). Chitosan films containing tetracycline in three different concentrations were developed by the solution casting method. A burst release of the drug was achieved followed by a progressive fall up to 7 days in non-cross-linked films, and also extended drug release was represented for more number of days by using cross-linking agents. The advantages of this drug delivery system are composed of reducing the dose and increasing the concentration of the drug in the periodontal pockets with low systematic drug absorption. Chitosan wound healing property and antibacterial ability were also positive effects of the drug delivery system (Ahmed et al. 2009).

Buccal drug delivery systems have achieved a great attention as they could inhibit first pass metabolism of the drug to a great extent successfully. Buccal mucoadhesive films of a model opioid analgesic (tramadol hydrochloride) were prepared by forming interpolymer complex (IPC) between chitosan and carboxymethylated *F. limonia* fruit pulp mucilage in a solvent casting method. The drug was released over a period of 8 h from chitosan films through buccal mucosa which results in a controlled drug delivery system. The IPC system formed a gel by swelling in aqueous media leading to diffusion of the drug. Bioadhesion force, a necessary feature for holding the films at the site of application, is related to the swelling index. Swelling index was increased directly by increasing the concentration of IPC leading to a higher bioadhesion force (Patel 2016). Mucoadhesive bilayered films of chitosan and ethylcellulose were developed containing a drug-containing mucoadhesive layer and a drug-free backing layer for buccal drug delivery. The backing layer was

composed of ethylcellulose, and the mucoadhesive layer was made of a mixture of drug and chitosan with or without anionic cross-linking polymers. This drug delivery system could avoid loss of drug due to washout with saliva. These mucoadhesive bilayered films exhibited sustained drug release in a phosphate buffer (pH 6.4) (Remunan-Lopez et al. 1998). In another study, ibuprofen was released from chitosan films developed by supercritical solution impregnation (SSI) technology in rabbit buccal mucosa up to 70% in 40 min. SSI method provides a controlled release behavior in oral mucosal drug delivery systems (Tang et al. 2014).

Citrate cross-linked chitosan films containing riboflavin were developed by dipping chitosan film into sodium citrate solution. Riboflavin was released within 2 h completely in simulated gastric fluid at 37 °C as chitosan films were dissociated in the pH less than 3.5 and under the neutral conditions; the drug was released less than 40% in simulated intestinal fluid in 24 h. Citrate cross-linked chitosan films could be used as a choice in stomach-specific drug delivery systems as they exhibit pH-sensitive swelling and drug-controlled release properties (Hadisoewignyo et al. 2018). Chitosan-pectin polyelectrolyte complex (PEC) films were developed by blending two polymer solutions and then using solvent casting method. Eudragit RS as a water-insoluble was added to protect free films from high swelling in the aqueous media. Swelling process of chitosan-pectin film was pH-dependent and its swellability was decreased by adding Eudragit RS. Eudragit-chitosan-pectin films showed an initial controllable release of the drug followed by a burst release immediately after the change in pH. Eudragit-chitosan-pectin as a mixed-film blend could be applied in sigmoidal drug delivery systems (Ghaffari et al. 2007).

2.11 Hydrogel Drug Delivery Systems

From yesterday to today, polymers have attracted specific attention to themselves in drug delivery systems. However, one of the main challenges of using polymers is their biocompatibility and biodegradability. In regard to this, natural polymers such as chitosan have been highly pursued due to their low toxicity, biocompatibility, and biodegradability. Hydrogel systems are high water content materials that are able to provide controlled and local delivery of therapeutic agents. In hydrogel drug delivery systems, therapeutic agents are released from a polymeric network in a defined time and manner. Therefore, using controlled drug delivery systems such as hydrogels can lead to regulate the bioavailability of drugs (Bhattarai et al. 2010).

Hydrogels are cross-linked polymeric networks that trap a large amount of water without dissolving their hydrophilic groups in their structure. The chemical or physical bonds between polymer chains prevent them from being dissolved. When hydrogels are fully swelled, they achieve some properties such as soft and elastic consistency and low interfacial tension with biological fluids that reduce immune reactions in body. The size of hydrogels can be ranged from nanometers to centimeters (Bhattarai et al. 2010). There are two types of bonds (non-covalent and covalent) that can be accomplished between polymer chains in hydrogel systems to form a stable structure. There are some differences between these types of bonds

that affect the biodegradability of the system and drug release kinetics (Wichterle and Lim 1960). High molecular weight polymers form multiple cross links per polymer resulting in more robust hydrogel systems than small molecular weight polymers (Anseth et al. 1996). The equilibrium swelling ratio is a key parameter in using hydrogel systems that affect surface wettability, solute diffusion coefficient, and mechanical properties of hydrogel systems (Peppas et al. 2000). The pore size of the polymer and hydrodynamic size of the therapeutic agent are important factors in diffusion manner of hydrogel systems (Lin and Metters 2006). Drugs could be added by direct method or covalent attachment to the hydrogel-forming polymers. In direct addition there is a burst release of the drug that can lose a large amount of the agent. This could be prompted by increasing polymer cross linking (Khan et al. 2009). In covalent attachment, the release of drug is controlled by chemical or enzymatic cleavage rate of the polymer-drug bonds. By covalent attachment, the release of drug from hydrogel systems is more controlled over direct addition, and it can extend the release of drug from weeks to months (Kohane and Langer 2008).

2.12 Chitosan Hydrogels

Chitosan has a variety of positive features such as biocompatibility, biodegradability, functional amine groups, hydrophilicity, and a net cationic charge that have made chitosan a suitable polymer for use in hydrogel systems. Different formulations of chitosan hydrogels including tablets, films, beads, powders, capsules, liquid gels, microspheres, sponges, nanofibrils, microparticles, and inorganic composites have been developed successfully (Denkbas and Ottenbrite 2006). There are two methods of forming chitosan hydrogels: physically associated and cross-linked networks.

2.12.1 Physically Associated Chitosan Hydrogels

There are four major interactions between polymeric chains including ionic, polyelectrolyte, interpolymer complex, and hydrophobic associations that can form chitosan gels. Physically associated chitosan gels meet some features such as (Bhattarai et al. 2010):

(a) Short lifetime that makes them proper candidates for short-term drug release applications.
(b) Safe drug delivery carriers as they do not include any toxic covalent linkages in their structures.
(c) Weak mechanical strength that leads to uncontrolled dissolution and limited application.

In a study, a 3D hydrogel was formed by mixing the polyethylenimine with chitosan leading to chain-chain polymer interactions. This hydrogel system could be

useful in growth of primary human fetal skeletal cells. When the system is prepared at pH=7.5, chitosan is insoluble and forms crystallites between its chains leading to hydrogel structure (Khan et al. 2009).

2.12.2 Chitosan Hydrogels Formed by Polyelectrolyte Complexes (PEC)

PEC hydrogels are biocompatible alternatives to cross-linked networks that are formed by ionic interactions (Berger et al. 2004a). However, the main drawback of them is their preparation methods. Polymers with broad molecular weight distribution and anionic groups react with the cationic amino groups of chitosan in an aqueous environment leading to PEC formation. These interactions in PEC hydrogels are stronger than most secondary binding interactions such as chitosan/polyvinyl alcohol complexes. Ionically cross-linked hydrogels have similar features to PEC, although the entities reacting with components are ionic molecules and they are prepared in a more simple way. The advantages of PEC hydrogels over covalently cross-linked networks are represented as follows (Berger et al. 2004b):

The reaction is mostly performed in aqueous solution.
The reaction needs no catalysts or initiators.

These two advantages lead to biocompatibility and avoid purification before administration. Therefore, they can be can be used in various applications such as drug delivery systems, cell culture, enzyme immobilization, and tissue reconstruction. The most commonly used materials in PEC hydrogel systems include polyanions such as alginate, pectin, xanthan, carrageenan, collagen, synthetic polymers, chitin derivative bearing negative charges, nucleic acids, and positively charged chitosan derivatives. The main factors that are important in swelling of PEC hydrogel systems are represented as follows (Berger et al. 2004b): pH of the solution, negative charge of the additional polymer, positive charge of chitosan, temperature, order of mixing, flexibility of polymers, ionic interaction, nature of the solvent, and molecular weight and degree of deacetylation of chitosan. Swelling of PEC hydrogel systems occurs in acidic and also basic conditions. The charge balance inside the gel and the degree of interaction between the two polymers during pH changes play an important role in swelling mechanism (Chu et al. 1996). Therefore, they can be used for pH-controlled drug delivery systems in acidic and basic conditions.

2.12.3 Chitosan-Alginate Hydrogels

The amino groups of chitosan interact ionically with the carboxyl groups of alginate forming PEC hydrogels. Alginate is insoluble in low pH conditions; therefore, it prevents solubility of chitosan in low pH by forming chitosan-alginate PEC, and

alginate solubility in higher pH conditions is prevented by chitosan leading to develop a stable network at higher pH ranges. Chitosan-alginate polyelectrolyte complexes have attracted increasing attention in oral delivery of protein drugs due to their specific properties such as forming pH-sensitive hydrogels, decreasing the leakage of the encapsulated drugs, incorporating proteins in mild environment, and hence reducing the chances of protein denaturation. Carboxyl end groups of alginate make it a suitable mucoadhesive agent. Mixing of alginate with other polymers such as neutral gums, pectin, chitosan, and Eudragit could improve retaining of entrapped drugs and solving the drug leaching problem. Also chitosan covalent modification could change its physicochemical properties and improve its ability for controlling the release of encapsulated drug. Biocompatibility, biodegradability, pH sensitiveness, and mucoadhesiveness properties of chitosan-alginate PEC hydrogel systems have made them suitable choice for oral delivery of proteins and other therapeutic agents. Binding of chitosan to alginate could be enhanced by reducing the average molecular weight of chitosan below 20,000 Da and fraction of N-acetylations and by increasing the porosity of the alginate gel and pH in the range from pH 4 to 6 (George and Abraham 2006b). A hydrogel system composed of alginate blended with a water-soluble chitosan (N,O-carboxymethyl chitosan, NOCC) was developed to deliver a model protein drug (BSA) to the intestine due to pH-sensitive property. With increasing pH (at 7.4), the swelling ratio of the system increased significantly resulting in drug release in the intestine. The main advantage of this drug delivery system was protecting the protein from stomach environment due to chitosan-alginate polyelectrolyte complex's pH-dependent release mechanism (Lin et al. 2005)

2.12.4 Chitosan-Pectin Hydrogels

Chitosan-pectin polyelectrolyte complexes are developed with the contribution of Ca ions that react with amidated pectin to form hydrogel systems. Chitosan is entrapped in the hydrogel system and forms electrostatic interactions with the pectin chains at low pH conditions that make this drug delivery system protective for protein drugs in lower pH of the stomach. Chitosan-pectin hydrogel system swells as the pH increases and, therefore, releases the drug in intestine medium (George and Abraham 2006b).

2.12.5 Chitosan-Carrageenan Hydrogels

It should be noticed in chitosan-carrageenan polyelectrolyte complexes that the main mechanism of drug release is disintegration instead of swelling due to the high capacity of carrageenan that promotes the entry of water into the hydrogel system. Chitosan-alginate PEC has shown more prolonged drug release and higher mean dissolution time in comparison with chitosan-carrageenan hydrogel system (Tapia et al. 2004).

2.12.6 Chitosan-Collagen Hydrogels

Chitosan-collagen polyelectrolyte complexes are formed by two interactions. One of them is electrostatic interaction between chitosan and collagen polyelectrolytes. The second one is hydrogen-bonding type complex in the presence of a great excess of chitosan. Chitosan-collagen PEC can be used in drug delivery systems and other biomedical applications as it is a biocompatible and biodegradable complex (George and Abraham 2006b).

2.12.7 Cross-Linked Chitosan Hydrogels

Chitosan hydrogels can also be prepared by covalently bonded polymeric chains using (a) chemical cross linking, (b) secondary polymerizations, (c) photo cross linking, (d) enzymatic cross linking, and (e) grafting methods (Bhattarai et al. 2010).

(a) *Chemical Cross Linking*

Chemical cross-linking molecules react with the primary amines of chitosan leading to form irreversible bonds between chitosan chains. There are a variety of small molecules such as glutaraldehyde, diglycidyl ether, diisocyanate, diacrylate, and others that develop cross-linked chitosan polymers. Mechanical properties of these small molecules have been improved compared to physically associated chitosan hydrogels, but some of them have been found to be toxic. The main drawback of these hydrogel systems is limitation of choosing a safe and biocompatible cross-linking agent (Bhattarai et al. 2010). Genipin is a naturally derived chemical that acts as an effective cross-linking agent and is much less cytotoxic and exhibits a slower degradation rate than other cross-linking agents (Shalaby and El-Refaie 2018). One drawback of using genipin is its undesirable interaction with encapsulated drug in chitosan hydrogel systems (Tan et al. 2004).

(b) *Secondary Polymerizations*

Polymer chains with reactive functional groups can also be used to form covalently bonded hydrogels in situ. These hydrogel systems could be produced by Schiff bases or Michael addition reactions. In a study, N-succinylated chitosan and aldehyde-terminated hyaluronic acid reacted with each other through Schiff base reaction and formed a biodegradable hydrogel system that was stable for 4 weeks successfully (Tan et al. 2009). Chitosan-polyethylene oxide (PEO) hydrogel was produced by reacting acrylated chitosan with thiolated PEO through Michael addition reaction. Enhanced mucoadhesive properties could be achieved in the preparation of polymers using active thiols that make them an appropriate choice for oral drug delivery systems (Kim et al. 2007). There are some disadvantages of using polymer-polymer systems including multistep preparation, purification processes, and functionalization of polymers with cytotoxic reactive groups.

(c) *Photo Cross Linking*

Chitosan hydrogel systems can also be developed by using photosensitive functional groups that form cross linkages upon irradiation with UV light (Bhattarai et al. 2010). Ono et al. prepared a photosensitive chitosan hydrogel system by functionalizing the polymer with azide groups. After UV irradiation, azide groups could be converted into reactive nitrene groups that bind with chitosan's free amino groups and form gelation within 60 s (Ono et al. 2000).

(d) *Enzymatic Cross Linking*

Enzyme-catalyzed cross-linking reactions have been developed as an in situ hydrogel forming method for biomedical applications (Jin et al. 2007). Chitosan-gelatin hydrogel was developed by using tyrosinase that oxidizes the tyrosyl residues of gelatin, forming quinone residues that react with chitosan's amino groups leading to intermolecular cross linkages (Chen et al. 2003).

(e) *Chitosan-Grafted Hydrogels*

Chitosan-grafted hydrogels are formed by covalent binding of a molecule, the graft, onto the chitosan backbone. Free amino groups and hydroxyl groups of chitosan could be grafted. It should be noted that grafting of chitosan could improve its properties such as complexation, chelating, solubility, bacteriostatic effect, absorption, mucoadhesivity, biocompatibility, and biodegradability. Chitosan-grafted hydrogels maintain some advantages such as pH-controlled release and enhancing the solubilization of lipophilic drugs in aqueous conditions. They can be used in oral or nasal drug delivery systems. Drawbacks of chitosan-grafted hydrogels are using potentially toxic molecules for their preparation and exhibiting pH-sensitive swelling only in acidic conditions (Berger et al. 2004b).

2.13 Chitosan Hydrogels in Oral Drug Delivery Systems

pH sensitivity and mucoadhesive properties are two main advantages of chitosan hydrogel systems that make them appropriate choices for oral drug delivery systems. Specifically, thiolated chitosan hydrogels form disulfide bonds between the thiomers and mucus glycoproteins resulting in prolonged residence time in the GI tract (Bernkop-Schnürch 2005).

2.13.1 Chitosan Hydrogels in Oral Cavity

Chitosan hydrogels could enhance drug penetration within the mouth cavity and maintain high levels of drugs such as antimicrobial agents in the crevicular fluid with minimal systemic uptake (Bernkop-Schnürch et al. 2003). In addition to release of

drugs in the oral cavity by chitosan hydrogels, the chitosan polymer itself has shown antifungal and antibacterial activity. Adhesion of *Candida albicans* to human buccal cells was restricted by chitosan hydrogels and films that were also able to provide sustained release of chlorhexidine gluconate in the oral cavity (Şenel et al. 2000). In another study, chitosan hydrogels consisting of chitosan and PLGA were able to deliver ipriflavone, a lipophilic drug that promotes bone density, into the periodontal pockets (Perugini et al. 2003).

2.13.2 Chitosan Hydrogels in GI Tract

There are a variety of challenges in drug delivery systems to GI tract such as destructive enzymes, pH changes, and low residence times that can limit localized administration of therapeutic drugs (Bhattarai et al. 2010). Chitosan hydrogels can be an ideal drug delivery system in the GI tract as they can be prepared with pH-sensitive or enzyme-specific release triggers. Chitosan is insoluble at basic pH, but it dissolves at low pH easily. The amine groups of chitosan protonate under low pH conditions that lead to chain repulsion and diffusion of water inside the gel (Yao et al. 1994). Therefore, chitosan has been used in delivery of drugs to the stomach, but there is a limitation for delivery of therapeutic agents to the intestine because as the matrix gets dissolved in the stomach, the released drugs (especially proteins) will get denatured. Modifications can be used to overcome this problem and increase stability of chitosan in the stomach leading to controlled release of the drugs in the intestine. For example, ionic gelation of chitosan solution containing the drug could be achieved by cross-linking agents such as TPP that interacts via electrostatic forces to form stable ionic cross-linked networks. Therefore, release of the drug in higher pH in the intestine could be controlled instead of rapidly releasing the drug in the stomach (George and Abraham 2006b). Another way for improving the application of chitosan hydrogel for intestinal delivery of drugs (including protein agents) is chitosan interaction with other polymers such as alginate forming PEC. Swelling of such hydrogel systems is minimal in the stomach making them suitable pH-sensitive hydrogels for releasing the drug in intestine medium as the pH increases(Lin et al. 2005). In a study, amoxicillin and metronidazole antibiotics were delivered locally to the stomach by chitosan-PEO semi-IPN hydrogel system (Sogias et al. 2012). Chitosan-based bioadhesive PEC hydrogels loaded with 5-fluorouracil and insulin exhibited selective release in the intestine successfully (George and Abraham 2006a). In another study, nitrofurantoin encapsulated in chitosan-alginate hydrogel microcapsules was released selectively in intestinal medium compared to gastric medium due to the pH-dependent swelling properties of the hydrogel drug delivery system (Hari et al. 1996b). Chitosan-based hydrogel systems loaded with acetaminophen, mesalazine, sodium diclofenac, and insulin have also exhibited controlled release of the drug in the colon due to degradation of chitosan by the microflora (Tozaki et al. 2002; Sinha and Kumria 2002).

2.14 Conclusion

Chitosan is one of the most well-studied natural polymers in oral drug and gene delivery systems. Besides its safety, the polymer possesses properties making it a suitable candidate for many delivery aims. The mucoadhesive feature of chitosan chains is another promising intrinsic characteristic of the polymer. Many efforts have been put to oral delivery systems with the aid of chitosan as carrier. Conventional drugs have been trapped within chitosan capsules and tablets in order to prevent the drug release within the small intestine and to deliver the drug into the inflammatory site of the colon. In more novel delivery systems, chitosan was employed to orally deliver genes and nucleic acids since the polymer can successfully protect the trapped gene as well as increase its absorption in the gastrointestinal tract. Another challenging field in chitosan delivery systems is oral delivery of peptides and proteins which has attracted considerable attention in recent years. Derivatization of chitosan is another interesting field in the polymer oral delivery system, since the modified chitosan chains possess better and improved properties for oral delivery intentions. Finally chitosan hydrogels with its rich history of use are safe, easy-to-make solutions with the advantage of controlling drug release making them another promising option in oral delivery systems.

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Transdermal Delivery of Chitosan-Based Systems

3

Arshiya Praveen and Mohd Aqil

Abstract

Transdermal drug delivery has offered a promising alternative to other routes of delivery especially for the lipophilic drugs with limited oral bioavailability. Transdermal systems are acceptable because of their noninvasiveness, ease of application and removal, controlled drug release for long duration of time, avoidance of hepatic first-pass metabolism, and improved bioavailability. The predominant challenge in transdermal drug delivery is the skin barrier in the form of stratum corneum. Various approaches have been employed for breaching this barrier including penetration enhancers, iontophoresis, electroporation, and sonophoresis to name a few. Chitosan is a biodegradable, biocompatible polysaccharide polymer with anti-infective, antidiabetic, anticancer, and antihyperlipidemic effect. It has also been used as an adjuvant in transdermal drug delivery for its skin penetration enhancing properties. It is polycationic in nature and shows strong mucoadhesive property by interacting with negatively charged entity of skin moieties, thus prolonging contact time. This chapter deals with the synthesis of chitosan and application of chitosan-based drug delivery systems in transdermal drug delivery.

Keywords

Chitosan · Transdermal · Penetration enhancer

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_3

3.1 Introduction

The transdermal drug delivery has achieved much attention for a few decades in pharmaceutical research in comparison to other routes of delivery (Kim et al. 2013). Transdermal deliveries are generally accepted because of their ease of application in which drug carriers are able to directly enter into the systemic circulation via transdermal delivery, avoid hepatic metabolism, and get protected from acidic medium of the stomach, the enzyme effects of GIT, and the fluctuating plasma concentrations associated with the oral route (Al-Kassas et al. 2016). The important advantages of transdermal delivery are its ability to maintain the drug plasma concentration to a predetermined rate and reduce the side effects. Patient compliance of transdermal formulations is high because they are noninvasive and can be self-administered and provides flexibility to terminate delivery of drug carriers by simply removing the patch from the upper skin surface (Tanner and Marks 2008). One of the superior advantages of transdermal delivery is that they can provide therapeutic effect for extended time periods (up to 1 week) (Abnoos et al. 2018). It also offers an alternative to oral administration in the case of drug with erratic bioavailability. However, the challenge for transdermal route of drug delivery is the barrier of the skin called the stratum corneum (Ali and Hanafy 2016). The major obstruction of these delivery systems is either skin irritation or sensitization by the drug molecules or the carriers used in formulation (Hafez et al. 2018).

Miscellaneous strategies have been proposed to improve transdermal delivery through the skin by the use of vesicles, polymeric particles, and micelles in nano-range. Among these, the use of chitosan polymer provides great opportunity as a carrier in transdermal delivery (Arai et al. 1968). Chitosan is nontoxic and biodegradable and converts into harmless amino sugar which is absorbed by the body. Chitosan is also acknowledged as a mucoadhesive agent and permeation enhancer. Chitosan binds with the epithelial cell of stratum corneum, and its positive charge shows depolymerization of F-actin and tight junction disbandment leading to enhanced permeation across the skin (Agnihotri et al. 2004). These chitosan polymeric carriers are used to prevent drugs from degradation and check interaction with biological surrounding. Moreover, it enhances penetration, absorption, and bioavailability into the target tissues. For topical application, chitosan systems are dispersed into suitable vehicles to enhance adherence on the skin (Kassas et al. 2016).

3.2 Transdermal Drug Delivery System (TDDS)

Transdermal drug delivery system (TDDS) is a fascinating drug delivery route that is used as an alternative to parenteral and oral administration of drugs to minimize and avoid their limitations. These oral and parenteral delivery systems tolerate certain constraints including peak and valley fluctuations in drug plasma levels and inability to maintain sustained effect (Mudshinge et al. 2011). However, TDDS meets the requisitions to dispense the delivery of drug in steady-state and prolonged way to reduce the peak-valley fluctuation-related side effects and to ensure the level of drug

between the effective therapeutic ranges. As a controlled delivery, transdermal route is a tremendously user-friendly application with ease of termination at the time of systemic toxicity along with minor pain sensation (Magnusson et al. 2001).

Transdermal delivery enables drug permeation either topically or across the skin for systemic circulation that tries to avoid the hepatic degradation which can be usually observed in the case of oral administration and provides protection from inconvenience and pain of parenteral administration (Alexander et al. 2012). Transdermal drug delivery has good application for pharmaceutically active entity for topical administration to achieve systemic treatment through healthy skin. Pharmaceutical researchers gave attention to transdermal drug delivery systems for decades when systemic drug delivery got popular in transdermal patches. However, the drug penetration across the skin to achieve percutaneous delivery is hindered by the barrier property of immensely organized composition of stratum corneum (SC) (Brown et al. 2006).

3.2.1 Advantages of Transdermal Drug Delivery Systems (TDDSs)

1. It represents a noninvasive route of delivery.
2. It avoids the exposure of active molecules to first-pass metabolism (hepatic exposure).
3. It can maintain the therapeutic window of active molecules for extended periods by sustaining the plasma levels.
4. Transdermal patches are usually well accepted and easy to apply with the possibility of an ease and immediate cessation of drug administration by patch removal.
5. TDDSs are also providing an appreciated alternative to oral administration that is not feasible or may cause erratic bioavailability.
6. It has lower variation in drug responses.
7. It considerably improves patient compliance.
8. Dose termination is easy in the case of adverse reactions including either systemic or local.
9. It is the most convenient and painless drug administration.
10. Treatment costs of TDDS may be less in overall health care by reducing dose frequency (Brown et al. 2006).

3.2.2 Disadvantages of Transdermal Drug Delivery System (TDDS)

1. Transdermal routes are still under challenge for delivery through the skin because of impermeable epithelium (Ali and Hanafy 2016).
2. This route of delivery shows pre-systemic metabolism by the presence of skin enzymes including peptidases and esterases which might metabolize the active

entity into therapeutically inactive form and reduce the drug efficacy (Brown et al. 2006).

TDDS is used to deliver drug dose with controlled rate through the skin for prolonged time period. It has been reported that a well-designed transdermal formulation supplies the drug with sustained rate to achieve the required therapeutic concentration in plasma without much fluctuation (Delgado-Charro and Guy 2014). Nowadays, transdermal route has been accepted as an innovative research in drug delivery that is proved in quantitative research with approximately 40% of the drug moieties under clinical evaluation and approved by the FDA (Food and Drug Administration). The research involving transdermal products has a bright future due to its interesting upward trend. Transdermal products have continuously provided therapeutic benefits in patients all over the world.

3.3 Skin

The skin is the largest organ in the human body with appropriate biological barrier. The thickness of the skin is normally 2 mm and accounts for around 4% of total body weight (Cevc and Vierl 2010). The important function of the skin is its protective barrier ability against foreign particles including chemicals and microbes. The barrier ability of the skin is reflected because of its multilayered structure (Brown et al. 2006). This multilayered skin protects the internal organs of the body from surrounding environment and helps in maintaining the ideal physiology of the body (Godin and Touitou 2007).

3.3.1 Skin Structure

The skin is a multilayered organ which is composed of numerous histological structures generally divided in two tissue layers as shown in Fig. 3.1:

1. The epidermis
2. The dermis

The Epidermis

Epidermis is the outermost layer of the skin composed of 95% of keratinocyte cells which form a "brick and mortar" arrangement with intercellular lipids along with Langerhans cells, melanocytes, and Merkel cells. The corneocytes of hydrated SC constitute the "bricks," implanted in a "mortar" made of multiplex lipid bilayers including ceramides, cholesterol, fatty acids, and their esters. The epidermis is mainly composed of the layers of stratified keratinocytes in which stratum corneum (SC) cells are dipped in a protein-rich medium with outer lipid medium that is

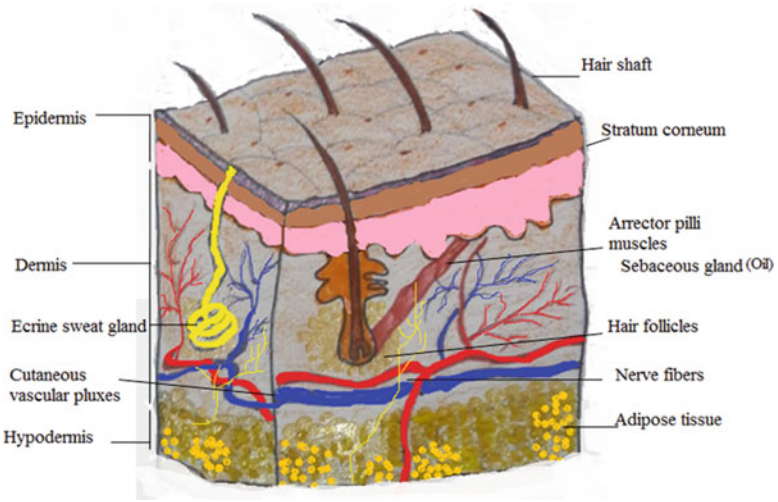

Fig. 3.1 Skin structure

surrounded by a matrix of extracellular lipid (Alexander et al. 2012). In the process of keratinization, keratinocytes undergo cell differentiation and move upward from the innermost layer (stratum basale) to the outermost layer (stratum corneum (SC) or horny layer) passing through the stratum spinosum and stratum granulosum. Upon reaching the SC, cells are eventually sloughed off and become flattened and anucleated. Cells of the viable epidermis that are interspersed in the keratinocytes show some important roles including sensory perception (Merkel cells), melanin production (melanocytes), and immunological function (Langerhans and other cells) (Monteiro-Riviere 2010). Other than the cellular structured components of the skin, some appendages considered as the apocrine glands, eccrine sweat glands, and pilosebaceous units (hair follicles and associated sebaceous glands) are also present to regulate the P^H and temperature (Prow et al. 2011).

The Dermis

The dermis layer is found beneath the epidermis. It is made of structural fibrin and very few cells that look like a normal histological tissue. The thickness of the dermis layer ranges from 2000 to 3000 μm. It consists of loose connective tissue matrix that is fibrous in nature. Below the dermis, another layer is considered as subcutaneous tissue or hypodermis which is made of connective tissue, and it is composed of loose and white fibrous connective tissue containing secretory pores of the sweat gland, blood and lymph vessels, and cutaneous nerves. Many researchers considered that in transdermal drug delivery, drug permeates through the skin and enters into systemic circulation before entering to the hypodermis, where the fatty tissue serves as a depot of the drug (Pathan and Setty 2009).

3.3.2 Skin Barrier Properties

The transport of many drugs to deliver through the skin is slow because of the large lag time to achieve the steady-state fluxes. Therefore, it is difficult to attain the effective therapeutic drug level without improving skin permeation. Consequently, there are many strategies to mitigate the permeability barrier property of the SC. To enhance the transport of a wide range of drugs across the skin, many techniques have been developed. These techniques employ chemical and physical means to increase either skin permeability or apply driving force on drug molecules (Alexander et al. 2012). Due to complex and multiplex properties of mammalian skin, its major roles are to prevent the entry of the foreign substances by providing a defensive barrier to external environment. The skin has enormous defense mechanisms that give physical, metabolic, UV-protective, and immunological barriers to inhibit attacks by toxic chemicals, UV radiation, particulate matter, and microbes. In the other way, the skin can be used as a medium for entry of therapeutic content considered as drugs and vaccines by clarifying the mechanisms of barrier properties (Sinha and Kaur 2000). The purpose of TDDS is to improve drug's ability to invade the barrier of the skin and reach its targeted site. For penetration of drug molecules through the skin, many factors are responsible including species differences, age of skin and its site, nature of the skin (normal or diseased), temperature of skin, contact time period with skin, hydration of the skin, skin pretreatment, and physical properties of the penetrant (Bolzinger et al. 2012). The drug penetration mechanism through the skin is primarily diffusion that is concentration dependent. Many molecules that permeate across the SC follow intercellular, transcellular, and follicular pathways (Alexander et al. 2012).

3.3.3 The Barrier Property of Stratum Corneum (SC)

The SC of the skin acts as an important physical barrier, so that the diffusion and permeation of substance from the skin across the SC show the rate-limiting step. Even for diffusion of water out from the skin, the SC also acts as the main barrier. These protein-rich, anuclear, and flattened corneocyte cells of SC are packed densely within the matrix of extracellular lipid arranged in bilayer form, "bricks and mortar" fashion. The corneodesmosomes are what holds corneocytes together and help in forming the tough outer layer by regular packing and maintaining cellular shape. However, proteolytic enzymes cause corneodesmosome degradation leading to desquamation. Transport of any particles or molecules across the SC mainly occurs by passive diffusion. The brick and mortar structure of the SC along with appendages shows three possible routes for inner transport of entity (Roberts et al. 2002).

- The transcellular
- The intercellular
- The appendageal routes

The corneocytes of the SC surface are covered with a very fine thin and irregular layer consisting of sweat, sebum, bacteria, and dead skin cells.
The intercellular space is the favorable route for most penetrants. Through the intercellular spaces, small molecules move freely, and their diffusion rates largely depend on their lipophilicity and also their physicochemical properties such as molecular weight, solubility, volume, and hydrogen bonding ability. However, large macromolecules and particles are unable to move freely and may be restricted physically through the lipid channel.

3.3.3.1 Skin Turnover as a Barrier

The outer layers of the skin, so-called stratum disjunctum, undergo desquamation, allowing the SC for its turnover in a period of around 14 days depending on the age and anatomical site. Due to which, the cells of SC might move constantly toward renewable barrier and provide an inherent mechanism to prevent foreign bodies to penetrate the skin. This continuous upward migration property and deadness of corneocytes from the upper surface might help in eliminating pathogens, cancerous cells, and solid particulate matter (Marks 2004).

3.3.3.2 Transportation of Exogenous Substances from Stratum Corneum

Originally, it is thought that polar and nonpolar solutes permeate through the SC by different routes. Polar solutes follow the transcellular route, while lipophilic (nonpolar) solutes enter through the intercellular lipids. However, a repeated partitioning between hydrophilic and lipophilic compartments of SC is considered as mechanism in most cases, and that was supported by histochemical evidence showing that intercellular lipids show more diffusion for most of the solutes. Besides the transcellular route showing permeation for lipophilic solutes, its work is still under controversy. Most of the research that focuses on the delivery of drugs or particles through hair follicles, via the appendageal route, is considered as an alternative to delivery through the SC (Scheuplein 1965). These hair follicles extend into the deepest layer of the skin, and the thickness of the SC layer is progressively reduced in this region. Moreover, these follicles are rich in blood capillaries that increase the transport of solutes which diffuse out from the follicle. This route of delivery is considerably increasing interest in follicular targeted delivery with novel drug formulations or nano-bound drugs (Grice et al. 2010).

3.3.3.3 Acidic Nature of Stratum Corneum

For 70 years, the skin surface has been recognized as acidic with pH of 4.2–5.6 in humans which is considered to be influenced by sweat, sebum, sex, hydration, and anatomical site. The pH of skin in the upper layers of stratum granulosum reaches neutrality, providing a sharp gradient across the SC. This acidic pH of the skin has a number of enormous functions including taking action on extracellular lipid processing and organization, maintaining their permeability barrier, having antimicrobial defense, regulating pH-sensitive proteolytic enzyme, protecting corneocyte cohesion and integrity, and preventing inflammation by restricting pro-inflammatory

cytokine release (Wendtner and Korting 2006). Clear evidence is found between diseases and elevated skin pH in the case of atopic dermatitis patients that shows effective pH differences between unaffected and affected skin. Interestingly, penetration of nanoparticles is also controlled by the acidic pH of skin surface that supports the SC barrier property. Nanoparticles of carboxylated polystyrene were shown to aggregate as the lowering of pH of solution decreases their electrostatic forces. Aggregation of particles shows less penetration across the SC. Other than that, embedded particles have less probability to get sloughed off during the periods of desquamation by maintaining SC integrity and cohesion (Prow et al. 2011).

3.4 Overcoming the Barrier of Transdermal Delivery

Since the last 25 years, numerous works have been carried out to sort out the problems related to skin delivery. Due to the growth of technologies, recently many techniques have emerged to deliver drug carrier through dermal or transdermal route. These are divided into passive or active methods for transdermal delivery.

3.4.1 Passive Methods

These are the conventional methods for applying drugs through skin with vehicles including ointments, creams, gels, and patches. Recently these dosage forms are modified in such a way to improve the drug diffusion by enhancing the driving force and increasing the permeability through the skin (Williams and Barry 2004). These approaches include using penetration enhancers, prodrugs or drug metabolites, liposomes, emulsion, and different lipid vesicles. However, delivery of drug with these methods offers limited therapeutic application because of the skin barrier properties that are not changed fundamentally. In the market, many transdermal patches are available for a limited number of drugs. However, these systems do not minimize the physicochemical restrictions of the skin. Vesicular gel system shows dose control improvement and good patient acceptance and compliance compared to patches that encounter problems of poor adhesion and irritancy (Godin and Touitou 2003).

3.4.2 Active Methods

The advancement in biotechnology offers efficient delivery of new generation of large molecular weight therapeutically active, polar, and hydrophilic molecules including peptides and proteins. These materials are degraded extensively by enzymes of gastrointestinal tract on oral delivery. Hence some alternative routes are required for administration of these molecules with suitable delivery systems. These large solutes are incapable to permeate through skin by passive methods, thus necessitating alternative ways known as active methods. These active methods to

enhance permeation through skin entail some external energy which applies driving force to reduce the SC barrier property. Such approaches provide advancement in the potential of transdermal drug delivery system. These technologies progressed recently as a result of development in bioengineering, material sciences, and chemical engineering that helped to create many powerful devices which generate the required clinical effect (Brown et al. 2006).

3.5 Skin Permeability Enhancement by Active Methods

3.5.1 Electrical Method

These methods of delivery utilize electric charge to enhance the diffusion of active molecules across biological barriers and allow permeabilization through skin. These include iontophoresis and electroporation (Helmstadter 2001).

3.5.1.1 Iontophoresis

Iontophoresis enhances the permeation of therapeutic agent applied topically by applying a low-voltage electric current in the skin either directly or through dosage form. The increase in drug permeation is attributed to either one or a combination of mechanisms including electroosmosis (in case of uncharged solutes), electrorepulsion (in case of charged solutes), and electropertubation (in case of both charged and uncharged). Some important parameters that are responsible for iontophoretic delivery system through the skin include type of electrode, intensity of current, pH of the medium, and ion effect. The first iontophoretic device "the PhoresorTM device" (launched by Iomed Inc.) was approved by the Food and Drug Administration (FDA) in the 1970s. The electric current of iontophoretic systems used in humans is reported to be 0.5 mA cm^{-2} that causes irreversible damage to the barrier properties of the skin. Another drawback of iontophoresis is that it is unable to deliver the macromolecules larger than 7000 Da from transdermal route (Guy et al. 2000; Kalia et al. 2004).

3.5.1.2 Electroporation

The method of electroporation causes skin perturbation by inducing high-voltage pulses. It has been reported that electroporation generates transient pores that increase the skin permeability. High voltages (more than 100 V) are frequently employed for short duration of time (milliseconds). Other parameters of electroporation include pulse properties, waveform, rate, and number of pulses which affect delivery (Denet et al. 2004).

Molecules of different sizes and lipophilicity (i.e., proteins and peptides) above 7 KDa in molecular weight have been successfully delivered by this technology through the skin. In vivo it has been shown that the use of electroporation enhances the delivery of DNA through hairless mouse skin, and 100-fold more gene expression was observed compared to intradermal injection. It is documented that the use of iontophoresis and electroporation in combination gives more effective result than

either one of the techniques used alone for molecules delivered across the skin (Zhang et al. 2002).

3.5.2 Mechanical Methods for Transdermal Delivery

These methods use some mechanical means to bypass the barrier of SC.

3.5.2.1 Microneedle-Based Delivery

This transdermal device design is based on microneedle projections with length of 50–100 μm along with drug reservoir that delivers the drug by penetrating the stratum corneum of epidermis. The various layers of membranes used in this device control drug release from its reservoir. The reservoir of this device contains drug in either solution or gel form or may be in solid particulate form. The inventors of this device claim its ability to hinder the barrier nature of SC by the use of projected microneedle and deliver the active molecules at a controlled rate to achieve either local or systemic effect (Trautman et al. 2000).

A recently marketed product of microneedle device is the Macroflux_R developed by ALZA Corporation. The MacrofluxR patch delivers the drug either from drug reservoir or by dry drug coating on the microprojection (Matriano et al. 2002). These microneedles create pores in the skin to allow the easy movement of drug applied topically. Clinical evidence has been reported with minimum discomfort, erythema, and skin irritation (Kaushik et al. 2001). This technology provides advancement in transdermal delivery with ability to deliver medicaments with extreme variation including vaccines, hydrophilic drug, and low molecular weight drugs. In mice, a microneedle device is enabled to deliver gene transfer topically up to 2880-fold greater than topical controls. The microneedle device has been coupled with electrotransport system that provides controlled delivery (Prausnitz 2004).

3.5.2.2 Skin Abrasion Method

Another mechanical method includes abrasion techniques which facilitate the permeation of drug molecules applied topically by involving direct disruption of skin upper layers. These devices are used by dermatologists for removing superficial skin surface and for the treatment of skin diseases including scars, acne, skin blemishes, and hyperpigmentaion. This technique of skin abrasion is not potentially affected by the physicochemical parameters of the drug. The previous work has explained the advantages of this method for controlled delivery of hydrophilic molecule, transdermal vitamin C vaccines, and a wide range of biopharmaceuticals. Mikszta and his co-worker explained the use of one device that comprises an abrasive layer coated with another reservoir layer that is attached with the patch. Sage and Bock filed a patent in which they invented a method of skin pretreatment for transdermal delivery by a device that consists of plastic microneedles to remove the upper portion of SC without piercing the remaining layer; however it causes patient discomfort (Mikszta et al. 2002).

3.5.2.3 Skin Perforation Technique

These devices involved the use of blades or needle-like projections to damage the barrier of the skin by forming cuts and holes to develop a movement in the deepest layer when it comes in contact with the skin. It looks like a microneedle that causes reversible disruption of epidermis and enhances penetration in the epidermal region (Godshall and Anderson 1999). The microprotrusions make cuts in the skin with device movement and after disruption allow passive (patch, gel, ointment) or active (electroporation, iontophoresis,) delivery through the skin. A skin perforating machine made of alternate needle disks and spacers enhances the transdermal permeation (Jang 1998). The movement of this unit forms small cuts on the skin uniformly. It has been reported that the skin perforation effectively facilitates the skin delivery of DNA. An ImprinterTM device used for drug delivery and made of blunt needles has been invented by Imprint Pharmaceuticals. It is a handheld device that accelerates up to 60 mph in 1/20,000 of a second. The rapid application of this device causes no pain and bruising and is able to deliver solid particulates and a wide range of viscous formulations in various layers of the skin and scalp (Crocker et al. 2001).

3.5.2.4 Needleless Injection

It has been reported that needleless injection is a painless method for drug administration via the skin. For many years there have been numerous devices available for both liquid and powder delivery such as Ped-O-Jet_R, Medi-Jector_R, Dermajet_R, and PMEDTM device systems. Later it has been reported that this device is suitable for successful delivery of testosterone, lidocaine, calcitonin, and insulin (Muddle et al. 1997). This delivery system avoids the issues related to pain, fear, and safety in comparison to hypodermic needles. The delivery is achieved by firing of solid or liquid particles with supersonic speeds to outer layers of the skin by using a sufficient energy. The device consists of a helium gas cylinder, drug powder reservoir, and supersonic nozzle along with silencer. In this device, compressed gas (helium) passes through the nozzle with high speed that results in drug particles delegated within the jet flow to travel with sufficient velocity and penetrate the skin. Its use depends on particle size and its morphology, helium pressure, and types of nozzle geometry. Problems associated with needleless injection systems are high cost and it is not suitable for regular administration (Longbridge et al. 1998).

3.5.2.5 Suction Ablation Techniques

By the application of a vacuum or suction pressure form, a blister in the epidermis is removed. This method is also called as skin erosion by removing the skin barrier. This technique of delivery provides protection from bleeding and pain. One commercial patch, cellpatch_R (Epiport, Sweden), is available based on this approach. It is made of suction cup, reservoir, and epidermatome (to make blister on the skin) and is used to deliver morphine through the skin. In vivo study of this patch shows that the plasma levels of morphine were found comparable to intravenous infusion.

It has been also demonstrated that in vivo transdermal delivery of antidiuretic vasopressin achieved 100% bioavailability compared to direct intravenous infusion.

It has been found that epidermis removal by suction causes hyperemia as confirmed by microscopy. The authors explained that hyperemia may also enhance permeation. The drawback with this suction method is that it takes prolonged time to develop blister (2.5 h), and chances of epidermal infections cannot be ignored (Svedman et al. 1996).

3.5.2.6 Skin Stretching Techniques

The device under skin stretching techniques is designed in such a way that skin is stretched in multidirectional manner to enhance the permeation. It applies the tension of ~0.01 to 10 mP to develop reversible micro-pathways and to facilitate the diffusion across the SC. Removing this tension allows the skin to return back to its original feature. The delivery of small peptide around 1 kDa was monitored by the help of microprotrusion array on hairless skin of guinea pigs and illustrates the efficiency of stretching process. The results of this study examined that the stretching of the skin in bidirectional way opens the delivery pathway after microprotrusion piercing. The delivery device used for stretching method is based on electrotransport, osmotic pressure, and passive mechanisms (Cormier et al. 2001).

3.5.2.7 Ultrasound (Sonophoresis and Phonophoresis)

Sonophoresis or phonophoresis uses ultrasound with the help of ultrasonic energy to improve the transdermal delivery of drug solute. Different parameters of ultrasound required for percutaneous absorption are frequency and intensity of ultrasonic energy and its treatment duration. The frequencies of ultrasonic energy required to improve skin permeation have been reported between 20 kHz and 16 MHz. The frequencies of up to 100 kHz are reported to show significant effect on delivery of macromolecules (48 kDa) on transdermal delivery. The proposed mechanism to increase the skin permeability involved in sonophoresis and phonophoresis is formation of gaseous cavities in between the intercellular lipids on exposure to ultrasound which causes disruption in tight junction of SC. This technique causes reversible disruption in human skin and promotes the delivery of insulin and water. There is one commercial device SonoPrep_R (Sontra Medical Corporation) with frequency of 55 kHz used to enhance the permeability of the skin. This device reduces the onset of action for about 15 s and promotes the dermal delivery (Mitragotri et al. 1996).

3.5.2.8 Laser Radiation and Photomechanical Waves

In dermatological science, laser treatment is used frequently for treatment of acne and facial rejuvenation to damage the target cells with the help of laser radiation in short time periods (~300 ns). Direct exposure to laser radiation in controlled way on the skin surface causes ablation of SC significantly without harming the underlying layer of the epidermis. It enhances both the lipophilic and hydrophilic drug molecules' delivery by removing the SC. Some parameters including pulse nature like its length, energy, number, repetition rate, and wavelength affect the barrier disruption. Laser treatment offers many advantages in transdermal therapy including safe removal of tissue in a controlled way, less treatment time, painless, and with

minor adverse effects. For the last few decades, laser treatment has been used clinically, and its effects on biomembranes are well known. One portable laser device was invented by Norwood Abbey Ltd. (Victoria, Australia). In this study they considered human volunteers and used Norwood Abbey laser device to find the action of lidocaine in 3–5 min, while the control group attained similar effect in 60 min. Intense laser radiation generated by pressure waves causes direct ablation on the skin and is able to enhance the skin permeability. Pressure waves avoid the problems related to direct laser radiation (Jacques et al. 1988). It is realized that pressure waves create continuous hydrophilic pathway through the skin by expansion of SC. It has been reported that along with sodium lauryl sulphate, pressure waves show synergistic effect. The molecules delivered successfully include insulin, 40 kDa dextran, and 20 nm latex particles (Doukas and Kollias 2004).

3.5.2.9 Magnetophoresis

In this technology, a magnetic field is applied to develop external driving force that enhances the permeation of diamagnetic solute through the skin. Exposure of the skin to magnetic field brings the structural alterations and increases its permeability. Murthy et al. observed that in vitro increment in flux rate of benzoic acid induced magnetically increases with the strength of magnetic field. This technique is only used for diamagnetic materials which serve as limiting factor in application (Murthy and Hiremath 2001).

3.5.2.10 Thermophoresis

The method involves the use of elevated temperature on the skin surface to enhance the percutaneous absorption. Recently, there is some interest in improving the topical medicament delivery. Normal skin temperature is maintained at 32 °C by the human body homeostatic functions. In vitro studies revealed that transdermal flux increased 2–3-fold for every rise in surface temperature of up to 7–8 °C. The heat improving effect helped in increasing drug diffusion of dosage form by increasing the lipid fluidity of the stratum corneum (Clarys et al. 1998). In vivo results demonstrate that increasing temperature of the skin surface results in increasing blood supply which plays an important role in improving the delivery of topically applied drug. Previous work demonstrated the in vivo delivery of drugs such as nitroglycerin, lidocaine, testosterone, fentanyl, and tetracaine through transdermal patches along with heating devices. However, the elevated temperature does not enhance the delivery of molecules over 500 Da (Brown et al. 2006).

3.5.2.11 Radio Frequency

Radio frequency has been used extremely to cause thermal ablation of malignant tissues. This involves exposure of the skin to high-frequency alternating current of approx. 100 kHz , which creates heat-induced microchannels in the epidermal layer. This transdermal delivery method has been practically developed by TransPharma Ltd. to design a ViaDerm TM device. It is an electronic device that contains microprojection of 100 microelectrodes/cm^2 and a drug reservoir. This electronic device when kept on the skin exposes radio frequency from its microelectrode array

and facilitates the microchannels, through which the drug reservoir releases drug in treated area. Treatment time for this delivery method is less than a second and development of microchannels increases its reproducibility. Delivery of granisetron HCL in rats experimentally shows that it enhances the blood plasma level up to 30 times in 12 h than untreated skin. This device does not cause any skin damage with the radiofrequency-induced microchannels that return to original feature within 24 h (Sintov et al. 2003).

3.6 Chitosan

Chitosan is the most promising natural polysaccharide and is obtained as the second large biomaterial after cellulose. Naturally it is prepared from chitin which is found as the structural element from exoskeleton of crustaceans including crabs, lobsters, and shrimps. Chitin is a natural polymer containing 2-acetamido 2-deoxy-β-D-glucose linked through a β (1-4) linkage. Chitin like cellulose functions as structural polysaccharide (Paulino et al. 2006). Natural production of chitin is inexhaustibly obtained from 10^6 species of arthropods constituting a permanent large biomass source. It is an inelastic, hard, and white nitrogenous polysaccharide that is the major surface pollutant in coastal areas. Chitin is isolated from crustacean's exoskeletons particularly from crabs and shrimps to produce α-chitin. Another important source of chitin is squid that produces the β-form which is more prone for deacetylation (Pawadee et al. 2003). β-chitin shows high solubility and reactivity along with more swelling than α-chitin because of the weaker intermolecular hydrogen bonding. Many researchers face a problem in extraction of chitin from natural sources to obtain useful material chitosan (Abdou et al. 2008).

Chitosan is also found naturally in fungi and yeast. Although structurally chitosan looks similar to cellulose with some additional group including acetyl amine group, hydroxyl groups, and free amino groups, it shows entirely different properties from cellulose. Nowadays, chitosan has achieved more attention because of its important biological activity and applications in agriculture, medicine, and food industries (Elgadir et al. 2015). It has been estimated that there are more than 200 potential applications of chitosan and its derivatives. It has a wide range of applications in cosmetics, agriculture, food, and biomedical domians. The chitosan production from crustacean shells is economically feasible. The shells of crustacean contain carotenoid astaxanthin, which is used in aquaculture as a fish food additive. Chitosan is also produced directly from fungi by chemical method. Some scientists have invented these methods to deproteinize the crustacean chitin with the help of proteolytic enzyme microorganisms for more economic production of chitin and chitosan (Chassarya et al. 2005). The normal procedure is deacetylation of chitin by alkaline solution which helps in obtaining chitosan. Isolation of chitin from different sources is also important based on crushing raw material and washing with detergent and water (Abdou et al. 2008).

3.6.1 Physicochemical Properties of Chitosan

Chitosan is structurally identified as a heteropolysaccharide composed of linear binary β-1,4- glucosamine linked with N-acetylation. It is procured from chitin by its deacetylation with the help of concentrated sodium hydroxide (NaOH) solutions for a certain period of time at high temperatures. Another method for the chitosan production is N-deacetylation with enzymes under optimum conditions. Commercially, chitosan is easily obtained by alkaline N-deacetylation from chitin of crustaceans (Elgadir et al. 2015).

3.6.2 Isolation of Chitosan

The chitosan production involves a two-step process as shown in Fig. 3.2 and discussed below:

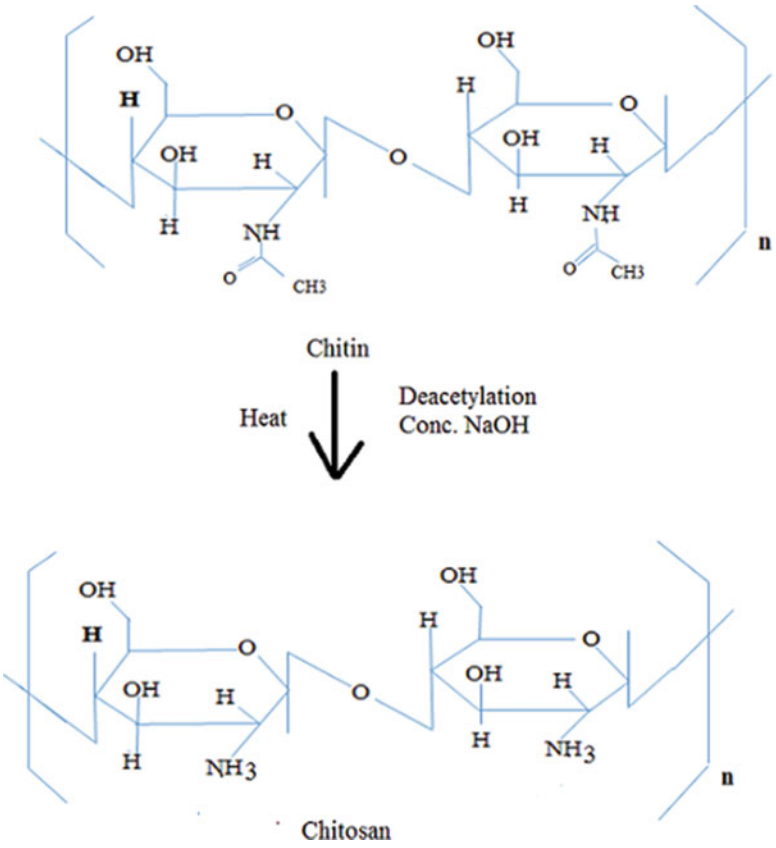

Fig. 3.2 Synthesis of chitosan from chitin

Step 1: Extraction of Chitin
The sources of isolation of chitin are shrimp shells, squid pens, crab shells, and lobster. The obtained raw materials in solid form are washed with water and dried at room temperature and then cut in small pieces. Demineralization of raw material is performed using 1 M hydrochloric acid bath at room temperature. The next step is deproteinization, which is performed with alkaline treatments of 1 M sodium hydroxide solutions maintained at 105–110°C. This process is repeated many times until the protein gets removed and solution becomes clear at the end stage. In the next step, washing is carried out with distilled water to achieve their neutrality after that sample was dried. Chitin obtained from squid pens shows a white color, while the one collected from other sources shows a pink color. Mild oxidizing agent (including KMnO4 + oxalic acid + H_2SO_4) is used to remove any trace of pigments. The remaining protein and color are also eliminated from raw materials by refluxing in ethanol (Kurita 2001; Abdou et al. 2008).

Step 2: Deacetylation of Chitin
Deacetylation of chitin is a preliminary experiment performed by refluxing with a strong sodium hydroxide solution at normal atmospheric pressure. This step takes time up to 20 h to perform deacetylation to obtain the final chitosan. This step takes a long time in deacetylation to achieve the chitosan. Another method can be applied to avoid lengthy heating times by refluxing with alkaline solution in an autoclave maintained at two different pressures. This process requires heating for 10–15 h to obtain the resulting chitosan. It has been found that deacetylation of chitin can be highly attained by treating with concentrated sodium hydroxide solution before heating at room temperature (Abdou et al. 2008). Deacetylation of chitin has been done with 40–50% of aqueous NaOH at temperature of 110–115 °C for several hours in the absence of oxygen. Chitosan is produced when the degree of deacetylation of chitin exceeds 50%. Around 75% of deacetylated chitin is usually identified as chitosan (Prashanth and Tharanathan 2007).

The function and properties of chitosan depend upon two fundamental parameters including degree of deacetylation and molecular weight. Chitosan is insoluble in water and alkaline solutions but soluble in aqueous acidic solutions using glacial acetic acid. Usually many of the polysaccharides show neutral or negative charge in acidic medium, but chitosan shows positive charge by conversion of amino groups ($–NH_2$) of the glucosamine protonated to $–NH_3^+$. This cationic polyelectrolyte shows electrostatic interactions with other anionic groups. Therefore, cationic chitosan shows bonding with negatively charged entity of the biological membranes which explains the unique functional properties of chitosan (Muxika et al. 2017; Elgadir et al. 2015).

3.6.3 Chitosan Derivatives

The property of chitosan has been changed by bringing modification in its backbone to improve its solubility, stability, and mucoadhesion (Fig. 3.3). In the backbone of

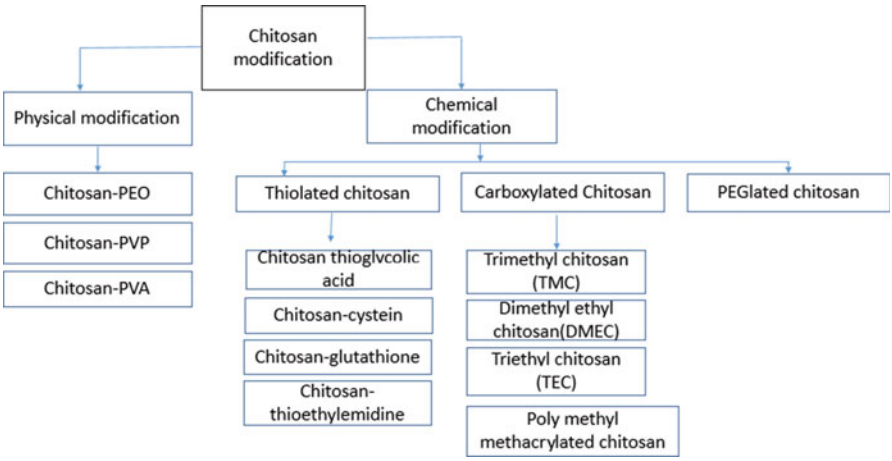

Fig. 3.3 Diagrammatic presentation of chitosan and its modification

chitosan, the –NH2 and –OH groups are the active sites that are responsible for modification. Some important techniques used to bring modification in chitosan polymers are blending, curing, and graft copolymerization (Shukla et al. 2013).

The process of blending involves the simple blending or mixing of two polymers. In case of graft copolymerization, there is covalent bond attachment of two polymers. However, curing changes the combined polymers into a solid mass by making three-dimensional bonds between the polymers by application of thermal, ultraviolet radiation, and electrochemical process.

3.6.3.1 Physical Modification of Chitosan

Physical modification of chitosan involves a simple blending process. It is the oldest, easiest, and most economical technique for polymer tailoring to specific applications. The ratios of the polymers used in the process of blending affect the quality and performance of modified polymer (Park et al. 2001). Some common hydrophilic polymers that have been used in the blending process along with chitosan include poly(ethylene oxide) (PEO), polyvinylpyrrolidone (PVP), and polyvinyl alcohol (PVA). Blending of chitosan along with PVA improves the tensile strength and permeability property of chitosan. Chitosan forms intermolecular interactions with PVA in the process of blending to form PVA-chitosan that has been used for controlled drug delivery (Risbud et al. 2000).

3.6.3.2 Chemical Modification of Chitosan

Chitosan modification by chemical method involves alteration in the functional groups of compound. There are several ways to do chemical modification which include enzymatic and photochemical plasma-induced grafting and chemical and radiation methods (Shukla et al. 2013).

Several derivatives of chitosan are prepared after chemical modification that results in formation of thiolated chitosan, carboxylated chitosan, quaternized chitosan, amphiphilic chitosan, lactose-modified chitosan with chelating agents, and PEGylated chitosan (Furlani et al. 2017).

The reaction site for chitosan is primary amine (–NH2) groups that provide chemical modification by reacting with phosphates, sulphates, and citrates to achieve many applications. This modification enhances drug entrapment inside the polymer and the stability of formulation. N-Trimethyl chitosan (TMC) is a quaternized chitosan which has been produced by chemical modification and enhances the transdermal permeation of hydrophilic molecules (Thanou et al. 2000).

The mucoadhesive property of chitosan nanoparticle has been imparted by thiolated chitosan. Several derivatives of chitosan are prepared by quaternization process including N-trimethyl chitosan (TMC), dimethylethyl chitosan (DMEC), and triethyl chitosan (TEC). Quaternization of chitosan helps in opening the tight junctions and improving permeability through the skin surface. Presently used chitosan in many research works are thiolated chitosan derivatives including chitosan-thioglycolic acid, chitosan-cysteine, chitosan-glutathione, and chitosan-thioethylamidine. Higher mucoadhesion and permeation of TMC-cysteine nanoparticles have been reported compared to TMC-NPs. The pH-sensitive properties can also be achieved by grafting the carboxylated chitosan with poly (methyl methacrylate) (Sadeghi et al. 2008).

3.6.4 Therapeutic Properties of Chitosan

Chitosan has enormous therapeutic properties that have been reported by many researchers including growth inhibition of microorganisms, pain alleviation, hemostasis promotion, and epidermal cell growth. However, its potential applications are also found in medical and pharmaceutical research. The reason for the increasing chitosan interest in pharmaceutical area is due to its favorable attributes such as biocompatibility, bioadhesivness, and biodegradability. These properties of chitosan make it amenable in controlled and targeted drug delivery along with wound healing, cartilage, and tissue engineering.

From the past few years, chitosan has achieved much more attention as an important excipient for drug delivery in biomedical applications due to its physicochemical and biological properties, recognizing chitosan as a promising material for drug delivery (Muxika et al. 2017). In this aspect, chitosan-based drug delivery systems have been explored from microparticles to nanoparticles and also for different routes of delivery. However, there are many drawbacks available with the use of chitosan in drug delivery (Howling et al. 2001). The main reasonable drawback is its poor aqueous solubility at physiological pH because of their partial protonation of amino groups that result in pre-systemic metabolism of drugs. These drawbacks of chitosan were overcome by the use of its different derivatives including thiolated, carboxylated, and acylated chitosan in different drug delivery systems (Elgadir et al. 2015).

3.7 Important Properties of Chitosan

3.7.1 Controlled Drug Release

Controlled release for cationic drugs can be attained by coating a number of anionic polymers such as alginate, polyacrylates, and sodium carboxymethyl cellulose. But in the case of anionic drugs, chitosan is only one choice of polymer to design the sustained-release systems using chitosan as a drug carrier matrix (Tapia et al. 2005). Chitosan forms the stable complexes from which the drug can be released for a prolonged time period. In the case of chitosan nanoparticle delivery, it provides very stable complexes and significantly improved the drug uptake (Bhise et al. 2008). In addition, chitosan has the ability that it can be homogenized with another anionic polymer such as alginate, carrageenan, polyacrylates, and hyaluronic acid resulting in formation of very stable complexes, from which incorporated drugs are released in a sustained manner by the process of erosion and diffusion from these complexes. In the case of polyvalent inorganic anions including sulfate or tripolyphosphate, cationic polymers show the same effect as that of anionic polymers.

3.7.2 Mucoadhesive Properties

The mucoadhesive properties of chitosan are mainly based on their cationic nature. The mucus of mucosa layer shows sulfonic acid and sialic acid like anionic substructures. When chitosan is applied on mucosal layer, its cationic amino groups form strong electrostatic interactions with anionic substructures of the mucus, and a strong mucoadhesion can be obtained (Grabovac et al. 2005). In addition, its hydrophobic interactions also explore its mucoadhesive properties. However, the mucoadhesive properties of chitosan are weak in comparison to various anionic polymers including carbomer, hyaluronic acid, and polycarbophil. Furthermore, to achieve more mucoadhesive properties, the polymer should show high cohesiveness in the form of adhesive bond between the mucosa layer and polymer. Chitosan has comparatively weak cohesive property that can be enhanced by forming its complexes with polyvalent anionic polymer, anionic drugs, and inorganic anions, but formation of this complex is quite limited by hindering the cationic character of chitosan that is responsible for mucoadhesion with the mucus. Cationic character of the chitosan polymer can be enhanced by trimethylation of its primary amino group. PEGylation of trimethylated chitosan (TMC) additionally increases its mucoadhesive properties up to 3.4-fold (Jintapattanakit et al. 2009). Mucoadhesive properties of chitosan can also be strongly improved by immobilization of thiol groups because thiolated polymer is able to form disulphide bonds with glycoproteins of the mucosal layer. Thiolated chitosan substantially enhances cohesive properties by forming intra- and interchain disulfide bonds within the chitosan itself (Werle and Bernkop-Schnürch 2008).

3.7.3 In Situ Gelling Properties

From a formulation point of view, the pH-dependent hydrate ability of chitosan enhances its in situ gelling properties. Gupta et al. combined chitosan and polyacrylic acid to develop in situ gelling delivery system. The prepared formulation was present in liquid state at pH of 6.0 and at physiological pH of 7.4; it underwent rapid phase transition from liquid to viscous gel phase (Gupta and Vyas 2010). Thiolation can also improve the in situ gelling properties of chitosan. Application of chitosan solution on oxygen-rich nasal and ocular mucosa enhances their viscosity strongly by forming disulfide bond via cross-linking process (Sakloetsakun et al. 2009).

3.7.4 Transfection Enhancing Properties

Much like the small molecules, chitosan can also be used for delivery of large polyanionic molecules such as DNA-based drugs and RNA by forming a stable complex. In this complex, nanoparticles show a positive zeta potential by using high ratio of the cationic chitosan polymer. Delivery of these particulates can be achieved by the mechanism of endocytosis in particular because of the net positive charge and small size of these particles which is below 100 nm in size (Mao et al. 2010). Toxicity of chitosan is less compared to other cationic polymers including polyethyleneimine, polyarginine, and polylysine. Therefore, it is considered as a promising excipient for gene delivery. The bioavailability of DNA-based drugs delivered into the body can be improved by forming chitosan-DNA-based drug complexes that show protection against degradation by DNAses (Lee and Mohapatra 2008). Conventional chitosan generally shows less transfection efficiency so its properties can be improved by bringing certain modification in polysaccharide. Some researchers find that the self-branching in chitosan structure enhances its gene transfer properties and also shows that self-branched chitosan enhances the gene expression levels by two and five times higher (Martien et al. 2007). Another approach is the thiolated chitosan forms the intra-chain disulfide bonds and promotes the stability against nuclease. This property can be further improved by trimethylation of thiolated chitosan to raise their cationic character. Furthermore, chitosan/cyclodextrin nanoparticles and PEGylated chitosan were considered as efficient tools for DNA-based drug delivery (Malhotra et al. 2011).

3.7.5 Permeation Enhancing Properties

One of the important mechanisms of chitosan is its permeation enhancing effect due to its positive charges that interact with cell membrane and bring structural change in tight junction proteins. The more cationic character is achieved after trimethylation of the primary amino group of chitosan which enhances the permeation properties. Chitosan with high molecular weight and higher process of deacetylation

comparatively shows high rate of epithelial permeability (Kotze et al. 1998). A high degree of deacetylated chitosan shows maximum permeation with minimal toxicity. A combination of chitosan with polysaccharide shows synergistic effect and enhances bioavailability of drug with 2–4-fold permeation across the mucosa. Recent research has shown that addition of cyclodextrin along with chitosan nanoparticles enhanced the permeation effect throughout the entire duodenum. However, thiolation of chitosan enhanced the permeation properties up to 30-fold through certain mucosal membranes (Kast and Bernkop-Schnürch 2002).

3.8 Transdermal Application of Chitosan

Nowadays, polysaccharide nanoparticles are getting much more attention in transdermal drug delivery. The list of polysaccharides includes β-cyclodextrin, carboxymethyl cellulose, chitosan, and propyl starch as a matrix constituent for drug delivery; shows biodegradable, biocompatible, and mucoadhesive properties; and possesses mucosal permeation. These polymers are nontoxic and have the ability to interact with the skin to fluidize the lipid layer of epidermis and to promote drug diffusion through transdermal route.

Among all the polysaccharides, chitosan has emerged as a favorable biopolymer in drug delivery system. Chitosan shows distinctive polycationic character. It shows strong mucoadhesive property by interacting with negatively charged entity of skin moieties. It is reported that chitosan is biologically active as anti-infective, antidiabetic, anticancer, and antihyperlipidemic agent (Taveira et al. 2009). Chitosan as a nanoparticulate matrix improves the transdermal drug delivery and shows synergistic therapeutic action for local disorders including skin malignant melanoma or infection and systemic complications like hyperlipidemia and diabetes (Nawaz and Wong 2009). Transdermal delivery of many drugs with polysaccharide chitosan could be improved by its mucoadhesive properties and positively charged nature. Several researches have showed that drug-loaded chitosan nanoparticles improved the transdermal delivery. Chitosan shows many important properties, such as bioadhesion, biodegradability, and nontoxicity. Chitosan and its modified forms promote the transdermal delivery in many studies.

3.8.1 Penetration Mechanism of Chitosan Through Transdermal Route

Previous research illustrates the penetration enhancing mechanism of chitosan through skin surface by loosening the tight junctions of the SC of epithelium. It facilitates the delivery of drug molecules by both paracellular and transcellular transport of drugs (Mohammed et al. 2017).

Chitosan and its derivatives carry a positive charge which interacts with negatively charged cell of the SC and provide successful transdermal delivery by widening the keratin layer of SC. It has been reported that hydrated chitosan

shows penetration by hydrating the stratum corneum. Some other reports also show the delivery of chitosan-based formulation through hair follicular appendageal route. Below, several formulations of chitosan and their derivatives are shown to have different mechanisms to deliver the bioactive molecules through transdermal route (Abdel-Hafez et al. 2018).

3.8.2 Drug Release from Chitosan Nanoparticles

Chitosan nanoparticles show drug release by several mechanisms including polymer swelling, drug diffusion from chitosan matrix, erosion or degradation of chitosan polymer, or by creating pores (Fig. 3.4). Initially, the drug shows burst release through chitosan nanoparticles due to swelling of chitosan polymer causing the drug to diffuse over the surface (Liu et al. 2017). The solubility of chitosan depends on pH due to which it shows pH-dependent release of drug. Derivatives of chitosan change the release rate of drug from nanoparticles, show effective drug release, and also affect the pharmacokinetic parameters of the loaded drug. In diffusion-controlled mechanism, the drug permeates from the interior region of polymeric matrix to the outer surrounding medium (Yuan et al. 2013). The chains of chitosan form the barrier to create difficulty in passing of drug and to maintain the rate-limiting drug release from chitosan membrane. Diffusion of drug is usually related with swelling or erosion of chitosan polymer. That can be shown by Fick's law of diffusion.

The chitosan swelling is represented by absorbance of water in polymeric matrix until it gets dissolved. The release mechanism of the drug is characterized by the polymeric solubility in surrounding water or biological medium (Siafaka et al. 2015). When the polymer swells in surrounding medium, polymer chains detangle which is followed by drug release from swelling region of the chitosan polymer

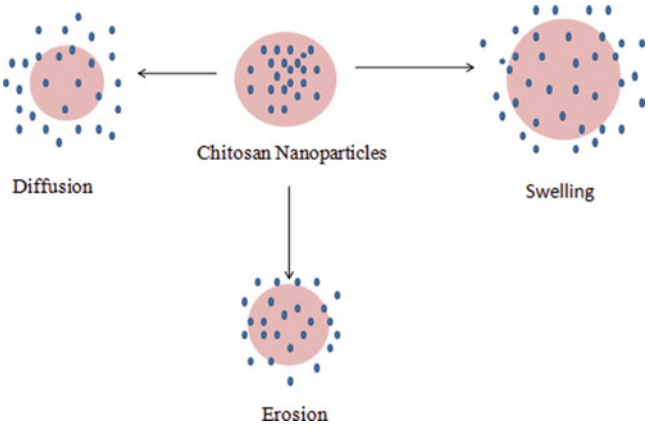

Fig. 3.4 Drug release from chitosan nanoparticles

matrix. The resultant study showed that the drug release mechanism depends on the polymer hydrophilicity, the swelling rate of polymer, and the polymer chain density. Alternatively, it will depend on the rate of drug absorption from the in vivo site of delivery that will affect the drug availability for cellular uptake (Siepmann and Siepmann 2012).

Other interrelated features of polymers are erosion and degradation. Sometimes, polymer degradation involves physical erosion by breaking of bonds. Polymer erosion presents a complex phenomenon involving swelling, dissolution, and diffusion. The phenomenon of erosion shows either homogenous or heterogeneous way of erosion. Homogenous erosion shows erosion of polymer throughout the matrix at the same rate, while heterogeneous erosion involves polymer erosion from the outer surface to the inner core (Fonseca-Santos and Chorilli 2017). The reason behind polymer degradation may be the surrounding media, pH, or the enzymes present in cellular media. The polymer degradation also depends on the copolymer bonding and water uptake of polymer. The release of drug depends on the polymer type and its internal bonding and derivatives of chitosan. It also depends on size and shape of the nanoparticles that affect the surface area and its free energy (Pawar et al. 2013).

Generally, chitosan nanoparticles show pH-dependent drug release mechanism. One study has proposed exendin-4-loaded chitosan-PLGA nanoparticles for transmembrane permeability through MDCK (Madin-Darby canine kidney) cells in male Wistar rats. Generally, exendin-4 is used to treat type 2 diabetes. The in vitro permeability studies explained that exendin-4 showed more permeability through cell layer by active transport loaded in chitosan-PLGA nanoparticles compared to having a drug solution. The permeability coefficient of chitosan-PLGA nanoparticles shows significantly greater permeation for exendin-4. The positively charged nature of chitosan-PLGA nanoparticles forms electrostatic interactions with negatively charged cell membrane representing higher partition coefficient (Manca et al. 2008; Mohammed et al. 2017).

3.9 Chitosan-Based Formulations for Transdermal Delivery

Various chitosan-based formulations for transdermal drug delivery are presented and discussed below.

3.9.1 Trimethylated Chitosan Nanoparticles

Trimethylated chitosan (TMC) is frequently used in transdermal studies because of its stability, well-defined structure, high penetration, and better drug absorption from the skin and mucosa. It has been found that in nasal delivery, TMC nanoparticles increase the subunit antigens' immunogenicity. In the same way, TMC nanoparticles also enhance the delivery of drug through transdermal route. The investigated mechanisms suggest chitosan and their derivatives promote the transdermal delivery by loosening the dense structure of keratin of stratum corneum and expanding the

tight junctions, or its net positive charge gets combined with negatively charged entity of the stratum corneum (Smith et al. 2004).

It has been hypothesized that chitosan polymers also enhance the transdermal delivery of protein. TMC nanoparticles for delivery of protein drugs were prepared by ionic cross-linking method. This method is reported to maintain the efficiency of protein drugs. The advantage of this method of preparation for chitosan-based nanoparticles is that it does not require any extra organic solvents that may be harmful to human health (He et al. 2009). TMC-NPs also present excellent stability and its size is significantly maintained for 30 days at 4 °C. Tu et al. showed that TMC-NPs significantly increased the skin permeation of proteins compared to free protein drugs. It is very purposeful that TMC-NPs can promote the delivery of proteins through transdermal route with greater extent which indicates that TMC-NPs show deep penetration in the skin. The penetration mechanism proposed for TMC-NPs is that it may extend the dense structure of keratin in the SC, loosen the tight junctions in the skin, and perforate the skin through hair follicles (Tu et al. 2016).

3.9.2 Chitosan/Cyclodextrin (CS/CD) Nanoparticles

However, the hydrophilic nature of chitosan has limited use for delivering only hydrophilic drugs. Currently, new nanocarriers including CS/CD were synthesized to successfully encapsulate hydrophobic drugs with the help of CDs (Grenha et al. 2008). The CS/CD nanoparticles show the advantages of high solubilization and good permeability to enhance the bioavailability of hydrophobic drugs. CDs make the hydrophobic cavity at the center and hydrophilic area in the outer layer to encapsulate substrates and form guest host complexes. Formation of this complex results in modulation of properties of drug as guest molecule by increasing its solubility and enhances their physicochemical stability along with absorption enhancing effect (Loftsson and Brewster 1996). Further some researchers have explained drug release enhancement and sustained drug release in systemic route through vehicles. Due to low cost and availability, CD is extensively used. The most popular method of formation of CD/CS polymer nanoparticles is previous incorporation of drug-cyclodextrin inclusion complex into chitosan nanoparticles. In this complex, CDs increase the loading efficiency of lipophilic drugs (Ceschel et al. 2003). For transdermal delivery, the key factor to design dosage is to enhance the loading of drug in the vehicles and improve permeation via the skin without any sensitization. Formation of CS/CD complex provided benefit for transdermal delivery because chitosan acts as a biocompatible penetration enhancer and controlled release polymer in nanoparticle, while CD acts as a solubility enhancer that hikes solubility and stability of hydrophobic drugs, e.g., warfarin (Khalil et al. 2012).

3.9.3 Chitosan-Based Hydrogels for Transdermal Delivery

Chitosan hydrogels are hydrophilic cross-linked polymers capable of absorbing water in large quantity by forming a gel-type structure. They act as a carrier matrix for delivery of drug through transdermal route. The nature of hydrogels is that they are soft and smooth and have high adhesion, and drug carrier gel entails penetration through the stratum corneum by hydrating and loosening the compact keratin layer. Chitosan-based hydrogels are also fabricated along with other polymers (polyvinyl alcohol (PVA)) to enhance their high hydrophilicity and elasticity. Chitosan-PVA blend polymer hydrogels are suitable for controlled release with low toxicity and high biocompatibility. The nano-insulin chitosan-PVA-based hydrogels were used for delivery of nano-insulin through transdermal route to provide controlled release of insulin and maintain its systemic circulation. The transdermal delivery of insulin can protect the drug from pre-systemic metabolism and hepatic first-pass effect with sustained release. The in vitro result shows that insulin release from hydrogel is suitable for transdermal drug delivery in diabetes therapy. This hydrogel entails elongation and higher tensile strength along with flexibility and better deformability by forming strong physical interactions between the chains of two polymers which are suitable for transdermal delivery. The morphologies of prepared mixed polymer hydrogel indicated porous honeycomb-like structure showing high accessibility of water in the porous region of hydrogel (Zu et al. 2012).

Chitosan-based hydrogel-thickened nanoemulsions have also been prepared in which chitosan was used as a thickening polymer to enhance the viscosity of nanoemulsions. Nanoemulsions are translucent colloidal vesicles that favor penetration of drug in different layers of the skin, being suitable for transcutaneous delivery. However, low viscosity of nanoemulsions is a limitation in general application on the skin, which can be overcome by incorporating nanoemulsion in chitosan-based hydrogel that is suitable to enhance topical application. In hydrogel-thickened nanoemulsions nanometric micelles are dispersed in three-dimensional network of hydrogel. The increase in viscosity of nanoemulsions improves its stability against flocculation and coalescence. In addition, hydrogel-thickened nanoemulsions also show controlled release of drug and optimize therapeutic effects. The reason for improvement in therapeutic effects is related to adhesive property as it increases the residence time of drug at the target site which favors the intimate contact of emulsion with the skin (Barradas et al. 2018).

3.9.4 Chitosan Nanoparticles

Chitosan nanoparticles are capable of significantly enhancing transdermal drug absorption by binding with negatively charged entity of epithelial cell membranes and tight junctions in which its positive charges show depolymerization of F-actin and opening the tight junctions (Jana et al. 2014). Chitosan yields effective skin permeation in different formulations including transdermal films, membranes, patches, micro-gels, nanogels, nanofibers, and nanoparticles. Chitosan enhances

the water content level in stratum corneum, modifies the accessory structure of keratin, decreases the potential of cell membrane, and enhances the fluidity of cell membrane (Budhian et al. 2007). Polymeric chitosan nanoparticles enhance the penetration, absorption, and bioavailability of drugs, protect the drugs from premature metabolism, and control the release rate of drugs. Previous studies report the potential role of nanoparticles through transdermal application. Nanoparticles of less than 500 nm in size show better penetration through the skin. This small size of nanoparticles makes close contact with stratum corneum and increases the permeation of drug inside the skin. The follicular route of skin appears as a key for penetration pathway in case of nanoparticulate drug delivery. For a long time, it was believed that the appendageal route shows less contribution in transdermal penetration as compared to intercellular route because skin appendages occupy only approximately 0.1% of total skin surface area (Alvarez-Roman et al. 2004). However, later research suggested that hair follicles deeply extend into the dermis and show more permeation by significantly increasing the definite surface area. It was also found that the hair follicles are encircled with dense network of blood capillaries, and it was proposed that the stem cells of hair follicle are responsible for regenerative medicine. It is reported that nanoparticulate systems deeply penetrate the hair follicles more efficiently. In the case of topical delivery, the hair follicles act as long-term reservoirs in variance to stratum corneum of the skin, where the chances of drug depletion are more because of textile contact washing or desquamation. The storage time of nanoparticles was found to be 10 days, compared to 4 days in the case of non-particulate systems (Hafez et al. 2018).

3.9.5 Chitosan Nanocapsules

Chitosan nanocapsules have been developed for treatment of cancer and many infectious diseases and for vaccine delivery through transdermal route because chitosan is able to open the tight junctions of epithelial cells of stratum corneum and improves permeation for many medicines, hormones, and immune response molecules. Bussio et al. fabricated chitosan nanocapsules for transcutaneous vaccination. The size of nanocapsule is an important parameter for transcutaneous penetration. The structural morphology of chitosan nanocapsule is an oil-core structure encapsulated by shell of chitosan in nano- range. In the above study, workers used ovalbumin as a model antigen loaded in oil core of nanocapsule. Their ex vivo studies prove that the new nanocapsules are able to penetrate and retain in the skin. The method of preparation for chitosan nanocapsules was same as that for nanoemulsion but 0.05% w/v of chitosan solution in place of water. The prepared nanocapsules show shell-nucleus structure which allows keeping active molecules in the core that are surrounded by shell of chitosan. The rational approach of nanocapsule for transcutaneous delivery considers smaller particle size for delivery of carrier. For delivery of antigen, smaller-size chitosan nanocapsules are considered necessary for transdermal approach. In this experiment, chitosan was

chosen as the corona because of its proven action as an adjuvant and penetration enhancer (Busio et al. 2018).

3.10 Conclusion

Chitosan is one the most important polysaccharides which has been extensively used in drug delivery technology because of its good adhesive properties on biosurfaces. Cationic amino groups in chitosan structure form strong electrostatic interactions with anionic biomembranes. It shows strong mucoadhesive property by interacting with negatively charged entity of skin moieties. Chitosan has been aptly modified for specific motives. Accordingly, N-trimethyl chitosan (TMC), a quaternized chitosan prepared by chemical modification, enhances transdermal permeation of hydrophilic drugs. Deacetylation of chitosan offers maximum permeation with minimal toxicity. Thiolated chitosan provides better skin and mucoadhesive potential compared to the parent compound. Chitosan facilitates the delivery of drug molecules through the skin surface by loosening the tight junctions of the SC epithelium and promotes both paracellular and transcellular transport of drugs. By virtue of its favorable features, chitosan has been and will be further harnessed in the development of new transdermal drug delivery systems (Table 3.1).

Table 3.1 Application of chitosan and its derivatives in transdermal delivery system

Chitosan derivatives	Formulation	Drug	Treatment	References
Chitosan	Nanoparticle	Curcumin	Tumor	Hafez et al. (2018)
Chitosan	Nanoparticle	Propranolol HCl	High blood pressure	Kassas et al. (2016)
Chitosan-egg albumin	Nanoparticle	Aceclofenac	Pain and inflammation	Jana et al. (2014)
Chitosan-cyclodextrin conjugates	Nanoparticles	warfarin	Coagulation	Khalil et al. (2012)
Chitosan-hyaluronic acid conjugates	Controlled-release particles	Lidocaine	Anesthesia	Anirudhan et al. (2016)
Chitosan	Nanoparticles	Minoxidil sulphate	Hair fall	Matos et al. (2015)
Chitosan-gellan conjugates	Nanogel	Ibuprofen	Pain	Abioye et al. (2015)
Chitosan-alginate complex	Nanocarrier	Pirfenidone	Pulmonary fibrosis	Abnoos et al. (2018)
Chitosan-lecithin conjugates	Nanoparticle	Melatonin	Circadian rhythm disorder	Hafner et al. (2011)
Chitosan	Patch	Glibenclamide nanocrystal	Type 2 diabetes	Ali and Hanafy (2016)

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Chitosan-Based Ocular Drug Delivery Systems

4

Subramanian Natesan, Venkateshwaran Krishnaswami, Saranya Thekkila Veedu, Dhilin Pathayappurakkal Mohanan, K. Ruckmani, and Rajaguru Palanichamy

Abstract

Chitosan is widely used in ocular drug delivery systems due to its biocompatibility, biodegradability, and favorable physicochemical characteristics. Chitosan-based ocular drug delivery systems are widely reported to improve the bioavailability at the anterior/posterior pole of the eye due to its mucoadhesive property that helps to increase efficacy of existing ocular drugs, affords stimuli responsive/targeted-based delivery regimen, enhances the corneal permeability, and improves the accumulation of drugs in the corneal/conjunctival epithelia for an extended period of time. This review summarizes the major ocular diseases affecting the eye, ocular delivery systems, novel ocular drug delivery systems, intraocular drug transport barriers, and ocular transporters. The utilization of chitosan toward the ocular drug delivery systems such as stimuli responsive systems, targeted delivery systems, and gene-based delivery systems is also discussed.

Keywords

Chitosan · Ocular drug delivery · Cornea · Ocular diseases

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_4

Abbreviation

AMD	Age-related macular degeneration
BBS	Bardet-Biedl syndrome
CS	Chitosan
CS-NP	Chitosan nanoparticle
JNK	C-jun NH_2 terminal kinase
CSL-NPs	Core-shell liponanoparticles
CMV	Cytomegalovirus
DR	Diabetic retinopathy
DAG	Diacylglycerol
DES	Diethyl squarate
EPR	Enhanced permeability and retention effect
EGDE	Ethylene glycol diglycidyl ether
HA	Hyaluronan
LCA	Leber congenital amaurosis
LCA2	Leber congenital amaurosis type 2
LCST	Lower critical solution temperature
NPs	Nanoparticles
NF- κB	Nuclear factor-kappa B
PLGA	Poly(lactic-co-glycolic acid)
PARP	Poly(adenosine diphosphate-ribose) polymerase-1
PEG	Polyethylene glycol
PKC	Protein kinase C
QUR	Quercetin
RAS	Renin-angiotensin-aldosterone system
RES	Resveratrol
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
ROCK	Rho-associated protein kinase
SEM	Scanning electron microscopy
SARM	Selective androgen receptor modulators
SERM	Selective estrogen receptor modulators
SNP	Self-assembled nanoparticles
TDDS	Targeted drug delivery system
VEGF	Vascular endothelial growth factor

4.1 Introduction

The unique anatomy and physiology of the eye serves as the major site for vision. Eye is classified into two segments, the anterior segment and the posterior segment. The anterior segment is composed of pupil, cornea, iris, ciliary body, aqueous humor, and lens, whereas the posterior segment is composed of vitreous humor, macula, retina, choroid, and optic nerve (Jiang et al. 2018). Various factors such as tear fluid turnover, nasolacrimal drainage, corneal epithelium, blinking, reflex lacrimation, and blood ocular barrier limit the ocular bioavailability of drugs to the eye upon topical application (Dubald et al. 2018). The anatomical positioning of various tissues along with the barriers associated with the eye creates a great challenge to deliver drugs to the eye. Various constraints for the ocular drug delivery are shown in Fig. 4.1.

The most common ocular diseases affecting the posterior segment include age-related macular degeneration (AMD), diabetic retinopathy (DR), cytomegalovirus (CMV) retinitis, proliferative vitreoretinopathy, Stargardt disease, and retinoblastoma. Conjunctivitis, trauma, dry eye syndrome, cataract, and glaucoma are the common diseases affecting the anterior segment of the eye. Even though the drug-containing formulations are instilled by various routes including intravitreal, periocular, systemic, topical, subconjunctival, and subretinal routes, topical administration is the widely preferred delivery route for most ocular drugs as it permits self-administration and localizes dosing into the ocular tissues and minimizes the risk of side effects. Poor bioavailability, rapid metabolic degradation, and clearance are the major disadvantages of this route (Davis et al. 2018). The impermeable nature of tight corneal barrier comprised of corneal epithelium (lipophilic), stroma (hydrophilic), and endothelium (hydrophilic) restricts the entry of drugs through the cornea (Harikumar and Sonia 2011). As a result, less than 5% of administered drug

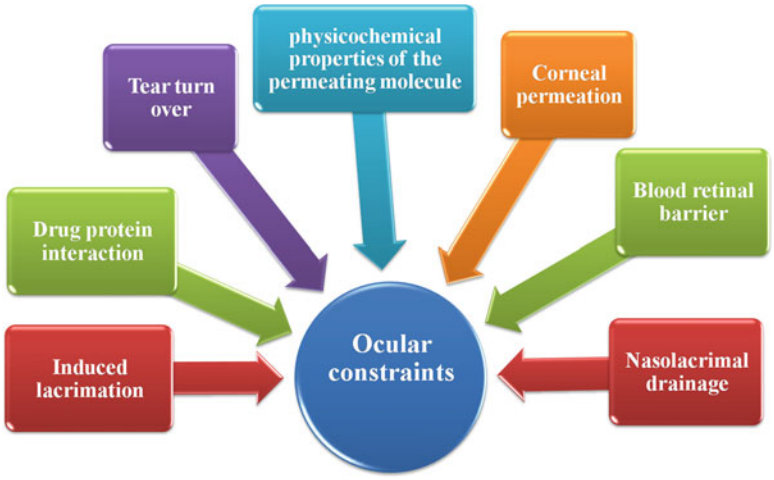

Fig. 4.1 Constraints for ocular drug delivery

penetrates the cornea and reaches intraocular tissues. The transcellular (lipophilic drugs) or paracellular pathways (hydrophilic drugs) are the preferred routes for the transport of drug-containing formulations across the corneal epithelium through passive or altered diffusion through intercellular spaces.

4.2 Major Ocular Diseases

Several diseases affect the anterior or posterior segment of the eye. Age-related macular degeneration (AMD) is a sight-threatening ocular disease affecting the posterior segment of the eye, associated with aging that gradually destroys sharp, central vision, and this is accompanied with angiogenic nascent blood vessels. Among the two types of AMD (dry AMD and wet AMD), the dry AMD is recognized as an important public health problem which is associated with dry eye syndrome (DES) and chronic eye pain with increased risk of ocular surface diseases, such as corneal ulcers and corneal abrasions. The key pathogenesis of DES is inflammation and oxidative stress, which leads to decreased tear production rate (Jee et al. 2016).

Glaucoma is the leading cause of irreversible blindness globally. Glaucoma is considered as a multifactorial neurodegenerative disease characterized by progressive loss of retinal ganglion cells and their axons in the optic nerve tract with elevated intraocular pressure. The various risk factors for glaucoma development include age, race, inflammation, oxidative and metabolic stresses, blood flow disturbances, and genetic background (Guo et al. 2018). The two types of glaucoma are open-angle glaucoma and angle-closure glaucoma. The pathogenesis of glaucoma includes excitotoxic damage caused by glutamate or glycine released from injured neurons, nitric oxide-based oxidative damage, and reactive oxygen species with profound morphological changes of thinning of neuroretinal rim and progressive cupping of the optic disc (Agarwal et al. 2009).

Cataract, the major cause of visual impairment especially in diabetic patients, is characterized with opacification of lens (loss of transparency). The different types of cataract include age related, traumatic, and metabolic cataracts. The major risk factors for cataract include cigarette smoking, alcohol consumption, lifestyle changes, genetic factors, socioeconomic status, etc. Sorbitol formation induced by aldose reductase pathway is considered as the major key factor for osmotic-related changes in the lens of cataract patients. This polyol accumulation leads to collapse/liquefaction of lens fibers, which supports the development of lens opacities in cataractous eye (Pollreisz and Schmidt-Erfurth 2010).

The microvascular complications of diabetes lead to diabetic retinopathy (DR), which is characterized by the growth of new blood vessels (retinal neovascularization). DR is further characterized by increased permeability of the blood-retinal barrier and accelerated loss of retinal neurons through apoptotic cell death. It is classified into proliferative and non-proliferative types. The pathogenesis of DR displays metabolic effects of chronic hyperglycemia, with profound vascular changes and subsequent retinal injury and ischemia. Neovascualrization may be

induced by vasoactive substances released during the inflammatory process (Tarr et al. 2013). The pathways that contribute to the development of DR are increased polyol pathway, activation of protein kinase C, increased expression of growth factors such as vascular endothelial growth factor and insulin-like growth factor-1, hemodynamic changes, accelerated formation of advanced glycation end products, oxidative stress, and activation of the renin-angiotensin-aldosterone system.

Conjunctivitis (pink eye) is an inflammation associated with infection of the conjunctiva, characterized by dilatation of the conjunctival vessels, hyperemia, and edema. The different types of conjunctivitis include bacterial, viral, and allergic conjunctivitis. Bacterial conjunctivitis is widely caused by coagulase-negative staphylococci, *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Micrococcus*, *Bacillus*, and *Lactobacillus* species (Richard and O'Callaghan 2018). Viral conjunctivitis is widely caused by adeno and herpes simplex viruses.

Ocular trauma that occurs due to inflammation of the iris/ciliary body mediated by inflammatory mediators (substance P, bradykinin, and histamine) results in blood vessel dilation with increased blood flow, blood plasma leakage, and breakdown of the blood ocular barrier. This often enhances photophobia with reddish eye, swollen appearance, pupillary miosis, decreased palpebral aperture with inflammation, and pain.

4.3 Ocular Delivery Systems

Conventionally, topical eye drop instillation into the lower pre-corneal pocket is the widely utilized route for ocular drug administration. Concentration of the administered drug present in the cornea serves as a driving force for the passive diffusion (Patel et al. 2013). Owing to poor pre-corneal retention and penetration across the cornea, the ocular bioavailability for topical drops was found to be less than 5%. In order to improve the drug contact time, permeation, and bioavailability, various excipients such as viscosity enhancers and permeation enhancers are incorporated in the topical formulations. Various properties of the formulations such as lipophilicity, solubility, molecular weight, charge, and degree of ionization affect the permeation rate of drug-loaded formulations within the cul-de-sac (Richard et al. 2018). Benzalkonium chloride, polyoxyethylene glycol ethers (lauryl, stearyl, and oleyl), sodium taurocholate, saponins, and cremophor EL as permeation enhancers and viscosity-enhancing agents such as cellulose derivatives and polyalcohol are utilized for ocular drug delivery (Patel et al. 2010). Emulsion-based formulations are also topically used in order to improve the corneal residence time and corneal permeation, to sustain drug release, and to enhance the ocular bioavailability (Liang et al. 2008). The optimal activity for these topical dosage forms depends upon the particle size of the formulations.

4.4 Novel Ocular Delivery Systems

In order to improve the ocular bioavailability of the drugs and to overcome the disadvantages associated with the conventional ocular drug delivery formulations, novel ocular drug delivery formulations such as nanomicelles, nanoparticles, nanodispersion, nanosuspension, liposomes, dendrimers, in situ gelling systems, contact lens, and microneedles are attempted by researchers. The physiological considerations taken for the development of novel ocular drug delivery systems are lipophilicity, solubility, molecular weight, charge, degree of ionization, route of administration, and permeation rate. The challenges associated toward the development of novel ocular drug delivery systems are corneal barriers, anatomical/physiological constraints, ocular toxicity, pre-corneal loss, and blood-retinal barrier. To improve the corneal residence time, various developments such as ocular inserts, presoaked hydrogels, iontophoresis, and phase transition systems are attempted. Delivery of drugs to the back side of the eye has been also achieved using development of drug-releasing devices for chronic vitreoretinal diseases. These nanoparticulate systems are also attempted to improve the solubility of most lipophilic drugs. Intravenous route-based ocular delivery nanoparticulate systems may avoid the quick clearance and improve the retention in the posterior pole for sustained delivery (Xu et al. 2013). Several proof of concept studies were conducted in delivering the drugs to the eye using these nano-based formulations. Mucoadhesive polymeric-based systems were reported to overcome the problems of quick elimination from the pre-corneal pocket and help to improve pre-corneal residence time. The interaction occurs between anionic polymer surfactant and mucins, may elicit hydrogen bonding at the anterior segment, and affords mucoadhesiveness (Bowman et al. 2009). The amphiphilic nature of surfactant/polymer-based nanomicelles was also reported to improve the solubility of lipophilic drugs along with pre-corneal retention. Due to the biocompatible nature, the liposomes were also used as a delivery system to encapsulate both hydrophilic and hydrophobic drugs. The novel nanoparticulate drug delivery systems are shown in Fig. 4.2.

4.5 Intraocular Drug Transport Barriers

Several barriers such as tears, corneal layers, conjunctiva, sclera, Bruch's membrane, retina, and blood-retinal barriers are associated with the intraocular drug transport mechanisms. Binding of the drugs to the tear protein results in tear dilution and clearance. The epithelium, stroma, and endothelial layers of the cornea restrict the entry portal of drugs/exogenous substances into the eye. Conjunctiva is the thin/transparent membrane. In the vascular endothelial layers, the drug molecules present in the conjunctiva may enter through pinocytosis/paracellular route. The lymphatic system in the conjunctiva acts as an efflux for drug elimination from the conjunctiva. The scleral permeability depends upon the hydrophobicity of the drug molecules utilized. The poor permeability of the positively charged drug molecules may be due

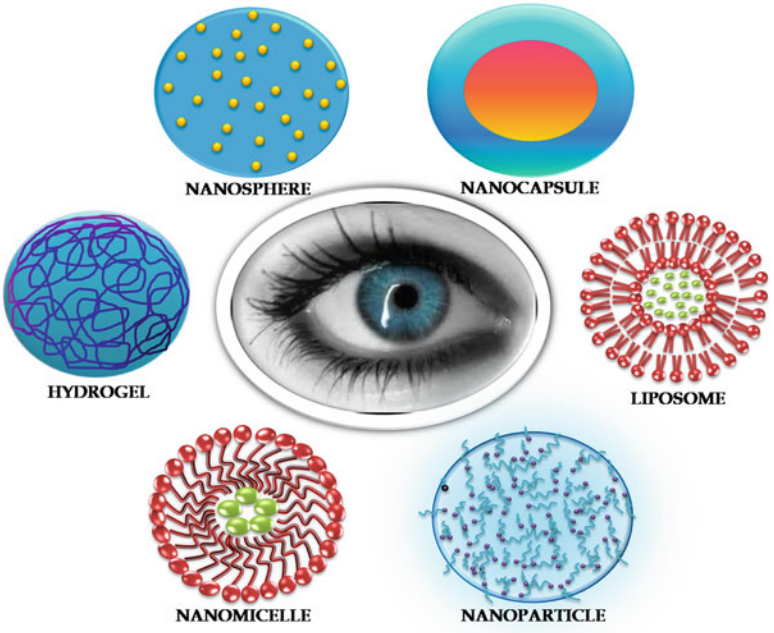

Fig. 4.2 Novel nanoparticulate drug delivery systems

to the highest binding efficacy to the negatively charged proteoglycan matrixes. The accumulation of glycation end products and lipofuscin in the Bruch's membrane may encounter difficulties toward the permeation of drug molecule across Bruch's membrane.

4.6 Ocular Transporters

At the corneal epithelium, several transporters have been observed to be located. While the multidrug resistance protein 1 (MDR1) efflux transporters are well characterized in the cornea, other multidrug resistance proteins (MRP1 to MRP5) act as additional efflux protein transporters in the cornea. In addition to corneal transporters, conjunctival tansporters have also possess impact on the drug transport. Even though non-corneal-based absorption of drugs is supported by conjunctiva, only a limited permeation is achieved through this route due to extensive clearance at systemic circulation. The MRP1 and MDR1 efflux protein transporters are also observed predominantly in conjunctiva. The iris-ciliary body is occupied with blood-aqueous barrier which includes nonpigmented epithelial cell layer, iris, and ciliary muscle vessels. The blood-aqueous barrier inculcates several efflux and influx transporters. The efflux transporters of neural retina are poorly understood. Organic

anion transporters OATP1A2, OATP1B3, and OATP2B1 and the organic cation transporter OCT3 are observed to be expressed in neural retina especially in photoreceptors (Vellonen et al. 2017). Basolateral influx transporters may support the uptake of drugs from the systemic circulation in certain cases through the retina.

4.7 Chitosan-Based Drug Delivery Systems

In the recent decades, pharmaceutical research is focused toward various drug delivery approaches by using polysaccharides from various sources owing to their ability to form gel-based and/or micro-/nanoparticulate systems. The potential transformation of polysaccharides from a simple excipient to active substances made their lead in cutting-edge researches (Morris et al. 2013; Liu et al. 2008).

Chitosan, a linear polysaccharide derived from fully or partially deacetylated chitin which forms the skeletal component of crustaceans and arthropods, has been established as a hallmark for novel drug delivery applications on account of its nontoxicity, biodegradability, biocompatibility, and inexpensiveness. It is a polymeric derivative of N-acetyl D-glucosamine with hydroxyl and amino group backbone. Its highly cationic and complex forming behavior renders chitosan as a versatile agent for a wide range of applications including cosmetic, food, and pharmaceutical purposes (Kean and Thanou 2010; De et al. 2010).

The chitosan has been produced from crustacean's shield through various chemical processings, viz., acid-mediated demineralization, alkaline-mediated deproteinization, solvent/oxidant-assisted depigmentation, and hydroxyl-based deacetylation (Chawla et al. 2014). The primary amino and hydroxyl groups of chitosan allow chemical modification to produce thiolated chitosan (improved mucoadhesion), quaternized chitosan (improved solubility), PEGylated chitosan, etc. (Mohammed et al. 2017).

The mucoadhesive property of chitosan contributes to its application in ocular drug delivery system. The polycationic groups of chitosan interact with the negatively charged sialic acid in the mucin present on the mucosal membrane at the ocular anterior surface. The electrostatic forces developed will retain the chitosan-based drug delivery system at the site of application, thereby enhancing the permeation of drug from the corneal membrane to the posterior segment of the eye (Fig. 4.3) (Sheetu Wadhwa et al. 2009).

4.8 Chitosan-Based Responsive Drug Delivery System

The responsive biopolymers are versatile drug delivery carriers with a promising capability of physicochemical transition in response to stimuli including exogenous (temperature, magnetic, radiation, ultrasound, light, etc.) or endogenous (pH, ionic, temperature, redox system, enzyme, etc.) origin (Mahlumba et al. 2016; Lopes et al. 2018). Chitosan is a well-established stimuli-sensitive polysaccharide with an ability to fabricate as a pH and thermoresponsive ocular drug delivery system that amplifies

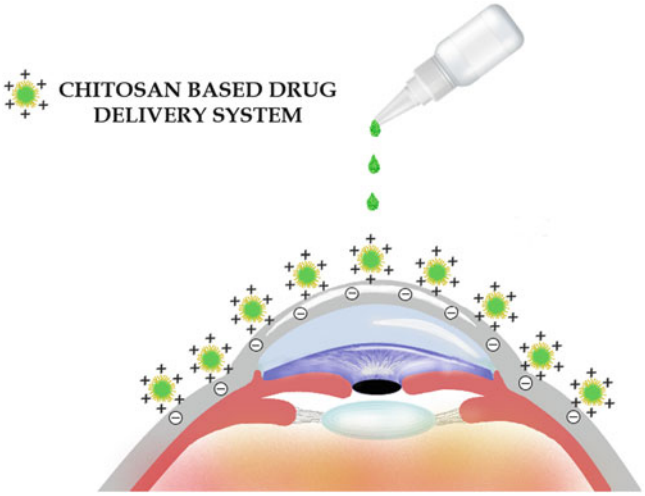

Fig. 4.3 Bioadhesive nature of positively charged chitosan on ocular mucosal membrane

a site-specific and tunable delivery of therapeutic agents. The in situ gelling property of chitosan effectively alleviates the rapid lachrymal clearance, pre-corneal loss, and low penetration capacity of classical ophthalmic delivery solutions by demonstrating a rapid sol to gel transition upon topical or injectable application (Kirchhof et al. 2015; Otero-Espinar et al. 2018). The chitosan solution converts in to in-situ gels after administration depending on temperature, pH or ionic strength through phase separation and covalent crosslinking. The types of mechanism of chitosan In situ gelling is represented in Fig. 4.4. The behavior of conventional ophthalmic solution and chitosan in-situ gel systems on administration in to ocular surfaces is represented in Fig. 4.5.

4.8.1 Phase Separation

The involvement of physical gelation through hydrophobic, electrostatic, and hydrogen bond interactions alters the solubility of polymer with response to various stimuli. These hydrogels are applicable to sustain the drug release for a few days to months due to low mechanical strength because of the absence of toxic covalent chemical cross linkers in their fabrication (Yumei Wu et al. 2018) (Fig. 4.4).

(a) Thermoresponsive Gelation

Thermoresponsive gelation is a fascinating technology for the delivery of sensitive biological molecules that are devoid of any chemical cross linkers or organic solvents but utilize gelling agents like low molecular weight polyol phosphates. The neutralizing effect of polyol phosphates (glucose-1-phosphate, glucose-6-phosphate,

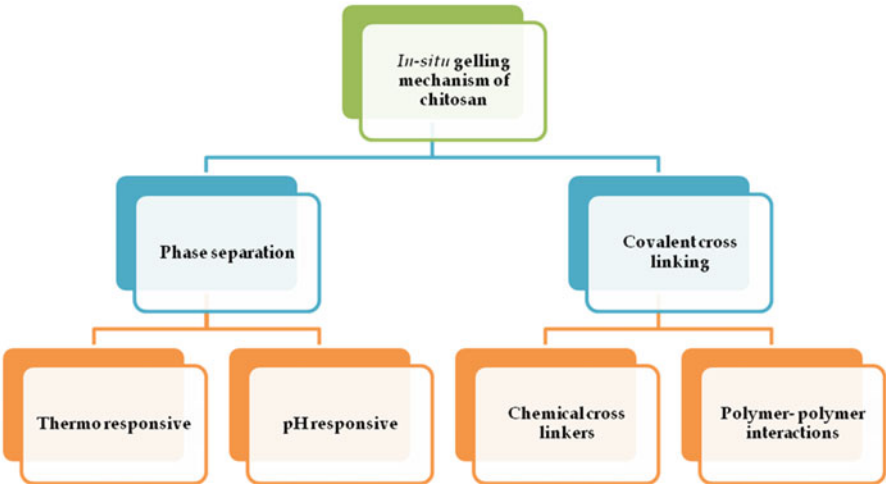

Fig. 4.4 In situ gelling mechanism of chitosan polymer through phase seperation and covalent crosslinking

β-glycerophosphate, etc.) on acidic solution of chitosan plausibly prevents the phase transition while maintaining isotonicity of the formulation at ophthalmic pH, but exhibiting a sol-gel transition at body temperature. The protective hydrated layer of polyol phosphates over chitosan polymer gets disrupted at higher temperature, allowing the gelation of polymer through strong hydrophobic interactions (Fig. 4.6). The thermosensitive polymers are capable of retaining a solution behavior below their LCST (lower critical solution temperature) and attain a gel phase above the LCST. Also the mechanical and thermogelling property of the delivery system is tunable with respect to derivatization (thiolated chitosan) and grafting of chitosan (Yumei Wu et al. 2018).

A novel in situ gelling ophthalmic delivery system of chitosan-poloxamer hydrogel as developed by Tsai Gratieri et al. (2016) that exploited the mucoadhesive property of chitosan and thermoresponsiveness of poloxamer as an in situ gel offers an improved mechanical property and sustained activity than individual polymer. The polymer concentration-dependent thermogelling, mucoadhesive, and mechanical properties at gelation temperature of 32 °C were observed. The gamma scintigraphy results revealed an improved ocular retention time with reduced lachrymal drainage of the in situ gel, exclusively due to the electrostatic attraction of cationic amino group of chitosan with anionic mucin and hydrophobic interaction of methyl group in mucine and acetylated chitosan (Gratieri et al. 2010). Based on the same principle, Gratieri et al. (2011) developed a fluconazole-loaded poloxamer/chitosan in situ gel and chitosan solution to treat fungal keratitis. They observed an improved mechanical strength and sustained-release kinetics following Fick's diffusion which supports the tightening of poloxamer gel by chitosan polymer (Gratieri et al. 2011).

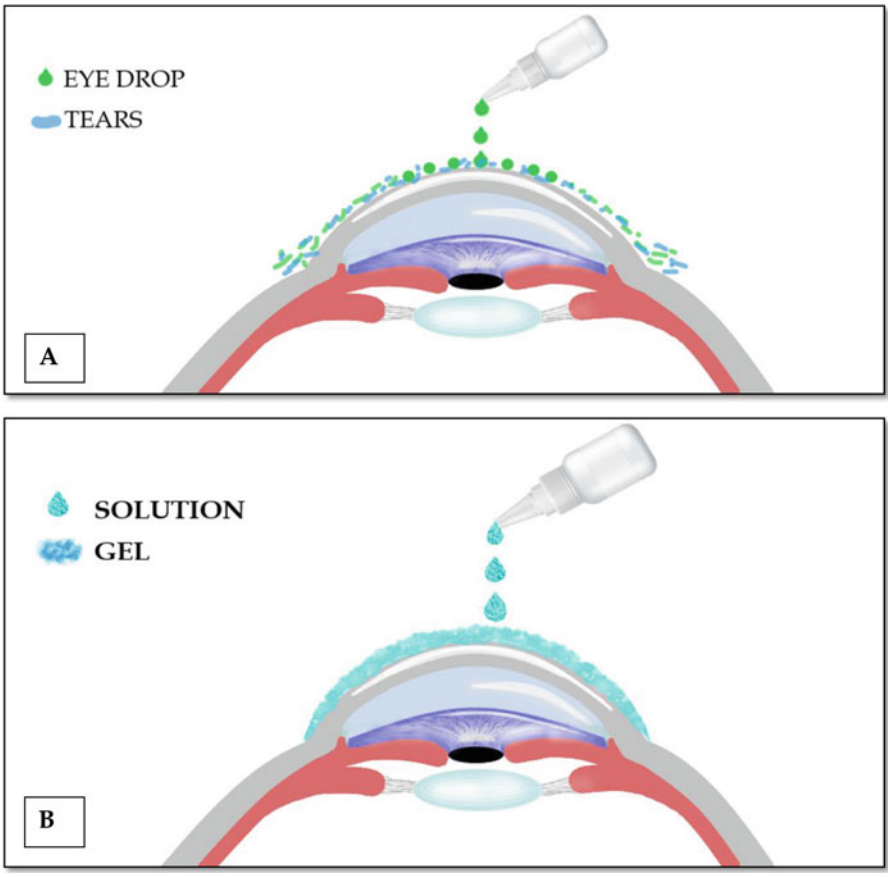

Fig. 4.5 Fate of conventional eye drop vs chitosan-based eye drop after instillation. (**A**) represents the pre-corneal loss of conventional eye drop in tear fluid and (**B**) represents the in situ gelling property of chitosan (sol-gel transition)

An effective thermoresponsive sustained-release subconjunctival injection of latanoprost-loaded chitosan-gelatin-glycerophosphate hydrogel circumvented the medication adherence failure of conventional glaucoma therapy with latanoprost. The intraocular pressure of rabbit was successfully reduced within 8 days in a triamcinolone acetonide-induced glaucoma model. The chitosan-gelatin-glycerophosphate hydrogel maintained a solution phase at 25 °C for 15 min and achieved a gelation at 37 °C within 1 min. A drug release of up to 70% was observed at the 28th day, not in a burst, but in a sustained-release manner at a dose of 1.2 μg/day. This revealed a prominent advantage of developed formulation in comparison with conventional topical latanoprost eye drops in a daily dose manner of 1.5 μ / drop, from which only 1–7% reaches the anterior segment. Moreover, the cytotoxicity and hemolytic assay results demonstrated the superior biocompatibility of

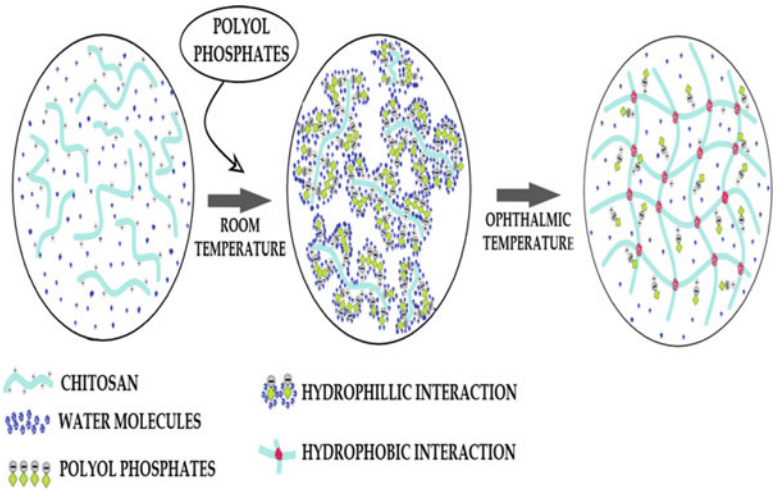

Fig. 4.6 Thermoresponsive sol-gel transition of chitosan drug delivery system. Protective layer of polyol phosphate gets depleted upon rise in temperature

chitosan-gelatin-glycerophosphate hydrogel due to the high degree of deacylated chitosan (>95%) (Cheng et al. 2014) (Fig. 4.5).

Later Cheng et al. (2016a, b) again proposed a novel, non-invasive, nonirritant delivery system of latanoprost-loaded thermoresponsive chitosan-gelatin-glycerophosphate eye drop for glaucoma by a similar method. This work aimed to check the feasibility of their previously developed chitosan-based hydrogel as a sustainable non-invasive topical formulation. The developed in situ hydrogel system formed through hydrophobic interactions of polymers escapes the drug from rapid nasolachrymal drainage in orbit. The SEM image revealed a lamellar structure of hydrogel with an abundant interconnected pores through which hydrophobic drug can be loaded and diffused. The higher degree of deacylation of chitosan improved mucoadhesive property which in turn enhanced the corneal permeation of drug. A cumulative percentage drug release of 51.7% was observed at the 7th day of instillation with an average dose of 0.9 μg per day. The level of latanoprost acid (active form) was diminished in aqueous humor after day 7, suggesting the need for a once a week application of developed formulation as a potential alternative of conventional eye drops for long-term management of glaucoma (Cheng et al. 2016a, b).

Alternatively, Ching-Yao Tsai et al. (2016) developed a topical eye drop of similar chitosan-based in situ hydrogel to deliver ferulic acid corneal wound healing in alkali-induced burns. They suggested that a cumulative percentage release of 28% up to the 24h may be attributed to afford strong bioadhesiveness, improved cross linking, hydrogen bonding capacity, and flexibility of high molecular weight chitosan and gelatin, along with the neutralizing capacity of glycerol phosphate to prevent the precipitation while adjusting pH of the formulation (Ching-Yao Tsai et al. 2016).

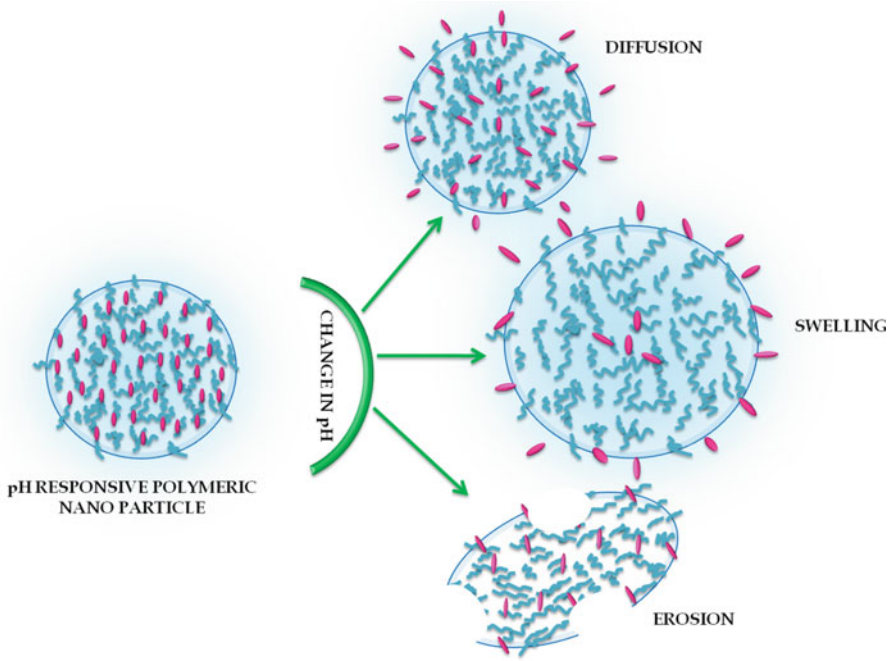

Fig. 4.7 Drug release mechanism from a pH-responsive polymeric drug delivery system

(b) pH-Responsive Gelation

The pH-responsive property of a polyelectrolyte greatly depends on the dissociation constant (pKa) of the polymer and pH of the surrounding environment. The ionizable groups in the polymer get protonated when pH < pKa and deprotonated when pH > pKa, and accordingly the drugs get released from the gel matrix by erosion/swelling/diffusion at recommended sites (Fig. 4.7) (Yumei Wu et al. 2018).

The pH-responsive behavior of chitosan is greatly attributed due to the presence of primary amino group ($–NH_2$) and hydroxyl group (–OH) in the polymer skeleton. Chitosan is a cationic polymer that holds pKa of 6.5 and gets deprotonated or deionized at ophthalmic pH of 7.4. In an acidic environment, the basic groups get ionized, forming an expanded structure due to the repulsion of ionized functional groups. Eventually the drug-loaded expanded polymeric gel matrix gets compressed or shrunk at ophthalmic pH due to deionization of polymeric chain, and hence the drug expected gets released (Fig. 4.8) (Elaref Ratemi 2018).

Levofloxacin-loaded dual stimuli (pH and ion sensitive)-responsive system was reported using chitosan and sodium alginate. The formulation exhibited a sol-gel transition at ophthalmic pH forming a stiff transparent gel with a first-order release kinetics extending up to 12 h. Chitosan forms hydrogel at pH of 7.4 as a result of hydrophobic force developed at alkaline pH. As an anionic polymer, sodium alginate gets cross linked with various divalent ions present in the lachrymal fluid forming

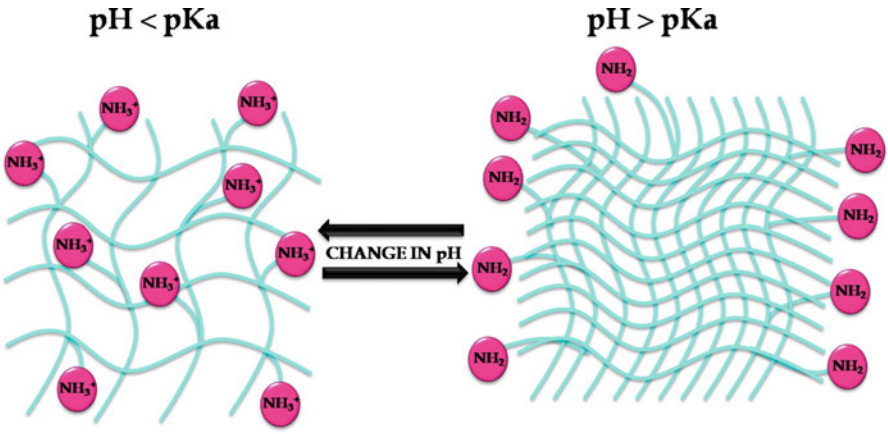

Fig. 4.8 pH-responsive behavior of chitosan polymer. Amino group gets protonated at acidic pH and deprotonated at basic pH

clear rigid gel with pseudoplastic behavior. The mucoadhesive property of chitosan also imparts an improved corneal penetration of the loaded levofloxacin which elicits better therapeutic action than conventional eye drops by preventing the lachrymal drainage with enhanced pre-corneal retention (Gupta et al. 2015).

4.8.2 Covalent Cross Linking

Even though cross linking involves utilization of toxic covalent cross linkers, it overcomes many drawbacks of physical gelation such as challenges during functionalization, uneven pore size, rapid in vivo degradation and drug release, etc. The covalent cross linking can be achieved either by means of chemical cross linkers (genipin, glutaraldehyde, formaldehyde, etc.) that exclusively target the primary amine and aldehyde group or by means of functionalizatioin of chitosan with light- or enzyme-sensitive moieties that cross linked upon UV irradiation or enzyme catalyzation, respectively (Yumei wu et al. 2018).

(a) Chemical Cross Linkers

The emerging utilization of chemical cross linkers in hydrogel preparations achieved a mechanically sound, controlled release formulation and at the same time demands stringent purification steps to eliminate toxic covalent linking agents and to ensure biosafety.

Diethyl squarate (DES), blocked diisocyanate, and ethylene glycol diglycidyl ether (EGDE) are some examples of small molecule chemical cross linkers which get cross linked at basic pH and higher temperature, while a long reaction time ruled out their application as in situ gelling system. But genipin is a nontoxic covalent cross

linker derived from gardenia fruit which is widely utilized in in situ gelling systems due to its nontoxicity, cytocompatibility, and self-polymerizing ability. The gelation period, degree of cross linking, and drug release behavior greatly depend on the type and amount of cross-linking agent used (Berger et al. 2004; Hennink and van Nostrum 2002).

(b) Polymer-Polymer Interactions

In this method, polymer chains are prefunctionalized to produce various polymer conjugates exhibiting in situ gelling properties. The reaction may involve Schiff base formation, disulfide bonding, Michael addition, etc. (Bhattarai et al. 2010).

Natesan et al. (2017a) developed resveratrol (RES)- and quercetin (QUR)-loaded nanoparticles holding potential ability to reduce intraocular pressure for the treatment of glaucoma. The enhancement of bioavailability of RES was observed by the concurrent administration of RES and QUR. For improved delivery and synergic effects on intraocular pressure reduction, QUR in RES-loaded chitosan (CS) nanoparticles (NPs) and polyethylene glycol (PEG)-modified CS-NPs has been developed. Using tripolyphosphate and CS, they prepared CS-NPs and PEG-modified CS-NPs by ionic gelation technique. They found that the prepared nanoparticles were amorphous with spherical morphology, and upon increasing the PEG concentration, the entrapment and particle size get enhanced. The presence of QUR enhances the radical neutralizing capacity. The corneal permeation of RES gets enhanced in RES- and QUR-loaded formulation compared to RES-NPs/free RES dispersion. Overall the RES- and QUR-loaded PEG-modified CS-NPs elicit a sustained-release profile with enhancement in the intraocular pressure reduction (5.5 ± 0.5 mmHg) as studied in normotensive rabbits (Natesan et al. 2017a).

4.9 Chitosan-Based Targeted Drug Delivery System (TDDS)

The recent commencement of nanoparticulate drug delivery system offered a breakthrough research for the development of functionalized or decorated nano-sized targeted drug delivery system to demonstrate a site-specific delivery of active payload. The evolution of TDDS starts from the first-generation, which is a mere delivery of the drug to proximity of the diseased site as it could be reached to the target site (microsphere, microcapsule). Later, second-generation TDDS emerged with an advantage of target attack even though administered via general routes and with the involvement of stimulus at the diseased environment (nanoparticle, liposomes, nanocapsules). The second-generation TDDS, functionalized with active ligands, accounts for the third-generation TDDS (peptide, monoclonal antibodies). When considering the physical form of drug delivery system, the TDDS can be classified as particulate type (nanoparticle, nanosphere, liposome, microsphere, nanomicelle, etc.), soluble type (proteins, peptides, monoclonal antibodies, siRNA, gene, etc.), and cellular type (viable cells, viral vectors) (Sen and Maiti 2017).

The conventional (invasive/noninvasive) drug delivery systems failed to achieve their therapeutic potential to treat various ocular diseases affecting the anterior and posterior segment of the eye due to inevitable loss during their passage through various anatomical-physiological ocular barriers to reach their target sites. The frequent and prolonged instillation of conventional eye drops, even though being the most convenient route for anterior segment diseases, is reported to be associated with corneal and conjunctival inflammation, tear film instability, etc. Similarly repeated intra-/periocular injections for posterior segment eye diseases also failed to achieve patient compliance toward conventional treatment modalities. In this regard, nanotechnology-based drug targeting by exploiting the exact location and pathogenesis underlying various ocular diseases like glaucoma, DES, AMD , DR, retinoblastoma, uveitis, etc. has been developed for the past few decades (Yuhua Weng et al. 2016; Diebold and Calonge 2010).

The active moieties can be chemically conjugated or passively absorbed to the nanocarrier molecule to demonstrate passive, active, or physical targeting approaches. The functional properties of carrier molecule greatly influence the fate of bioactive payload. The unique structure of chitosan with primary and secondary hydroxyl group (C_6 and C_3 positions, respectively) and primary amino group (C_2 position) makes them a versatile carrier with a broad range of functionalization to be fabricated as drug-encapsulated or ligand-conjugated nanocarriers. Moreover its greater biodegradability, cytocompatibility, non-immunogenicity, etc. made them a trump card for targeting delivery (Jae Hyung Park et al. 2010).

Chitosan-based nanotherapy demonstrated an enhanced permeability and retention effect (EPR) via passive targeting of some ocular diseases associated with inflammation-mediated leaky vasculature and angiogenesis such as choroidal neovascularization and DR. Hydrophobic drugs are conjugated with chitosan via a cleavable linkage that is stable at blood stream but cleavable at target site. Drug conjugates can also be encapsulated within cross-linked chitosan nanoparticles providing better lifespan expectancy (Feichin Hsiao et al. 2017; Natesan et al. 2017b).

Chitosan has also been reported to form polyelectrolyte complexes with polyions (hyaluronic acid, alginate, and heparin) through electrostatic attraction. An inflamed retinal pigment epithelium (RPE) was effectively targeted by a hyaluronan (HA)-modified core-shell liponanoparticles (HA-CSL-NPs) to treat autoimmune uveitis. The interaction of HA with CD44 cells in the RPE suggested the potential of developed system to treat intraocular inflammatory diseases. It was observed that intracellular trafficking is directly proportional to FA grafting and molecular weight (Gan et al. 2013). Due to the well-established biosafety, biodegradability, mucoadhesive property, and histocompatibility of HA, a simple HA-CS nanoparticle alone can be considered as a promising ocular targeting agent for corneal and conjunctival diseases (Contreras-Ruiz et al. 2010). A comparative study of the ocular bioavailability of dexamethasone phosphate from dexamethasone solution, chitosan nanoparticle (CS-NP), and HA-coated chitosan nanoparticle (HA-CS-NP) was evaluated. The results demonstrated a better pre-corneal retention of both nanoparticles due to mucoadhesive nature of polymers. But HA-coated CS-NP

showed an improved penetration and efficacy through receptor-mediated cellular uptake of nanoparticles. The results suggested the epithelial regeneration capacity of HA through CD44 binding and corneal-conjunctival motility receptor-mediated mechanism (Kalam 2016).

When linked with a hydrophobic moiety (bile acid, fatty acids) through primary hydroxyl and amine group, chitosan reoriented to form self-assembled nanoparticles (SNP) through hydrophobic interactions at physiological pH of 7.4. SNP can readily escape from the reticuloendothelial system providing a prolonged circulation lifespan (Hyung et al. 2010). A SNP of glycol chitosan or heteropolymer glycol chitosan-polyethyleneimine linked with 5β-cholanic acid has been reported to demonstrate the distribution of nanoparticle to vitreous chamber and retinal region after intravitreal injection. The nanoparticles were coated with fluorescent dyes for proper tracking. The cationic SNP easily crossed the vitreal barrier due to anti-fouling mechanism of glycol groups (Koo et al. 2012). Similarly, chitosan-cholesterol SNP loaded with cyclosporine A showed improved pre-corneal retention, offering a promising agent for external ocular diseases (Yuan 2006). PEGylated chitosan has also been reported as a better carrier for small drug molecule, bypassing reticuloendothelial system (Shi et al. 2015).

In the development of active targeted drug delivery system, the foremost concern will be the identification of overexpressed receptor or antigen at the diseased site. The ligand attached to the carrier should have high specificity for the target, and at the same time, the carrier should be stable at the targeted region preventing undesirable invasion to the normal cells and tissues. A high load of therapeutic agents is physically encapsulated within the carrier molecule which has been tethered with targeting ligand to bind with overexpressed receptors and further internalized to release active payload. This ternary system composed of encapsulated active drug, carrier molecule, and targeting moiety together elicits enhanced activity and preserved actual confirmation of ligand, when compared to simple binary system of drug-ligand conjugation (Allen 2002; Yoo et al. 2011).

The major targets involved in pathogenesis of ocular diseases at a glance are noteworthy prior to a comprehension on ocular targeted nanotherapy. Calcineurin inhibitors, interleukin-1β inhibitors, and inducible nitric oxide synthase (iNOS) inhibitors are major targeting agents to reduce inflammation (Chiou 2001; Colligris et al. 2014). Sex hormone deficiency-induced dry eye syndrome can be controlled through selective androgen receptor modulators (SARM) and selective estrogen receptor modulators (SERM). Anti-lymphangiogenic agents such as vascular endothelial growth factor (VEGF) inhibitors are another major target in treatment of DES, AMD, and DR, which prevents infiltration of antigen-presenting cells to lymphoid tissues (Dalton et al. 2008). C-jun NH_2 terminal kinase (JNK) inhibitors and lacritin peptides are other major targeting agents that improve tear fluid production in various pathways (Bennet et al. 2001).

Protein kinase C (PKC)-mediated synthesis of diacylglycerol (DAG) is a major risk factor for vascular dysfunction in DR. Aldose reductase enzyme inhibitors and poly (adenosine diphosphate-ribose) polymerase-1 (PARP) enzyme inhibitors reduce the pathogenesis of DR by reducing neuronal apoptosis, microaneurysm, retinal gliosis oxidative stress, nitric oxide production, etc. The nuclear factor-kappa

B (NF-κB) and renin-angiotensin-aldosterone system (RAS) are also reported as a promising target to treat DR. Anti-VEGF therapy using monoclonal antibody is also utilized for current therapy of DR (Chaira et al. 2009; Mohd et al. 2013).

A potent angiogenic factor VEGF is considered as a major target in age-related macular degeneration to arrest neovascularization. Anti-angiogenic effect is achieved by targeting integrin receptors ($\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$) overexpressed at endothelial cell surface, using delivery system with tripeptide (RGD) motif. The arginine has been attached to α subunit of $\alpha_v\beta_3$ receptor through guanidine linkage, while β subunit is attached via the carboxylate group of aspartate (Singh et al. 2010). Overexpressed transferrin receptor is also recognized as a potential target in AMD. A system-within-system PLGA microparticle loaded with a chitosan nanoparticle has been synthesized and evaluated for intravitreal delivery of ranibizumab as an anti-VEGF agent for AMD in a sustained manner (Elsaid et al. 2016).

Like all other ocular diseases discussed above, anti-VEGF treatment is a major strategy in glaucoma therapy also. Intraocular pressure reduction, which is a major treatment goal in glaucoma therapy, has been achieved via inhibition of adrenergic, cholinergic, and prostaglandin receptors, Rho-associated protein kinase (ROCK), and carbonic anhydrase enzymes. Similarly, inflammatory responses in glaucoma are alleviated by A_3AR receptor targeting (Ljubimov and Saghizadeh 2015). The possible targets for the delivery of drugs for the various ocular diseases are summerised in Table 4.1.

Table 4.1 List of major ocular diseases and possible targets for delivery of active payloads

Disease	Targets	Applications
Dry eye syndrome	Calcineurin, IL-1β, (iNOS), SERM, SARM	Reduce inflammation
	VEGF	Prevents infiltration of antigen-presenting cells to lymphoid tissues
	JNK, lacritin peptide	Improve tear fluid production
Diabetic retinopathy	PKC	Vascular dysfunction
	Aldose reductase enzyme, PARP	Reduces neuronal apoptosis, microaneurysm, retinal gliosis oxidative stress, nitric oxide production
	NF-κB, RAS, VEGF	Downstream inflammatory response
Age-related macular degeneration	VEGF, integrin, transferrin	Prevents neovascularization
Glaucoma	Adrenergic receptors, cholinergic receptors, prostaglandin receptors, Rho-associated protein kinase (ROCK), carbonic anhydrase enzymes	Intraocular pressure reduction
	A_3AR	Reduction in inflammatory response

4.10 Chitosan-Based Gene Delivery System

Gene therapy is a technique that utilizes a healthy copy of gene to correct a recessive or dominant disease either by introducing a new gene or by replacing a defective gene with a normal one or by "knocking out" a mutated gene to terminate its malfunctioning. Even though it is an emerging technique targeting the root cause of a disease rather than the symptoms, by curation at intracellular level, its applicability has currently been restricted only to major diseased conditions which are incurable. The major barrier of gene therapy in disease management system is due to probable safety concerns associated with delivery of genetic materials to an individual, having the potential to provoke host defense mechanism (Deng et al. 2017; Hanna et al. 2017).

Ocular gene therapy has now emerged as a promising technology for the permanent treatment of various diseases, creating a history of approved, progressing, and completed clinical trials of around 1.3% of the total number of gene therapy clinical trials reported globally as per publicly available database "Gene Therapy Clinical Trials Worldwide," provided by the *Journal of Gene Medicine* and updated on August 2018 (http://www.abedia.com/wiley/). The reports mainly involve diseases like retinitis pigmentosa (RP), Bardet-Biedl syndrome (BBS), Leber congenital amaurosis (LCA), glaucoma, AMD, diabetic macular edema, etc. Luxturna™ (voretigene neparvovec-rzyl) is the only FDA-approved (December 2017) gene therapy product for retinal dystrophy due to biallelic RPE65 mutation (Ginn et al. 2018). The eye is a well-known immune-privileged site in the human body characterized by ocular barriers. This peculiar character makes the gene delivery less immunogenic on intraocular administration than systemic route (Zhou and Caspi 2010). The critical factors to the eyes, being a potential target for gene therapy, to be considered are type of carrier system, route of administration, and applicability of genetic element.

4.11 Route of Administration of Gene Therapy

The genetic material-loaded carriers are administered at the anterior or posterior lobe of the eye through topical, periocular (subconjunctival, retro-/peribulbar, sub-tenon, etc.), intracameral, intravitreal, subretinal, and suprachoroidal injections. The application convenience of topical route is beaten by the low bioavailability of huge sized genetic material which could neither be penetrated through nor be retained long at ocular surface. In another way, simply we can say that topical route is the least invasive but most ineffective route for retinal targeting. The subconjunctival injections are less invasive, providing a sustained delivery of larger sized particles of > 200 nm to anterior segment without any undesired infiltration to systemic circulation. But poor transduction efficiency due to rapid aqueous humor clearance is circumvented by intracameral injections. The intravitreal route is reported to deliver small interference RNA (siRNA) against VEGF. Similarly subretinal injection also has been recently utilized for the treatment of leber congenital amaurosis

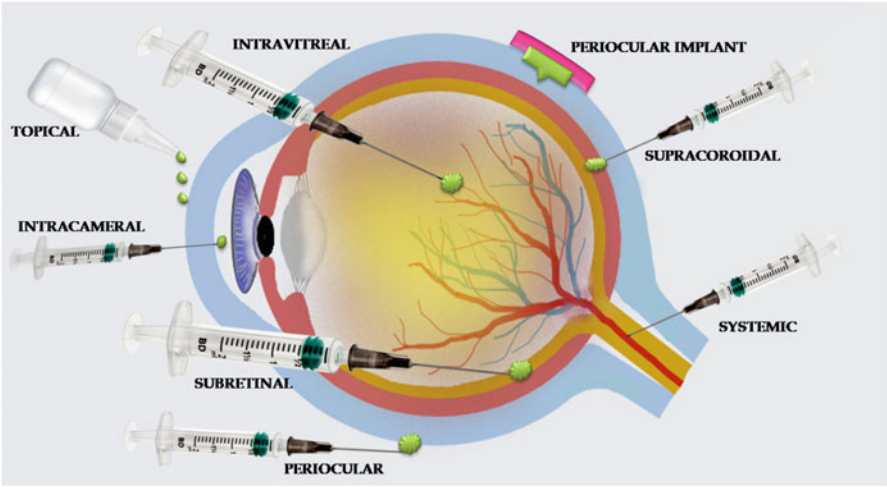

Fig. 4.9 The different routes of administration of gene therapy

type 2 (LCA2), with improved responses in clinical trials. But these two routes are associated with serious adverse effects such as retinal detachment, retinal tear, increased IOP, endophthalmitis, etc., while suprachoroidal route demonstrated an efficient transfection against a wide range of tissues at the posterior lobe, such as photoreceptors, retinal pigment epithelium, retinal ganglion, etc. The site is more prone to rapid systemic clearance, demanding gene delivery in sustained manner.

When considering all the severe damages to ocular tissues associated with the aforementioned route of administrations, the intravenous delivery of therapeutic gene is beneficial to some extent to target the retinal or deeper tissues. But the blood-retinal barrier deprives the entry of macromolecule into the retina. This can be bypassed by fabricating the delivery system with a targeting moiety to attack the transferrin receptor situated all over the retinal vascular cells. But the major drawbacks of this approach are the unavoidable loss of genetic material which are opsonized/phagocytosized by host defense system, requirement of large volumes to be administered, and chances of off-target delivery to normal tissues (Fig. 4.9) (Solinis et al. 2014).

4.12 Vectors in Gene Therapy

The therapeutic gene should be loaded in a suitable vector in order to elicit better transfection efficiency and sustained delivery without ectopic expression. At the same time the vector should not produce any immunogenic, inflammatory, or toxic responses to the host cells. The viral vectors are widely used owing to its potential transfecting efficiency (Conley and Naash 2010). Adenovirus vectors/recombinant adenovirus vectors are better agents utilized for transient gene expression to treat

DR, glaucoma, AMD, etc., but are associated with immunogenic risk factors. Similarly retroviral vectors are reported to develop oncogenesis in patients participating in clinical trials of SCID. Hence the critical problem associated with viral vectors such as high immunogenicity and mutagenicity paves a greater consideration toward the development of various nonviral vectors. The nonviral vectors comprise a wide range of lipid-based (solid lipid nanoparticle, liposomes) and polymer-based (polyethyleneimine, poly(lactic) acid, poly(glycolic) acid, chitosan) delivery system. Even though nonviral vectors produce a lower range of transgenic expression, their ability to be fabricated as functionalized system to improve cellular trafficking and enhance loading efficiency made them a prominent candidate for therapeutic gene delivery (Zulliger et al. 2015; Kompella et al. 2013).

4.13 Chitosan as Gene Therapy Vector

Chitosan, being a natural polysaccharide, is a promising vector due to its well-established safety, biocompatibility, and encapsulation efficiency. The cationic nature of chitosan allows efficient encapsulation of negatively charged genetic material to deliver at the target cells. The cationic amino group also enhances the mucoadhesion of the delivery system allowing sufficient time to penetrate through ocular cells. Chitosan also promotes protein structural reorientation at the cellular tight junctions promoting paracellular transport of payload.

The molecular weight and degree of deacetylation of chitosan molecule greatly affect the carrier properties. High molecular weight chitosan hinders the transfection due to strong complexation with DNA and higher viscosity properties. So a commercially available completely deacetylated low molecular weight ultrapure chitosan oligomer (NOVAFECT) has been widely utilized to fabricate a chitosan-DNA nanoparticle transfecting COS-7 cells to treat corneal diseases. The intrastromal injection of chitosan-DNA nanoparticles demonstrated a 5.4-fold increase in luciferase gene expression in corneal fibroblast when compared to polyethylenimine-DNA nanoparticles, which has been reported as a gold standard vector for nonviral gene therapy (Klausner et al. 2010). A pCMS-EGFP plasmid (enhanced green fluorescent protein)-loaded oligochitosan polyplexes demonstrated a pH-dependent transfection efficiency on HEK-293. Chitosan polymer also protects the plasmid from DNase I enzyme. The study concluded the effect of route of administration in response to transfection efficacy stating that subretinal injection produces localized transfection at retinal pigmental epithelium whereas intravitreal injection diffuses plasmid to deeper layers of retina such as photoreceptors and retinal ganglion cells (Puras et al. 2013a). The NOVOFECT-plasmid system demonstrated a critically nanometric positively charged polyplexes that facilitate the cellular binding due to electrostatic interactions (Puras et al. 2013b). A novel chitosan oligomer- DNA-SLN system also has been reported that produces better transfection efficacy than simple chitosan polyplex (Delgado et al. 2013).

4.14 Conclusion

Chitosan is a cationic biodegradable natural polysaccharide that established its goodwill in the field of drug delivery applications over the past few decades. The promising nature of chitosan to be fabricated as stimuli-responsive, targeted, and gene delivery system with cytocompatible, non-immunogenic, and mucoadhesive property has fixed its hallmark in the area of ophthalmic drug delivery system. Chitosan-based ocular drug delivery devices overcome many demerits of classical ocular drug delivery systems such as pre-corneal drug loss, frequent drug application, inefficiency to reach the posterior segment, etc. The ability of chitosan to bond with mucosal glycoprotein through disulfide linkage enhances the corneal retention, penetration, and sustained delivery of active payload for the effective treatment of various ocular diseases affecting anterior and posterior lobes of the eye. Moreover the appreciable transfection efficiency also ascertained its role as a nonviral vector for ocular gene therapy.

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Functional Chitosan Carriers for Oral Colon-Specific Drug Delivery

5

Nafisah Musa and Tin Wui Wong

Abstract

Chitosan is a polysaccharide consisting of D-glucosamine and N-acetyl-D-glucosamine units linked by β-(1,4) linkages. It is derived via deacetylation of chitin. Chitosan is a cationic polymer which is biodegradable, biocompatible, nontoxic, and mildly allergenic. It is characterized by antitumor, antimicrobial, and antioxidant activities which render a widespread research interest for pharmaceutical and biomedical applications. Used as a matrix and/or coat material, chitosan can protect drugs from chemical and enzymatic degradation with reference to oral delivery. Chitosan binds strongly to mucus and exhibits mucosal permeation-enhancing property that promotes drug absorption through intestinal epithelial cells. Oral colon-specific delivery systems have been explored for targeted drug administration for the treatment of colon cancer, ulcerative colitis, Crohn's disease, diverticulitis, irritable bowel syndrome, Hirschsprung's disease, antibiotic-associated colitis, and other colon diseases. This chapter gives an overview of relevant physicochemical and biological properties of chitosan and its derivatives and innovative formulations with respect to their use as oral colon-specific drug delivery systems.

Keywords

Chitosan · Colon specific · Drug delivery · Oral

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_5

5.1 Introduction

Chitin is a structural aminopolysaccharide that is obtained from crustaceans, mollusks, marine diatoms, insects, algae, fungi, and yeasts. The most commercially processed chitin is obtained from the outer skeleton of crustaceans such as crab, prawn, shrimp, and crayfish (Kaya et al. 2014, 2015). It is a semicrystalline polymer that appears as colorless and odorless flakes. Chitosan is a partially deacetylated polysaccharide of N-acetyl-D-glucosamine that can be obtained through alkaline deacetylation of chitin. It consists of β-(1,4)-linked D-glucosamine residues with the amine groups being acetylated randomly (Fig. 5.1). The degree of deacetylation of commercial chitosan is usually between 70 and 95% and the molecular weight between 10 and 1000 kDa (Gulbake and Jain 2012). Chitosan is nontoxic, eco-friendly, biocompatible, and biodegradable (Kaya et al. 2015; Aranaz et al. 2009).

Chitosan and its derivatives have gained attention as drug delivery carriers and systems in pharmaceutical and biomedical product development over the last few decades due to their excellent physicochemical and biological characteristics. Its functions as material for drug delivery vehicles are affected by molecular weight, degree of deacetylation, distribution pattern of acetamide groups, and solution viscosity. Higher-molecular-weight chitosan of approximately 1400 kDa demonstrates a stronger level of mucoadhesion than low-molecular-weight chitosan of 500–800 kDa, because the former has a higher level of viscosity (Werle et al. 2008). Chitosan possesses a good complexing capacity with an oppositely charged polymer such as alginate, pectin, xanthan, carrageenan, poly(acrylic acid), sodium salt of poly(acrylic acid), carboxymethyl cellulose, and others.

Chitosan has a pKa value close to 6.5 that makes it insoluble in water and quite soluble in acidic solutions since its primary amine gets protonated and forms a positively charged polyelectrolyte (Kumar et al. 2016). Being a cationic polyamine, chitosan has been widely investigated as a controlled and targeted drug delivery vehicle for mucosal, ocular, and topical administration (Netsomboon and Bernkop-schnürch 2016; Chen et al. 2016; Bansal et al. 2011). Crosslinking, complexation, and coacervation are possible processing methods to develop drug carriers made of chitosan (Ahmed and Aljaeid 2016). The presence of amine and hydroxyl functional groups along with chitosan chains enables chemical modification of the polymer which offers a wide range of derivatives to induce specific biological functions and solubility attributes (Croisier and Jérôme 2013; Jana and Maiti 2017). Derivatives

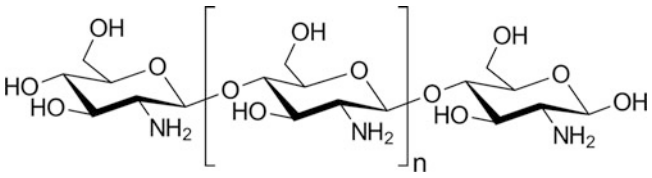

Fig. 5.1 Chemical structure of chitosan

such as quaternized chitosan (N,N,N-trimethyl chitosan), carboxyalkyl chitosan, thiolated chitosan, sugar-bearing chitosan, bile acid-modified chitosan, and cyclodextrin-linked chitosan have been produced (Zargar et al. 2015; Bansal et al. 2011).

Chitosan has attractive biological properties such as antitumor (Qin et al. 2004; Vin and Vav 2011), antimicrobial (Sudarshan et al. 1992; Liu et al. 2000), antifungal (Seyfarth et al. 2008), and antioxidant (Yen et al. 2008; Xing et al. 2005) activities that render a widespread research interest for oral drug delivery. Chitosan exhibits mucoadhesive (Lehr 1992; Dodane et al. 1999), analgesic (Aranaz et al. 2009), and haemostatic (Ong et al. 2008; Yang et al. 2007) properties that make it an outstanding candidate for pharmaceutical and biomedical applications.

Oral delivery is the most commonly used and readily accepted form of drug administration (Ensign et al. 2012). Drug administration by oral route is preferred as it offers patients less pain, greater convenience, higher likelihood of compliance, and reduced risk of cross infection and needlestick injuries (Liu et al. 2003). It is still the most popular way of drug administration because of high patient compliance and convenience of self-administration (Werle et al. 2008). Oral administration for chronic therapies, such as cancer chemotherapeutics, is expected to improve the quality of life of patients and increase the cost-effectiveness of treatment through reducing the duration of hospitalization (Wong et al. 2011). With reference to colon cancer and inflammatory bowel diseases, an oral colon-specific drug delivery system has its own advantages in improving local colonic drug concentration and reducing drug dose and systemic side effects. However, there could be some challenges in delivering drugs effectively to the colon as gastrointestinal tract physiology is complex with pH variations along the gastrointestinal tract, presence of digestive enzymes, and prolonged transit time (Amidon et al. 2015). These factors may influence the formulation/development of an oral colon-specific drug delivery system and the colonic bioavailability of drugs (Malayandi et al. 2014).

5.2 Chitosan

5.2.1 Physicochemical Properties

Chitosan with different physicochemical properties can be produced under different deacetylation reaction conditions and/or from different sources of starting materials (Yen et al. 2009; Aranaz et al. 2009; Panith et al. 2016; Bansal et al. 2011). Chitosan encompasses three types of reactive functional groups: an amino/acetamido group, a primary hydroxyl group, and a secondary hydroxyl group at the C-2, C-3, and C-6 positions (Xia et al. 2011). The amino content, degree of deacetylation, and molecular weight are the primary parameters that are responsible for the physicochemical and biological properties of chitosan (Zou et al. 2016). Random distribution of amino acid in chitosan molecules makes it easy to generate intra- and intermolecular hydrogen bonds (Jiali et al. 2010). Table 5.1 summarizes the relationship of

Table 5.1 Experimental studies on the relationship of physicochemical and biological characteristics with the amino content, degree of deacetylation, and molecular weight of chitosan

Experiment	Remark	Reference
Evaluation of the anticancer abilities of chitin, chitosan, and low-molecular-weight chitin using a human tumor cell line THP-1	Low-molecular-weight chitin has a higher tumor-suppressive activity	Salah et al. (2013)
Anticancer activities in three cancer cell lines, HeLa, Hep3B, and SW480, of differently charged chitosan oligosaccharide derivatives	Highly charged chitosan oligosaccharide derivatives significantly reduce the cancer cell viability mainly via interacting with cancer cells electrostatically, regardless of positive or negative charge status	Huang et al. (2006)
Investigation of the relationship between physicochemical characteristics (molecular weight and degree of deacetylation) and functional properties (viscosity, ability to form spherical gel, drug release behavior, and biodegradation) of chitosan from the perspectives of drug delivery	Chitosan species with a high degree of deacetylation are characterized by a low molecular weight Higher-molecular-weight chitosan translates to the formation of a more viscous polymeric solution Chitosan with a lower degree of deacetylation tends to be degraded more rapidly by means of enzymatic digestion The ease of spherical gel formation in aqueous amino acid solution or aqueous solution containing metal ions is affected mainly by viscosity of the chitosan solution Drug diffusion rate from the chitosan gel is controlled by density of the gel matrix structure, which is governed by viscosity of the chitosan solution prior to gelation	Kofuji et al. (2005)
Development of a method to prepare water-soluble chitosan and quantitative investigation of the dependence of water solubility on N-acetylation degree and molecular weight	N-acetylated chitosan with about 50% acetylation degree exhibits the highest level of water solubility The water solubility increases with a reduction in the molecular weight of chitosan	Kubota and Eguchi (1997)
Studies on the effects of chitosan molecular weight and degree of deacetylation on the rheology and film formation properties of gelatin-based films	The interaction between gelatin and chitosan is stronger in the blends made of chitosan of higher molecular weights or higher degrees of deacetylation than the blends made of chitosan of lower molecular weights or degrees of deacetylation Chitosan of larger molecular weights or higher degrees of deacetylation yields gelatin-chitosan films with longer junction zones or longer strands via the formation of strong bonds during film formation	Liu et al. (2012)

(continued)

Table 5.1 (continued)

Experiment	Remark	Reference
Effects of physicochemical characteristics (molecular weight, morphology, and form of chitosan particulates) of five commercial chitosan products on fat-binding capacities	The high-molecular-weight chitosan (2100 kDa) shows a higher fat-binding capacity than the low-molecular-weight chitosan (30 and 890 kDa)	Panith et al. (2016)
Drug release characteristics of matrix tablets prepared from chitosan-clay microparticles with various molecular weights of chitosan	An increase in molecular weight of chitosan microparticles provides the tablet with a higher level of hardness as a function of compression pressure Chitosan-clay tablets provide sustained drug release kinetics in both acidic and neutral media The rate of drug release from the tablets in neutral media decreases with an increase in the chitosan molecular weight	Khlibsuwan and Pongjanyakul (2016)

physicochemical and biological characteristics with the amino content, degree of deacetylation, and molecular weight of chitosan.

5.2.2 Biological Properties

Different chitosan sources and its derivatives have different chemical structures and physicochemical properties, which may result in novel bioactivities or novel findings of bioactive compounds. Chitosan has attracted considerable interests in pharmaceutical and biomedical applications because of its biological activities, namely, anti-inflammatory (Azuma et al. 2015; Abraham et al. 2017; Chaudhary et al. 2011), antimicrobial (Islam et al. 2016; Kong et al. 2010; Chien et al. 2016), antifungal (Seyfarth et al. 2008; Roller and Covill 1999), antiviral (Chirkov 2002), and antioxidant activities (Song et al. 2013; Wan et al. 2013; Yen et al. 2008), and hypocholesterolemic (Zhang et al. 2008b; Suganoa et al. 1988), antitumor (Chien et al. 2016; Rata-Aguilar et al. 2012; Zheng et al. 2015; Ouchi et al. 1989; Kim et al. 2008), and mucoadhesive effects (Shitrit and Bianco-Peled 2017; Dhawan et al. 2004; Roldo et al. 2004; Abruzzo et al. 2015; Xu et al. 2017).

Chitosan and its derivatives have been extensively used as colon drug delivery carriers, and a number of formulations are being developed for colon-specific drug delivery due to their mucoadhesive properties. The first study on mucoadhesive characteristics reports that many commercially available chitosans adhere strongly in vitro to the mucosa through hydration, hydrogen bonding, ionic interactions, and interaction between positively charged amino groups of chitosan and the negatively charged mucus gel layer (Lehr 1992). Electrostatic interaction between polycationic surfaces from the amino groups of chitosan and negative charges of the mucin layer

creates molecular attractive forces to generate the mucoadhesive effect. The mucoadhesion of chitosan increases with an increase in the degree of deacetylation, since deacetylation renders the availability of a higher number of free amino groups in the polymer (Kumar et al. 2016). Cationic chitosan derivatives such as N-trimethyl chitosan chloride and cyclodextrin-chitosan complexes can enhance the mucoadhesive properties (Thanou et al. 2000). Chitosan has an ability to protect bioactives from the hostile conditions of the upper gastrointestinal tract and release the entrapped bioactive specifically at the colon through degradation of the glycosidic linkages of chitosan by microflora present in the colon (Sinha and Kumria 2003). This introduces targeted colon delivery. Table 5.2 summarizes the studies focusing on the biological activities of chitosan and its derivatives.

5.3 Oral Colon-Specific Drug Delivery

Oral delivery is the most commonly used and readily accepted form of drug administration (Ensign et al. 2012). It is still the most popular way of drug administration due to high patient compliance and convenience of self-administration (Werle et al. 2008). Oral colon-specific drug delivery represents a possible approach toward efficient treatment of a range of local diseases such as ulcerative colitis, Crohn's disease, diverticulitis, irritable bowel syndrome, Hirschsprung's disease, antibiotic-associated colitis, and colon cancer. Some of the frequently used drugs for the treatment of these ailments include sulfasalazine, dexamethasone, hydrocortisone, metronidazole, prednisolone, and anticancer drugs such as 5-fluorouracil, paclitaxel, and doxorubicin. Chitosan has been extensively used as a colon drug delivery vehicle in various oral dosage forms including tablets, capsules, microparticles/microspheres, beads, nanoparticles, and hydrogels (Gulbake and Jain 2012). Chitosan, as a drug carrier responsive to environmental stimuli, is expected to maintain proper drug concentrations, over adequate time intervals in particular regions of the gastrointestinal tract with systemic side effects minimized.

5.3.1 Mode of Delivery

It is essential for an oral colon drug delivery system to protect the drug from being released in the stomach and small intestine. A number of oral colon-specific drug delivery approaches have been devised to improve the treatment of local diseases affecting the colon while minimizing systemic side effects. Table 5.3 summarizes the oral colon-specific drug delivery strategies.

5.3.2 Limitations

The development of an oral colon-specific drug delivery system is associated with persistent physiological challenges (Amidon et al. 2015). Chitosan has a low aqueous solubility at a physiological pH of 7.4, limiting its role as the drug absorption

Table 5.2 Experimental studies on biological activities of chitosan and its derivatives

Biological activity	Experiment	Remark	Reference
Antioxidant/ antimicrobial	An investigation of physical, mechanical, and antioxidant properties of gelatin and/or chitosan films	The antioxidant activity of gelatin-chitosan films decreases with an increase in the chitosan content The antimicrobial activity increases with a rise in the chitosan content of the composite films Mixing of gelatin and chitosan at a weight ratio of 3:1 or 1:1 may improve the physicochemical performance of the composite films, without altering the antimicrobial property of chitosan or the antioxidant effect of gelatin	Jridi et al. (2014)
Antioxidant	Grafting of phenolic acids (gallic acid, caffeic acid, and ferulic acid) onto N, O-carboxymethyl chitosan by a free radical-mediated reaction	The antioxidant activity in vitro of N, O-carboxymethyl chitosan is greatly enhanced by grafting with phenolic acids	Liu et al. (2013)
Antioxidant	Introduction of quaternized glycidyl trimethylammonium chloride and glycidyl triethylammonium chloride groups on different sites of high-molecular-weight chitosan (400 and 1240 kDa)	The quaternized chitosan (1240 kDa, 97% deacetylation) displays a good antioxidant activity Different forms of quaternized chitosan have different free radical scavenging activities and mechanisms, as an attribute of their molecular weights, contents of active hydroxyl and amino groups, positive charge, and steric effect	Wan et al. (2013)
Antioxidant	Characterization of solubility and antioxidant activity of chlorogenic acid-chitosan conjugates	Total antioxidant capacity increases with an increase in the chitosan content and degree of chlorogenic acid-chitosan conjugation	Rui et al. (2017)
Anti-inflammatory	Investigation of the effects of incorporating chitinase-hydrolyzed shrimp shell chitin into the diet of hybrid tilapia with regard to intestinal immune status and autochthonous gut bacteria and protection against bacterial pathogen	Dietary supplementation with chitosan oligosaccharides significantly reduces the inflammatory response in the intestine of tilapia	(Qin et al. 2014)

(continued)

Table 5.2 (continued)

Biological activity	Experiment	Remark	Reference
Antimicrobial	Development of an environmentally friendly, organic, antibacterial material from chitosan particles	The permanent positive charges in the form of quaternary ammonium groups are introduced to the surface of prefabricated chitosan particles under heterogeneous conditions via either a direct methylation or a reductive N-alkylation using aldehyde-propionaldehyde and benzaldehyde followed by methylation with methyl iodide All quaternized chitosan particles exhibit a higher antibacterial activity against *Staphylococcus aureus* than the chitosan particles at a neutral pH medium	Wiarachai et al. (2012)
Antimicrobial/ antitumor	Evaluation of the antimicrobial and antitumor activities of chitosan from shiitake stipes and crab shells prepared by different N-deacetylation treatments	Chitin/chitosan from shiitake stipes exhibits a more effective antimicrobial activity than that of crab shells	Chien et al. (2016)
Antitumor	A study on the anticancer activities of differently charged chitooligosaccharide derivatives using three cancer cell lines	The results suggest that highly charged chitooligosaccharide derivatives can significantly reduce the viability of cancer cells, regardless of their charge status Further studies by fluorescence microscopic observations and DNA fragmentation reveal that necrosis is the main cause of the anticancer effect of highly charged chitooligosaccharide	Huang et al. (2006)
Antitumor	Evaluation of the anticancer abilities of chitin, chitosan, and low-molecular-weight chitin using a human tumor cell line THP-1	Low-molecular-weight chitin has a higher tumor-suppressive activity, and the tumor suppression increases significantly with reduced molecular weight of chitin	Salah et al. (2013)

(continued)

Table 5.2 (continued)

Biological activity	Experiment	Remark	Reference
Antitumor	Effects of degrees of acetylation and polymerization of chitosan on its antiangiogenic activity	The inhibitory effect of chitosan on angiogenesis is dependent on its degrees of acetylation and polymerization	Wu et al. (2012)
Fat-binding and hypocholesterolemic effects	A comparative study on hypolipidemic activities of high- and low-molecular-weight chitosan in rats fed with high-fat diets	Chitooligosaccharide with a higher degree of polymerization exhibits a more pronounced effect on the increase of fecal fat and cholesterol in mice	Zhang et al. (2012)

enhancer due to reduced cationic character and mucoadhesiveness (Gulbake and Jain 2012). The highly crystalline structure of chitosan enhances inter- and intramolecular hydrogen bonding that in turn negates the solubility of chitosan in a wide pH range along the gastrointestinal tract (Amidon et al. 2015; Gulbake and Jain 2012). In order to resolve the challenges, chemical modification of chitosan has been exploited and evaluated regarding its potential for oral colon drug delivery through introducing additional functionality to chitosan.

5.4 Chitosan Derivatives/Formulations in Oral Colon-Specific Delivery

5.4.1 Design Features

What makes chitosan unique over other polysaccharides for colon-specific drug delivery is its chemical structure that permits specific modifications through alteration in the chitosan amine or hydroxyl functional groups. Quaternized, thiolated, hydrophobic, and chemically grafted chitosan derivatives are types that have been reported to improve properties or impart new properties to chitosan for oral colon-specific delivery.

Chitosan derivatives of N-alkyl or quaternary ammonium are characterized by their permanent cationic charge that confers an increase in the aqueous solubility of chitosan over a wide pH range, enhanced mucoadhesiveness, and drug penetration-enhancing properties (Changyong et al. 2016; Ahmed and Aljaeid 2016; Bose and Wong 2018; Sonia and Sharma 2011). N-Alkylated chitosan derivatives such as trimethyl, diethylmethyl, triethyl, and dimethylethyl chitosans are usually obtained by alkylation of the primary amine groups of chitosan with the suitable aldehyde in the presence of reducing agents (Ahmed and Aljaeid 2016). Trimethyl chitosan, for example, is obtained by reductive methylation of chitosan using methyl iodide in the presence of a strong base such as sodium hydroxide at 60 °C (Chang et al. 2009). A multi-particulate dosage form of pectinate gel containing trimethyl chitosan beads

Table 5.3 Oral colon-specific drug delivery strategies

Approach	Drug release-triggering mechanism	Comment	Reference
Prodrug	Prodrugs are inactive derivatives of a drug molecule Cleavage of the bond linkage between drug and carrier via reduction and hydrolysis by colonic bacterial enzymes releases the active drug. Typical enzymes include azoreductase, glycosidase, and glucuronidase	The extent of enzymatic hydrolysis of bond between drug and carrier should be minimized in the upper gastrointestinal tract to enable colon drug delivery	Amidon et al. (2015)
pH-dependent systems	Combination of polymers with pH-dependent solubility to take advantage of the pH changes along the gastrointestinal tract and release drug at the colon following polymer solubilization in situ	Unpredictable site specificity of drug release takes place with a wide inter−/intra-subject variation due to similarity of gastrointestinal pH with reference to disease states, fasted/fed states, sexes, and ages in humans	Xiao and Merlin (2012), Fallingborg et al. (1993), Nugent et al. (2001), Amidon et al. (2015), Ibekwe et al. (2008), and Jennifer B. Dressman et al. (1990)
Time-release systems	The drug is released in the colon after a specified duration of administration	The time-release approach is dependent on the transit time through the gastrointestinal tract Inconsistent gastric emptying time between individuals complicates this approach with respect to the prediction of accurate location for drug release Diseases associated with the colon, such as irritable bowel syndrome, can influence the transit time through the colon	Xiao and Merlin (2012), Amidon et al. (2015), Jennifer B. Dressman et al. (1990), and Fukui et al. (2000)

(continued)

Table 5.3 (continued)

Approach	Drug release-triggering mechanism	Comment	Reference
Microflora-activated systems	Drug release is initiated by primarily fermentation of non-starch polysaccharides of drug carrier by colon anaerobic bacteria	The strategy is highly promising as non-starch polysaccharides can only be degraded in the colon Enzymatic degradation of a polysaccharide matrix is a slow process, usually requiring over 12 h for complete degradation Anaerobic bacteria in the colon such as bacteroides, eubacteria, clostridia, enterococci, and enterobacteria produce numerous enzymes such as glucuronidase, xylosidase, nitroreductase, and azoreductase to ferment polymers of drug carrier and have drug release initiated	Yang et al. (2002) and Vandamme et al. (2002)
Bioadhesive systems	Bioadhesive systems allow a formulation to remain in contact within the colon for a longer period of time to assist the absorption process of poorly absorbable drugs	Polycarbophils, polyurethanes, and polyethylene oxides have been used as the matrix materials of bioadhesive drug carrier The use of a combination of polysaccharides has been found to be more effective for achieving colon-specific delivery compared to the use of a single polysaccharide Cellulose derivatives are frequently used in a combination manner as they are not absorbable systemically upon oral administration	Amidon et al. (2015), Ahmad et al. (2012), and Chourasia and Jain (2004)

(continued)

Table 5.3 (continued)

Approach	Drug release-triggering mechanism	Comment	Reference
Multi-particulate systems	Multi-particulate systems have a smaller particle size in comparison to single-unit systems. They can reach the colon more quickly since they pass through the gastrointestinal tract more easily The multi-particulate systems demonstrate a slower transit in the colon, enabling a greater level of drug exposure in the colonic region	Microparticles and nanoparticles in hydrogel are examples of multi-particulate systems used in oral colon-specific drug delivery	Xiao and Merlin (2012), Amidon et al. (2015), and Chourasia and Jain (2004)

has the capability to deliver the water-soluble high-molecular-weight drug in a colon-specific manner (Atyabi et al. 2005). N,N,N-Trimethyl chitosan/alginate beads containing gold nanoparticles exhibit an excellent biocompatibility with Vero and Caco-2 cells, while alginate beads/gold nanoparticles show a mild cytotoxic effect against both cell lines (Martins et al. 2015).

Carboxymethyl chitosan derivatives are attained through introducing a carboxymethyl group to the hydroxyl and amine moieties of chitosan. This modification increases chitosan's solubility in neutral and basic solutions without affecting other important characteristics. Among various methods of chitosan modification, carboxymethylation is the most attractive as such derivative is nontoxic, biodegradable, and biocompatible and exhibits antibacterial and antifungal bioactivities (Jayakumar et al. 2010). A study on chitosan-based nanogels prepared by electrostatic interaction between chitosan and carboxymethyl chitosan using tripolyphosphate and calcium chloride as ionic cross-linkers indicates that the nanogel exhibits prolonged contact with the intestinal mucosa and improves local drug concentration (Feng et al. 2015). Design of the polyelectrolyte complex composed of chitosan and O-carboxymethyl chitosan as a pH-responsive carrier for oral delivery of doxorubicin hydrochloride suggests that oral administration of such complex is effective in delivering doxorubicin hydrochloride, giving an absolute bioavailability of 42% (Feng et al. 2013).

Thiolated chitosans are obtained by modification of the chitosan amine groups with cysteine, 2-iminothiolane, thiobutylamidine, or thioglycolic acid. The thiolated chitosan derivatives have strong mucoadhesiveness and permeation-enhancing properties attributed to the formation of in situ gelling behavior through inter- and intramolecular disulfide bonds with cysteine-rich domains of mucus glycoproteins

(Ahmed and Aljaeid 2016; Hornof et al. 2003, 2004; Bernkop-schnürch et al. 2003). Curcumin/5-fluorouracil-loaded thiolated chitosan nanoparticles have been formulated and subjected to anticancer activity screening using HT-29 cell line and in vivo pharmacokinetics analysis using the mouse model (Anitha et al. 2014). The combination drug system shows enhanced anticancer effects on colon cancer cells in vitro and improved drug bioavailability in vivo.

Chemical grafting of chitosan is a process by which one or more species of blocks are connected as a side chain to the main chitosan chain, resulting in the formation of macromolecular copolymers with modified physical and chemical properties (Jayakumar et al. 2005). The properties of the resulting graft copolymers are broadly controlled by the characteristics of the side chains, including molecular structure, length, and number (Jayakumar et al. 2005). Chitosan-folate conjugate has been designed to encapsulate the hydrophilic 5-fluorouracil and to control its release (Li et al. 2011). The in vitro drug release study demonstrates that the chitosan-folate conjugate nanoparticles can reduce drug release to a greater extent than chitosan nanoparticles. The chitosan-folate nanoparticles, co-loaded with 5-fluorouracil and leucovorin and prepared by ionic gelation technology followed by encapsulation by enteric polymer, succeed to deliver the drugs to the colon (Li et al. 2015).

5.4.2 Preparation Methods of Chitosan-Based Oral Colon-Specific Drug Delivery System

A chitosan-based oral colon-specific drug delivery system can be prepared using different procedures, depending on the kind of drugs and excipients employed and the final purpose of the system. The commonly used techniques are ionic gelation, emulsification-solvent evaporation, and spray drying.

The ionic gelation method proceeds with ionic cross-linking of chitosan with low-molecular-weight counterions, hydrophobic counterions, and high-molecular-weight ions. Chitosan-based micro- and nanoparticles for colon-targeted delivery of vancomycin have been prepared by the ionic gelation method using tripolyphosphate as the gelating agent (Cerchiara et al. 2015). Generally, the chitosan is dissolved in acetic acid solution to produce the cationic phase. The anionic tripolyphosphate solution, mixed with drugs, is added dropwise to the cationic phase to form chitosan particles. The particles can be isolated by centrifugation and subjected to freeze-drying. In addition to tripolyphosphate, glutaraldehyde and polyaspartic acid (Zhang et al. 2008a) can be used as the hardening agent of chitosan.

The emulsification-solvent evaporation method refers to the preparation of an emulsion, with aqueous and oil phases blended intimately, followed by solvent evaporation to produce the particles (Inés Paños et al. 2008). In recent years, microencapsulation of nanoparticles has been performed by means of the emulsion-solvent evaporation technique (Wang et al. 2013). An example of such use is the Eudragit S-100 as the enteric polymer. The polymer is dissolved in an

organic solvent mixture (dichloromethane/ethanol/isopropanol in 5:6:4 volume ratio). The drug-loaded chitosan-folate nanoparticles (coat to core ratio of 5:1 and 10:1) are directly dispersed into the enteric polymer solution under magnetic stirring. The mixed suspension is subsequently added into liquid paraffin containing 1% w/w sorbitan sesquioleate as the emulsifying agent. The oil/oil emulsion is formed under stirring, and the organic solvent is completely removed by evaporation to produce chitosan-folate nanoparticles-entrapped microparticles that can be collected by vacuum filtration and freeze-drying.

Spray drying is a relatively simple process that has been used by the manufacturing industry since 1927. The process of spray drying involves spraying a solution of the polymer, in which the drug is solubilized, inside a chamber at high temperatures (Inés Paños et al. 2008). The drug is first dispersed in an aqueous acidic solution of chitosan with the addition of a suitable cross-linking agent when necessary. The liquid sample is then atomized in a stream of hot air to form small droplets of free-flowing particles. The chitosan based micro- and nanoparticles for colon-targeted delivery of vancomycin have been prepared using two different spray-drying processing methods (Cerchiara et al. 2015). The first method applies Mini Büchi Spray Dryer B-191 (Büchi Labortechnik AG, Flawil, Switzerland) under the following drying conditions: inlet temperature 120 °C, outlet temperature 60–70 °C, and air flow rate 700 NI/h to produce microparticles. Büchi Spray Dryer B-90 "Nano" (Büchi) is used as an alternative technological approach with the following drying conditions: inlet temperature 80 °C and outlet temperature 40 °C for the preparation of nanoparticles.

Chitosan and its derivatives have been exploited in the development of oral dosage forms such as tablets (Shao et al. 2015; Abruzzo et al. 2015), pellets (Wong and Nurulaini 2012), spheroids (Zolkefpeli and Wong 2013), hydrogels (Xu et al. 2017; Chang et al. 2009; Yu et al. 2017; Delmar and Bianco-Peled 2015), beads (Alfatama et al. 2018; Wong and Nurjaya 2008; Seth et al. 2014), microparticles (Elbaz et al. 2016; Du et al. 2014), and nanoparticles (Haziyah et al. 2016; Syed Mohamad Al-Azi et al. 2014; Tekie et al. 2016; Ji et al. 2012). Table 5.4 summarizes the experimental studies related to chitosan and its derivatives/formulations for oral drug delivery, specifically in the colon-specific mode.

5.4.3 In Vitro and In Vivo Experimental Outcomes

Table 5.5 summarizes the in vitro cell culture/drug release studies and in vivo experimental outcomes of chitosan and its derivatives/formulations pertaining to the use of the dosage forms for oral colon-specific drug delivery. Typically, a favorable in vitro drug release/cell culture profile with respect to colon targeting brings about positive in vivo biological outcomes.

Table 5.4 Experimental studies on chitosan and its derivatives/formulations for oral colon-specific drug delivery

Drug candidate	Dosage form	Experiment	Remark	Reference
Resveratrol	Nanosuspension	Resveratrol-polyethylene glycol-loaded pectin/zinc chloride/chitosan system is developed to form a food-grade colloidal nanosuspension	Nanosuspension shows a promising potential to protect resveratrol from being released in fortification of beverages	Andishmand et al. (2017)
Resveratrol	Microparticle	Zinc-pectin-chitosan composite microparticles are designed as a colon-specific delivery system of resveratrol	The cross-linking solution pH, cross-linking time, and chitosan concentration exhibit a major influence on the drug release pattern of microparticles Formulation prepared at lower pH (1.5) demonstrates very low drug release in the upper gastrointestinal conditions (<8% drug release after 5 h) followed by rapid but sustained drug release in the colonic condition (>86% drug release after 12 h) Drug release decreases with an increase in cross-linking time of particles. Formulation cross-linked for a longer duration (120 min) produces a sufficiently strong matrix which retards a major fraction of drug release in the upper gastrointestinal tract, but has drug released in the simulated colonic fluid in a controlled manner Formulation prepared with 1% chitosan exhibits a lower drug release propensity in	Das et al. (2011)

(continued)

Table 5.4 (continued)

Drug candidate	Dosage form	Experiment	Remark	Reference
			the simulated intestinal fluid (<8% drug release after 5 h) followed by enhanced and prolonged drug release in the simulated colonic fluid (>86% drug release after 12 h of dissolution)	
Enoxaparin	Nanoparticle	Development of alginate-coated chitosan core shell nanoparticles through ionic gelation method	Coating of alginate over the chitosan nanoparticles improves the release profile of enoxaparin from the nanoparticles where only 1.98 ± 0.17% of drug is released in the first 2 h in the simulated gastric fluid, whereas more than 40% of drug is released from the nanoparticles in the subsequent parts of the gastrointestinal tract	Bagre et al. (2013)
Coomassie brilliant blue G 250	Bead	Pectinate beads prepared with different formulation/processing variables, such as cross-linker type, cross-linking time, cross-linker concentration, trimethyl chitosan chloride/pectin weight ratio, pectin concentration, and voltage of bead generator, are produced with optimal physicochemical characteristics for colon-specific drug delivery of water-soluble drugs	Formulations with trimethyl chitosan do not release 100% of the loaded drug in the soluble form even after the beads are completely degraded The drug-trimethyl chitosan may complex and render incomplete drug release which masks the drug absorption-enhancing effect of trimethyl chitosan	Atyabi et al. (2005)
Icariin	Microsphere	Design of alginate-chitosan microspheres loaded with icariin by emulsification-internal	Slow icariin release (10%) from microspheres is demonstrated in the	Wang et al. (2016)

(continued)

Table 5.4 (continued)

Drug candidate	Dosage form	Experiment	Remark	Reference
		gelation technique with glutaraldehyde as a cross-linker	simulated gastric fluid and intestine fluid, whereas 65.6% icariin is released in the simulated colonic fluid	
Vancomycin	Microparticle	Design of spray-dried polyelectrolyte complex microparticles of chitosan and carboxymethyl cellulose with lauric acid coat introduced by fusion technique	The chitosan/carboxymethyl cellulose (1:3) microparticles show the best performance in terms of yield and drug encapsulation efficiency. Coating of microparticles facilitates the delivery of vancomycin to the colon	Cerchiara et al. (2016)

5.4.4 Unique Characteristics and Limitations

An oral colon-specific drug delivery system offers smart and systematic delivery of the drug specifically to the desired site of action. Targeting of a drug not only potentiates the efficacy but also limits the toxicity of the drug by altering its biodistribution and pharmacokinetics profile (Gupta et al. 2017). Unique characteristics owing to formulation designs are able to deliver the drug efficiently to the colon and reduce the systemic drug effects. Table 5.6 summarizes unique characteristics and limitations of chitosan and its derivatives/formulations in oral colon-specific delivery.

5.5 Future Perspectives

Various oral dosage forms have been developed with the use of chitosan and its derivatives for colon-targeted drug delivery. The introduction of chitosan as a part of a formulation enables delayed or sustained drug release, thereby targeting the drug release in the colonic region and increasing the drug availability to the target site for therapeutic actions or absorption. Nonetheless, toxicity evaluation of the oral colon-targeting vehicle is still lacking. Specifically, the relevance of chitosan derivatives as drug delivery carriers requires further toxicity analysis.

Table 5.5 In vitro and in vivo experimental outcomes of chitosan and its derivatives/formulations in oral colon-specific drug delivery

Drug candidate	Experiment	In vitro outcome	In vivo outcome	Reference
5-fluorouracil	Preparation of microspheres of chitosan cross-linked with polyethylene glycol by emulsion cross-linking followed by coating with cellulose acetate phthalate	The neat 5-fluorouracil produces an immediate cytotoxic effect on human HT-29 colorectal cancer cell lines The microspheres on the other hand inhibit the proliferation of tumor cells and induce apoptosis over an extended duration	Using microspheres, a higher 5-fluorouracil concentration is accumulated in the colon though its action to stop the growth of colon cancer cells is still detectable in the cecum and colon after 24 h of administration Albino male Wistar rats treated with microspheres show a marked lymphocytic infiltration in and around the tumor cells, which indicates that the body is responding to the 5-fluorouracil therapy. A significant reduction in tumor volume and multiplicity is witnessed Elevated levels of serum albumin, creatinine, leukocytopenia, and thrombocytopenia are observed in animals receiving standard 5-fluorouracil formulation. The possible systemic side effects are reduced with the use of microspheres	Ganguly et al. (2015)

(continued)

Table 5.5 (continued)

Drug candidate	Experiment	In vitro outcome	In vivo outcome	Reference
Levofloxacin	Development of levofloxacin-loaded glutaraldehyde-cross-linked chitosan microspheres through spray-drying method	Only 15% of levofloxacin is released from the microspheres in the first 2 h in pH 1.2 medium Levofloxacin is gradually released from the microspheres as time lapses (~50% release in 6 h, ~70% release in 12 h, and ~80% release in 20 h in pH 6.8 medium)	The chitosan microspheres can maintain levofloxacin concentration within target ranges in the colon for a long period of time Absence of the severe signs, such as appearance of epithelial necrosis and sloughing of epithelial cells, is detected through histopathologic studies	Jin et al. (2014)
Icariin	Development of icariin-loaded alginate-chitosan microspheres through emulsification-internal gelation technique	Slow release of icariin (10%) from microspheres in the simulated gastric fluid and intestine fluid with 65.6% icariin being released in the simulated colonic fluid	The microspheres loaded with icariin can decrease the rats' colon mucosa damage index by reducing the production and gene expressions of inflammatory mediators and cytokines The microspheres cross-linked by glutaraldehyde can increase the residence time of drugs in the colon and avoid drug loss in the upper and middle regions of the digestive system	Wang et al. (2016)
Progesterone	Development of progesterone-loaded zinc-pectinate/chitosan microparticles through ionotropic gelation approach	Minimal levels of progesterone are released in the simulated gastric fluid (3–9%) followed by burst release at pH 6.8 and a sustained-release phase at pH 7.4	In vivo study using healthy male New Zealand white rabbits reveals that the drug mean residence time in the plasma is more than twofold higher with microparticles than the drug alone	Gadalla et al. (2015)

(continued)

Table 5.5 (continued)

Drug candidate	Experiment	In vitro outcome	In vivo outcome	Reference
Ciprofloxacin	Preparation of methacrylate micro−/ nanoparticles by spray-drying technique	Microparticles with a drug/ polymer ratio of 1:2 release less than 60% of ciprofloxacin after 24 h of dissolution	Oral administration of microparticles to healthy white albino rabbits is characterized by peak plasma concentration of 1.38 mg/L at 5 h of administration compared to free drug which is 6.1 mg/L at about 1 h which is deemed critical to reduce systemic adverse effects The reduced peak plasma drug concentration of particles is associated with their sustained drug release behavior	Muñoz Ortega et al. (2016)
Ibuprofen	Development of polyelectrolyte complex nanoparticles based on chitosan and methoxy poly (ethylene glycol) methacrylate-co-poly(methylacrylic acid) to improve bioavailability of ibuprofen	Drug release study using dialysis bag indicates that after 24 h only 41.3% ibuprofen is released in pH 1.2 buffer and 55.4% is diffused into pH 7.4 buffer from the nanoparticles	After 6 h of oral administration, the body temperature of *Sprague Dawley* rats treated with ibuprofen-chitosan nanoparticles is lower than their initial basal temperature compared to ibuprofen suspension as positive control group Ibuprofen-chitosan nanoparticles do not result in obvious symptoms of piloerection, shivering, and diarrhea compared to negative control group	Shi et al. (2018)

Table 5.6 Unique characteristics and limitations of chitosan and its derivatives/formulations in oral colon-specific drug delivery

Drug candidate	Experiment	Unique characteristics	Limitation	Reference
5-fluorouracil	Oral formulation design of nanoflowers with cationic β-cyclodextrin as matrix core of inclusion complex with alginate and chitosan	The release of drug from cationic β-cyclodextrin in alginate/chitosan nanoflowers is less than 20% at initial 2 h in pH 2.3 medium and less than 60% over 24 h in pH 7.4 medium due to gradual swelling of chitosan and alginate that leads to a controlled and slow release of drug from the nanoflowers	The system is pH dependent. The drug release can be affected by pH variations along the gastrointestinal tract	Lakkakula et al. (2017)
Resveratrol	Resveratrol-polyethylene glycol-loaded pectin/zinc chloride/chitosan system as a food-grade colloidal nanosuspension	The nanosuspension can be an alternative oral dosage form for colon cancer chemotherapeutics In vitro release studies in different simulated gastrointestinal media show that 49% and 60% of resveratrol reach the colon from nanosuspensions prepared with and without polyethylene glycol, respectively	Changes in physiology of the gastrointestinal tract such as colon pH, transit time, and mucosal integrity will influence the efficiency of the nanosuspension system	Andishmand et al. (2017)

5.6 Conclusion

Chitosan has unique physicochemical and biological characteristics which permit its use in the development of oral colon-specific drug delivery systems. Many studies on chitosan formulations of drugs and biological active substances have been successfully conducted with respect to colon drug delivery.

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Chitosan-Based Hydrogels for Drug Delivery

6

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Abstract

The advances in the field of biomaterials have led to several studies on alternative biocompatible devices and to their development focusing on their properties, benefits, limitations, and utilization of alternative resources. Due to their advantages like biocompatibility, biodegradability, and low costs, polysaccharides have been widely used in the development of hydrogels. Among the polysaccharides, which are studied on hydrogel preparations, chitosan (pure or combined with natural/synthetic polymers) have been widely investigated for use in biomedical field, especially due to its biocompatibility and non-toxicity. Thus, the chitosan-based hydrogels play a crucial role in the development of new biomaterials. Many crosslinking (or polymerization) approaches have been developed to convert chitosan into smart hydrogels, with the aim of obtaining new drug delivery devices. Such hydrogels can also undergo changes in their physical-chemical properties in response to environmental

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_6

changes such as pH, ionic strength, temperature, magnetic field, and so forth. In view of potential applications of chitosan-based hydrogels, this chapter focuses on the most recent progress made with respect to preparation, properties, and their salient accomplishments in drug delivery.

Keywords
Hydrogel · Chitosan · Biomaterial · Drug release

6.1 Introduction

Developed from the necessity of biologically compatible materials, hydrogels (HGs) were first mentioned by Wichterle and Lim, in 1960, as hydrophilic gels for biological uses. The HGs were defined as a material with three-dimensional polymer networks, containing hydrophilic groups, which are able to absorb the desirable amount of water. In addition, such material may be absorbed by the organism without unfavourable reactions (Wichterle and Lím 1960).

Back then, synthetic polymers, such as poly(hydroxyethyl methacrylate) (PHEMA) (Wichterle and Lím 1960; Ratner and Miller 1973; Holly 1975), poly (ethylene glycol) (PEG) (Revzin et al. 2001; Koh et al. 2002), and polyacrylamide (PAAm) (Christensen et al. 2003; Lin et al. 2004; Akkaya and Ulusoy 2008; Mukhopadhyay et al. 2014), were used on medical devices, instruments, and implants.

However, most of synthetic polymers have low biocompatibility, and such differences, from biological systems, may lead to severe irritations causing even tumour growth. These responses can be seen in short or long times after the biomaterial contact with the human body (Wichterle and Lím 1960).

In this way, researchers started looking for potential alternatives to develop compatible biomaterials, finding their answer in natural polymers, which are biodegradable and non-toxic and can be found from renewable sources (Nair and Laurencin 2007). Yannas and colleagues were pioneers in that field by incorporating natural polymers into hydrogels for cell encapsulation (Yannas et al. 1989; Pillai and Panchagnula 2001). Among natural polymers, hydrogels based on polysaccharides, such as chitosan (Fukuda et al. 2006; Crompton et al. 2007; Murakami et al. 2010), alginate (Chen et al. 2004; Novikova et al. 2006; Tan and Takeuchi 2007; Jeon et al. 2009), agarose (Meilander et al. 2003; Luo and Shoichet 2004; Liang et al. 2006), and proteins, like gelatin (Tabata et al. 1994; Tabata and Ikada 1999; Yamamoto et al. 2001) and albumin (Hirose et al. 2010), are widely studied due to their unique advantages such as biocompatibility, biodegradability, non-toxicity, and low cost (Pitt 1990). Chitosan (CS), which is a natural cationic and hydrophilic polymer, has been the object of several studies by researchers in the areas of biotechnology. This polysaccharide, obtained from alkaline hydrolysis of chitin, is one of the most abundant natural amino polysaccharides extracted from the exoskeleton of crustaceans and insect, fungal cell walls, etc. (Pellá et al. 2018).

The presence of amine groups (-NH_2) and hydroxyl groups (-OH) along the chitosan chains, is useful for providing properties of the desired HG. In addition, such functional groups may be crosslinked by reacting with crosslinker agents (Xiao et al. 2016a). Moreover, the amine groups can be easily converted to ammonium groups, below pH 6.3, making chitosan an ideal candidate for use in the preparation of pH-responsive hydrogels. Besides non-toxicity and biocompatibility, chitosan can be degraded in vivo by several enzymes, mainly by lysozyme (a non-specific protease present in all mammalian tissues) (Szymańska and Winnicka 2015).

Furthermore, the products from degradation of chitosan are non-toxic oligosaccharides, which can be then excreted or incorporated to glycosaminoglycans and glycoproteins. These properties make chitosan suitable for clinical use. In addition, chitosan may enhance drug penetration by opening the tight junctions between epithelial cells (Mohammed et al. 2017). These properties make chitosan an ideal candidate for use in the preparation of new materials for biomedical applications.

Herein, we described important studies on the development of hydrogels based on chitosan, in combination with other polymers, as a biomaterial of high biological relevance and near-physiological approach. We addressed the issues of propriety-structure relationships, drug-loading techniques, applications, and future perspectives on using chitosan for producing biomedical devices.

6.2 Basic Concepts and Properties of Hydrogels

Hydrogels are soft materials, with three-dimensional structure, that are similar to the extracellular matrix. The crosslinked hydrophilic polymer network structure is able to swell absorbing biological fluids or water. The swollen hydrogels have elastic characteristic, and thus they can easily be applied to normal or damaged tissue (Salva and Akbuga 2017).

Such structure consists of either physically or chemically crosslinked polymers (Ahmed 2015; Michele K. Lima-Tenório et al. 2015). In addition, the crosslinking can be formed either in vitro (during preparation) or in vivo (after application at a specific location inside the body). Furthermore, HGs can be classified based on their source (synthetic or natural), configuration (crystalline or amorphous), physical appearance (matrix, film, or microsphere), network electrical charge (ionic, neutral, zwitterionic) (Eichenbaum et al. 1998; Horkay et al. 2005; Kim and Shin 2007; Ahmed 2015), or polymeric composition (homopolymer, copolymer, or interpenetrating polymeric network). Physically crosslinked HGs are obtained when the polymeric chains hold together by physical interactions, such as electrostatic forces, van der Waals forces, hydrogen bonds, or even entanglements between the polymer chains. On the other hand, the chemical crosslinking consists in the formation of covalent bonding between polymeric networks (Ahmed 2015). A detailed discussion of crosslinking methods, as well as their properties, is discussed in Sect. 6.3.

When a dried hydrogel gets in contact with solvents, especially with water, the solvent molecules start penetrating into the polymeric network, creating a rubbery region, while the dried regions remain in a glassy phase. The amount of water absorbed by the HG depends on the type and extent of its porosity, pore interconnections, and pore size (Hoffman 2012). Therefore, hydrogels can also be classified in four classes according to pore size: non-porous, microporous, macroporous, and super-porous hydrogels (Ganji et al. 2010).

A non-porous hydrogel has molecular-size pores and shows a slow swelling ratio due to their densely packed polymeric chains, which restricts the solvent transport through diffusion. Microporous hydrogels are the ones with pores ranging from 100 Å to 1000 Å. They show a densely packed polymeric chain and slow swelling rate, which is dependent on the sample size. Macroporous hydrogels have pores ranging from 0.1 μm to 1 μm and tend to swell faster than two the aforementioned. The solvent/solute transport occurs by the combination of molecular diffusion and convection through the pores. Finally, super-porous hydrogels, however, show high number of pores, and they swell very fast. Since the HGs with high porosity are able to absorb solvent molecules, bioactive agents may be "entrapped" into the pores for further release on desirable and/or specific environments. This property makes the HG a very interesting device to be applied as drug delivery systems (Alarcon et al. 2005; Mano 2008; Ashley et al. 2013).

In order to improve some hydrogel properties, including for controlled release, several alternatives were evaluated leading to the discovery of a brand-new type of material with stimuli-responsive properties, increasing significantly hydrogels' possible application. These ones have the ability to quickly respond in face of an external stimulus, such as pH, electric stimulus, magnetic field, and temperature (Alarcon et al. 2005; Lima-Tenório et al. 2015a, b).

Chitosan-based HGs can be prepared either directly from native chitosan (combined by itself or with anionic small molecules) or combined with other polymers. It is well reported that chitosan is soluble in acid medium owing to the presence of amine groups (N-free) from D- glucosamine units. At this pH, N-free units are positively charged limiting the interchain interactions (Sereni et al. 2017). Moreover, the ability to change the apparent charge density under certain experimental conditions (e.g. pH, ionic strength, temperature) has been exploited to produce hydrogels. Chitosan can be self-crosslinked either by increasing the pH or by dissolving in a nonsolvent. This kind of material has biocompatibility (intrinsic of chitosan) and can be considered safe for clinical applications, since no organic solvent or toxic crosslinker is needed (Kiene et al. 2018). In addition, it possesses a dense scale mesh-like network which only allows for passive diffusion nutrients and metabolic wastes being not suitable for biomedical applications. However, CS self-crosslinked has a weak mechanical properties and uncontrolled dissolution. This drawback can be avoided by combining chitosan with either natural or synthetic polymers for tuning the HG properties (Kiene et al. 2018).

6.3 Strategies for Preparation of Hydrogels Based on Chitosan

Several crosslinking alternatives have been developed for the formation of hydrogel matrices. The crosslinking methods can be divided into two major groups: (i) physical crosslinking and (ii) chemical crosslinking. Also, interpenetrating and semi-interpenetrating hydrogels have been reported for the formation of hydrogel matrices (Ahmed 2015).

The properties of the hydrogels, such as, self-healing, biodegradability, swelling degree, mechanical resistance, among others, are related such crosslinking method used to prepare hydrogels (Pellá et al. 2018). Depending on the desired application, it is important to determine the synthesis method. For example, in biomedical application, which it is necessary a biodegradable hydrogel, physical crosslinking is advisable. Otherwise, for environmental application, that used a pH range, the appropriated method is chemical crosslinking (Oladipo et al. 2015; Falco et al. 2017).

6.3.1 Physical Crosslinking

Several researches have been focused on developing the synthesis of physically crosslinked hydrogel, especially due to the relatively facile production. It does not require the use of crosslinker agent; therefore, the trapped materials (for instance, drug) are not degraded. In addition, such HGs have self-healing properties. As mentioned earlier, electrostatic forces, van der Waals forces, hydrogen bonds, or even entanglements between the polymer chains are employed to obtain physically crosslinked HG. In addition, it is important to point out the physical crosslinking methods that form fragile hydrogel compared to chemical ones (Liu et al. 2016; Mahinroosta et al. 2018).

An approach used to produce chitosan hydrogels without external crosslinker can be the freeze-melting-neutralization method. In this method, CS solution is frozen at controlled temperature. After freezing, the solution chitosan is placed in a gelling solution (ethanol and strong base) below the freezing temperature of CS that will induce degelation (Hsieh et al. 2007).

For example, Xu, Han, and Lin synthetized a multilayer gradient CS hydrogels which are capable of mimicking the tissue structure by the physical gelation of pure CS. Thus, the obtained hydrogel was applied for the release of bovine serum albumin (BSA) (model drug). By this approach, the hydrogel formation may be associated with the following factors: (i) neutralization of the $-NH_3^+$ sites of chitosan in $-NH_2$, resulting in loss of ionic repulsion between the chains, (ii) formation of crystallites, (iii) formation of hydrogen bonding, and (iv) hydrophobic interactions. The CS hydrogel shows a multilayered structure composed of nanofibres with interconnected pores and good mechanical properties (Hsieh et al. 2007).

Freeze-melting-neutralization method requires the use of strong bases, such as sodium hydroxide and ammonium hydroxide, during the synthesis of CS hydrogels. However, strong bases can affect the biocompatibility of hydrogels, since they are difficult to eliminate. In addition, the strong basic environment may be responsible for destroying the drugs in the hydrogel. In the same work mentioned above, Xu, Han, and Lin synthesized chitosan hydrogel by the freeze-melting-neutralization method, without the use of strong bases. In this case, the gelation solution was formed by phosphate buffer solution (PBS, pH = 7.4), NaCl, or both (PBS and NaCl), and the frozen CS solution had to remain immersed for 48 h at 4 °C.

Another alternative to obtain CS-HG without chemical crosslinkers is the freeze-thawing method. This method uses freeze-thaw cycles of aqueous polymer solutions to obtain robust and elastic hydrogels (Alves et al. 2011). Abureesh, Oladipo, and Gazia synthesized chitosan-poly(vinyl alcohol) (CS-PVA) hydrogels using boric acid like crosslinking and freeze-thawing. For this method, the CS-PVA hydrogel was frozen at −20 °C for 24 h. Subsequently, the hydrogel was thawed at 25 °C for 6 h. To obtain the hydrogel, three cycles of freezing and thawing were used, and BSA was used as the model drug for the release. The final material showed better stability with the addition of boric acid, and the experimental results demonstrated that pH and glucose concentration influence the release behaviour and the absorption capacity (Abureesh et al. 2016).

Complex coacervation is also used to obtain CS hydrogels (CS-HG). In complex coacervation method, ionic interactions occur between two or more oppositely charged polymers, usually using proteins and polysaccharides. The main determining factor for complex coacervation is the electrostatic interaction between charged macromolecules present in the reaction medium. Using this approach, Xiao and co-workers synthesized CS and carboxymethyl konjac glucomannan (CMKGM) hydrogels with potential for colon-targeted delivery. In such study, the effect of DS of chitosan, pH (2.5–7.0), temperature (25–75 °C), ionic strength (NaCl concentration, 0–50 mmol/L), CMKGM mixing ratio and chitosan (3:1, 2:1, 1:1, 1:2, and 1:3), and the concentration of the biopolymer (w / v 0.05%, 0.1%, and 0.15%) were investigated. The optimal condition for obtaining the hydrogel was at pH 6.5 with a mixing ratio of 1:1 with the total biopolymer concentration range of 0.05–0.15% (w/v). The influence of temperature was not observed, while ionic strength weakens the formation of the hydrogel. For this method, the electrostatic interaction and hydrogen bonds are involved in complex coacervation (Abureesh et al. 2016).

Polyelectrolyte complexation (PEC) is a safe, effective, and green alternative for the synthesis of chitosan hydrogels with interesting swelling characteristics. PEC can be formed by ionic interactions of two opposite charge polymers in solution, with cationic charges of CS, referring to the amino group and anionic group of another polymer. As an example of the use of PEC, Chen et al. obtained CS-HG in polyanionic polymers (alginate (SA) and poly(glutamic acid) (PGA)) in acidic atmosphere with application in colon-specific drug. The formation of the CS/PGA/SA hydrogel in acidic atmosphere facilitates the solubilization of chitosan and produces a homogeneous hydrogel with stable structure. The formation of the

hydrogel is due to the interaction of the -NH_2 groups of CS and -COO^- of PGA and SA. The hydrogels exhibited pH-sensitive properties, and in vitro experiments demonstrated that the composite hydrogel could control the piroxicam (PXC) release as a colon-specific drug delivery carrier and thus reduces the gastrointestinal irritation side effect of PXC (Chen et al. 2018).

The gel-casting technique was used by Konwar in the synthesis of hydrogels of chitosan with magnetic graphene. Variations in the concentration of magnetic graphene oxide (0.05%, 0.1%, and 0.2% w/w) and acid solution of glycerol (acetic acid/glycerol) were used in the synthesis of the hydrogel. In this method, the three hydroxyl groups of glycerol play a key role in interacting to the CS polymer. In this system, crosslinking is given by secondary interactions, such as hydrogen bonding, and by electrostatic interactions between the -NH_3^+ group of chitosan and the -OH groups of glycerol and magnetic graphene oxide. Hydrogels presented antimicrobial activity against many novice microorganisms, including MRSA, which commonly infects wounds and food products (Konwar et al. 2016). The combination of electrostatic interactions and the hydrophobic association can generate thermosensitive CS-HG, as suggested by Barragan and collaborators. They combined CS-HG with glycerophosphate and phosphorylated β-cyclodextrin with gelling time of less than 1 min. The material is suitable for injected solutions since the gelation temperature was close to the physiological conditions. Moreover, the ability to encapsulate hydrophobic molecules in the hydrogel is related to the addition of phosphorylated β-cyclodextrin in the hydrogel (Ramirez Barragan et al. 2018).

In addition, the chemical modification of the CS structure may induce gelation as a function of temperature. For example, Bhattarai and colleagues modified the chitosan structure with PEG. The formation of the hydrogels was dependent on the amount of PEG added to the chitosan chains with gelatinization temperature ranging from 10 °C to 37 °C (Bhattarai et al. 2010). Using 1,2-butene oxide, Sun et al. (2017) obtained hydroxybutyl chitosan at which gelation is obtained at 37 °C.

To obtain CS-HG by physical crosslinking with tuned mechanical properties, Li and colleagues synthesized a series of physical CS-Ag^+/NH_3 hydrogels with different concentrations of CS (0.5–3.0 wt.%) and Ag^+ (0.085–0.424 wt.%). In such work, the authors observed the mechanism of hydrogel formation was dependent on the ammonia atmosphere (Ramirez Barragan et al. 2018).

6.3.2 Chemical Crosslinking

Chemical crosslinking hydrogels are formed by irreversible covalent bonds, which provide good mechanical strength and preserve the properties of the hydrogel over time. Chemical crosslinking occurs due to the reactions of the functional groups such as -OH, -COOH, and -NH_2 with the crosslinkers. There are several methods reported in the literature for obtaining hydrogels by chemical crosslinking (Hamedi et al. 2018; Pellá et al. 2018).

To synthesize chemically crosslinked CS-HGs, the functional groups of chitosan (e.g. -NH_2 and -OH) must react with a crosslinker agent. Up to date, the most common crosslinking agents used to obtain chitosan hydrogels are genipin and

glutaraldehyde (Hamedi et al. 2018). Genipin is a chemical and bifunctional crosslinker, and it is the most popular and non-toxic to produce CS-HG, while glutaraldehyde is a synthetic chemical crosslinker and reported as highly cytotoxic; however, this disadvantage is addressed by ensuring that all functional groups of the aldehydes are actually crosslinked. A series of CS-HG are being prepared using these chemical crosslinkers in biomedical applications (Mirzaei et al. 2013; Dimida et al. 2015; Zhang et al. 2016, 2018b).

For example, hydrogels can be obtained by chemical reaction of hydroxylated carboxymethyl chitosan with glutaraldehyde. The mechanism of hydrogel formation is associated with inter- and intramolecular bonding of amino groups (from chitosan) with glutaraldehyde.

Using genipin, the formation of the hydrogel is achieved by the formation of heterocyclic amines. The amount of genipin influences both the gelation time and the biocompatibility. For example, as demonstrated by Zhang and co-workers, a hydrogel with 0.5% genipin exhibits excellent biocompatibility (Zhang et al. 2016).

Chemical crosslinking can be performed in the presence of ultraviolet (UV) light and with (or even without) the presence of a photoinitiator. The irradiation time is an important factor because it interferes with the degree of crosslinking, and when the exposure time is longer, the hydrogel presents a lower rate of swelling and high mechanical properties (Rickett et al. 2011; Hamedi et al. 2018). In this type of crosslinking, the chitosan must have polymerizable groups. The photopolymerization is initiated by free radicals, produced by photoinitiators, in the presence of UV light, in which the double bonds of the monomers and the propagation of the active radical are attacked, creating a network of crosslinked polymers (Hu et al. 2012). The photosensitive groups can also be attached to the -NH_2 groups of CS to obtain a monomer which may undergo photopolymerization (Zhou et al. 2018). Aycan et al. obtained chitosan-grafted glycidyl methacrylate, a precursor monomer with photopolymerizable groups. This monomer when combined with (ethylene glycol) diacrylate (EGDA) and bone ash gave a new HG that mechanically strengthened the hydrogels with no toxic effects on L929 cells (Aycan and Alemdar 2018).

The Diels-Alder reaction is a chemical reaction between a conjugated diene and a substituted alkene (dienophile). This reaction may also be used to obtain CS-HG. Chitosan must be functionalized with diene and/or the dienophile function. The main advantage, of using the Diels-Alder reaction to synthetize hydrogels, is related to fast reaction in aqueous solution (Montiel-Herrera et al. 2015). This approach was applied by Guaresti and co-workers. They grafted furfural groups (CS-Fu) in chitosan backbone. Then, CS-Fu and bismaleimide (BMI, crosslinker) were used. The hydrogel shows a pH dependence and low degradation in a lysozyme (Montiel-Herrera et al. 2015).

Schiff base and Michael addition are other examples of chemical reactions which may form CS-HG (Montiel-Herrera et al. 2015). In both cases, the reaction occurs in mild conditions. To apply Michael addition reaction, Kiene and co-authors modified the chitosan structure with maleimide and thiolate molecules. The hydrogel was formed instantly after solubilization of chitosan derivative in aqueous solution due to the strong interactions between the polymer chains. The hydrogel kept its physical chemical properties even after lyophilization and rehydration (Kiene et al. 2018).

6.3.3 Interpenetrating and Semi-Interpenetrating Polymer Networks

To show high mechanical and thermal properties and to be able to control the diffusion of solutes in hydrogels, multicomponent networks, such as semi- or interpenetrating polymer networks (IPN), have been developed (Ngadaonye et al. 2013). The IPN hydrogels are organized in two groups: (i) IPN (in which a blend of two polymeric network without covalent bonds to each other is formed) and (ii) semi-IPN (where a polymer diffuses into another crosslinked polymer network) (Myung et al. 2008; Dragan 2014; Priya et al. 2016).

The synthesis of IPN chitosan-based hydrogels represents a convenient way to modify the properties of CS-HG. IPN and semi-IPN chitosan hydrogels can be synthetized with a different synthetic monomer. The monomer most used are polyethylene glycol (PEG), poly(vinylpyrrolidone) (PVP), poly (N-isopropylacrylamide) (PNIPAM), vinyl pyrrolidone (NVP), poly (methacryloylglycylglycine) (MAGG), polyacrylamide (PAAm), poly(acrylic acid) (PAA), isopropylacrylamide (NIPA), poly(ethylene glycol diacrylate) (PEGDA), triethylene glycol dimethacrylate (TEGDMA), and methacryloyl gelatin (GelMA) .

For example, semi-IPN chitosan hydrogel is obtained in combination with acrylic acid (AA), oligo (ethylene glycol) methacrylate (OEGMA), and 2-(2-methoxyethoxy) ethyl methacrylate (MEO2MA) (Che et al. 2018). Other examples of IPN hydrogels based on chitosan are summarized in Table 6.1.

6.4 Drug-Loading Techniques

The drug delivery systems have emerged as an alternative method of disease treatment, once the active substance is loaded into a carrier or device, providing the controlled and sustained drug release at a specific site and at a specific rate, avoiding the overdosage, and reducing side effects (Pellá et al. 2018). Among the different controlled-release systems, the hydrogels, because of their particular properties, are being widely investigated for the design of the ideal future controlled-release systems. Because of the diversity in the chemistry and size of the delivered molecules, the drug loading for controlled release in any chitosan hydrogel can differ widely from one application to another. The method by which the drugs are loaded directly impacts the availability of the drugs during release. Thus, several approaches to incorporating the drug into hydrogel matrix have been proposed. The easiest method of drug loading consists of crosslinking the polymer chains in the presence of the therapeutic drug. Alternatively, the drug can be loaded after the crosslinking by diffusion into the pores of the hydrogel. Despite these methods being easy to perform, the release of the loaded molecules is not well regulated (Hoare and Kohane 2008). Depending on the drug-polymer interactions, the release profile may show a rapid burst release leading to losses of great amount of the therapeutic-loaded drug (Bhattarai et al. 2010).

Table 6.1 List of IPN chitosan-based hydrogels

Type	Network 1 ǀ Network 2	Technique	References
IPN	O-Carboxymethyl chitosan ǀ polyacrylic acid Crosslinking agent (glutaraldehyde)	UV light (photopolymerization)	Che et al. (2018)
	Carboxymethyl-chitosan (CMCS) ǀ gelatin-graft-polyaniline (GP) Oxidized dextran Crosslinked by Schiff base	Body temperature (37 °C) Time 1.5 s	Li et al. (2015)
	Chitosan/poly (ethyleneglycoldiglycidyl ether) ǀ poly (methacrylic acid-co-acrylamide/2-hydroxyethylmethacrylate)	Network 1–40 °C and pH 11 Network 2- cryogelation	Dragan et al. (2016)
	Chitosan (CS) ǀ 2-hydroxyethyl methacrylate (HEMA) CS was crosslinked with glutaraldehyde HEMA was crosslinked with N, N′-methylenebisacrylamide	50 °C for 24 h	Garcia et al. (2017)
	Gelatin and chitosan (CS) ǀ polyvinyl pyrrolidone (PVP) Gelatin and CS were crosslinked with 1,2-epoxy-4-vinylcyclohexane PVP was crosslinked with glutaraldehyde	Ultrasonic Microwave	Wang et al. (2018)
Semi-IPN	4-Azidobenzoic acid-modified chitosan (Az-C) ǀ polyethylene glycol (PEG)	UV light (photopolymerization)	Amoozgar et al. (2012)
	Carboxymethyl chitosan (CMCH) ǀ poly acrylamide (am) and maleic acid (MA) CMCH ionic bonds with ferric solution Am and MA free radical polymerization	55 °C for 5 h	Huang et al. (2016)
	Chitosan (CS) ǀ polydimethylsiloxane (PDMS)/poly(ethylene glycol) CS was crosslinked with hexamethylene-1,6-di-(aminocarboxysulfonate)	Casting forming film at 30 °C	Rodkate et al. (2010)
	Chitosan (CS) ǀ polyethylene oxide (PEO) CS was crosslinked with glutaraldehyde	Room temperature	Xiao et al. (2016b)
	Poly (methacryloylglycylglycine) (MAGG) ǀ chitosan MAGG chemically crosslinked with ethylene glycol dimethacrylate (EGDMA)	Freeze-thawing followed by free radical polymerization (40 °C, 4 h)	Dash et al. (2012)

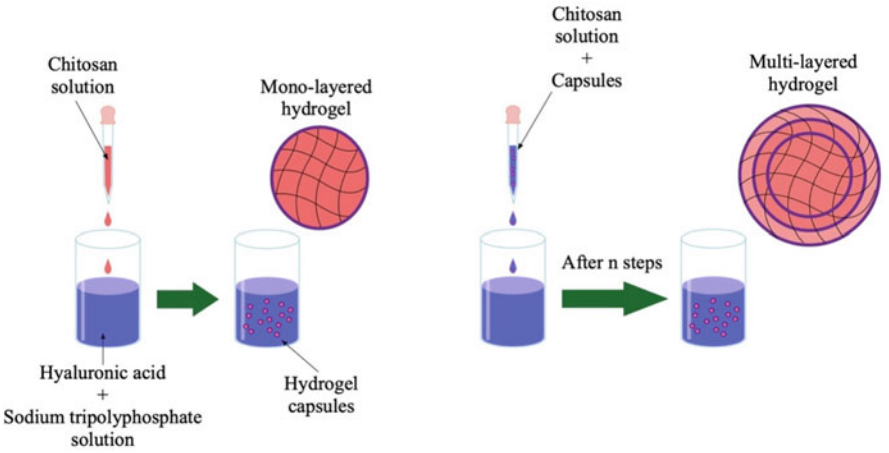

Fig. 6.1 A representative schema of the multilayered hydrogel preparation

With the attempt to overcome this drawback, numerous approaches have been proposed. They include modulating network structure of hydrogels (e.g. increasing the crosslinking density), modulating the drug-polymer interactions, and the incorporation of separated release systems in the hydrogel matrix. For example, Treenate P. and colleagues investigated drug release profiles of pH-sensitive hydrogels composed of hydroxyethylacryl chitosan (HC) and sodium alginate (SA) obtained by different crosslinker agents (e.g. Ca^{2+}, Zn^{2+}, and Cu^{2+}). In this work, the drug release profiles were studied using paracetamol as a soluble model drug. It was observed the burst release of paracetamol was decreased with increasing the HC content and/or applying the crosslinker (Hamedi et al. 2018).

On the other hand, Zhang W. and co-authors have prepared onion-like structure hydrogel capsules based on chitosan for doxorubicin release (Fig. 6.1). Using such method, they could avoid the burst release of doxorubicin. Due to the unique structure and functional groups of the hydrogel capsules, the hydrogel shows pH-responsive properties releasing doxorubicin faster in low pH by non-Fickian diffusion mechanism (Zhang et al. 2019).

Liposomes or nanoparticles containing the therapeutic drug may also be incorporated into the hydrogel. Such approach avoids the burst release, especially for long-term applications (e.g. when several weeks of sustained delivery is needed). For example, in comparison with the system where carboxyfluorescein is free in hydrogels, the release of such drug could be delayed when the drug is encapsulated in liposomes (Billard et al. 2015). O'Neill H. S. and co-authors entrapped drug-loaded liposomes in an injectable chitosan-based hydrogel. Using their approach, they developed a heat-responsive liposome-loaded hydrogel for controlling the release of pro-angiogenic therapeutics. The entrapment of drug-loaded liposomes in an injectable hydrogel provided a prolonged release in target tissues. Moreover, the authors could control the release of different drugs by alternating external stimuli (O'Neill et al. 2017).

The therapeutic drugs can also be covalently bounded to the hydrogel matrix. Thus, the release may be controlled by the cleavage of polymer-drug bond. The covalent conjugation of the therapeutic drugs to the polymer backbone can extend drug release from weeks to months. Usually, the attachment of drug may be performed by click chemistry or by disulphide bonds. However, in both cases, the process of forming covalent bonds must be reversible.

6.5 Chitosan Hydrogels for Drug Delivery Application

Over the past decades, a large number of pharmaceutical agents have been discovered. However, among the active compounds that could serve as therapeutics, very few candidates have shown clinical success. Most of them, although possessing good therapeutic effects, present low bioavailability and poor pharmacokinetics. In this context, drug delivery systems have emerged as an alternative method of disease treatment, once they can provide alternative approach to regulate the bioavailability of therapeutic agents (controlling and sustaining drug release, at specific site and at a specific rate) (Michele K Lima-Tenório et al. 2015). Much effort has been done on designing physical and chemical properties, of drug delivery systems, in order to produce smart devices, for biomedical application. Hydrogels, as a drug delivery device, have grabbed the attention of many scientists, for the design of the ideal future controlled-release systems (Hamidi et al. 2008).

The hydrogel-based delivery systems may be divided into two major categories: (i) the conventional (or time-controlled systems) and (ii) the stimuli-responsive release systems. The difference between them is the last one undergoes changes in response to external stimuli, such as pH, temperature, electric field, ionic strength, magnetic field, and so on, being thus called smart materials. In the last decade, much interest has been done in the development of polysaccharide-based hydrogels, as smart biomaterials. Biocompatibility, biodegradability, non-toxicity, and low cost are some of the intriguing properties of polysaccharides, for the development of biomaterials (Lima-Tenório et al. 2015b). Among the polysaccharides commonly utilized to prepare hydrogels for biomedical applications, chitosan has proved to be very efficient for the delivery of biologically active molecules, especially because of their pH sensitivity (Liu et al. 2016). Moreover, the cationic nature due to the presence of amine group in chitosan makes it a bioadhesive polymer (it can adhere to negatively charged biological surfaces), thus prolonging the residence time of drug-loaded systems and providing localized drug delivery.

In situ forming chitosan-based hydrogels are very attractive for local delivery of drugs, once they can enhance the drug bioavailability, reducing systemic toxicity and improving patient's compliance, and show sustained release of drug. Furthermore, these hydrogels have attracted an increasing interest for decades owing to its many advantages, including the simplicity of preparation (Fang et al. 2018).

Injectable hydrogels (pH-sensitive) are of great interest, for example, for anticancer drug delivery. The intratumoural injection and the in situ forming HGs can enhance the drug bioavailability to the tumour and reduce systemic toxicity. An in

situ forming hydrogel system based on N-carboxyethyl chitosan (CEC) and dibenzaldehyde terminated poly (ethylene glycol) (PEGDA) was developed by Qu et al. (2017). The developed system has demonstrated potential as delivery vehicle of doxorubicin (DOX) for hepatocellular carcinoma therapy. In addition, the HGs exhibited, in vitro, pH-dependent gel degradation and DOX release, being suitable for tumour therapy. Zhang et al. (2018c) have also reported the preparation of injectable hydrogels based on chitosan, hyaluronic acid, and sodium glycerophosphate (GP) for pH-sensitive drug release and adhesion to cancer cell. The results of in vitro DOX release showed the hydrogels are pH sensitive. The introduction of hyaluronic acid depressed the initial burst release of doxorubicin: the higher the HA content, the better the sustained drug release behaviour of the hydrogel, especially at acid media. Moreover, when incubated with human cervical cancer cells (Hela), the hydrogels reveal the remarkable influence of HA on modulating cancer cell adhesion.

More recently, another study which focused on the chitosan-based HGs for anticancer therapy was reported by Peng et al. (2019). In this work, the authors have developed a chitosan/cis-dichlorodiamineplatinum (CS/DDP) hydrogel-based drug delivery system for the in situ treatment of nasopharyngeal carcinoma (NPC) in combination with chemoradiotherapy (RT) and investigated their synergistic antitumour efficacy and underlying mechanism of action. This study showed that CS/DDP hydrogel was a safe drug delivery system due to its biocompatibility and biodegradability and significantly inhibited tumour growth and prolonged the survival of nude mice with NPC xenografts, compared to the control group. The main mechanism was likely the increase in cancer cell apoptosis.

Jalalvandi et al. (Jalalvandi and Shavandi 2018) have also investigated injectable hydrogels based on chitosan. Their HGs were formed via Schiff base linkages and were designed to function as inserts for intravaginal delivery of therapeutics. The matrices were loaded with DOX and a non-hormonal contraceptive (iron (II) gluconate dihydrate, FeGl). The authors have concluded the hydrogels are pH-sensitive and the fast release of the spermicide and sustained release of DOX from these hydrogels make them promising candidates for localized delivery of therapeutic agents through intravaginal administration.

Despite the considerable potential of in situ forming HGs, oral delivery is a commonly used route of drug administration due to its non-invasive nature and the fact that it avoids patient pain and discomfort in compression with the intravenous administration. In this context, great attention has been paid on developing drug delivery systems capable of releasing their cargo in response to pH variation (depending on the different therapeutic purposes, these formulations can be differently designed). The aforementioned pH sensitiveness of chitosan, due to the different functional groups (amino and hydroxyl groups) on its structure, makes it chemically active and facilitates the effective encapsulation of several biomolecules. However, the wide application of chitosan in the biomedical field is restricted by its poor solubility.

To overcome this problem, a variety of chitosan derivatives have been synthesized. For example, Mukhopadhyay et al. (2014) have prepared succinyl chitosan (S-chitosan), pKa 4.48 $\pm$ 0.2, by the introduction of succinyl groups at

the N-position of the glucosamine units of chitosan to improve its water solubility and pH sensitivity. Then, the efficiency of S-chitosan-grafted polyacrylamide hydrogel (PAA/S-chitosan) was investigated for successful oral insulin delivery. As a result, compared to the native PAA-chitosan, the PAA/S-chitosan was found to be more effective in releasing insulin in a sustained fashion in the intestine while protecting it from harsh stomach environment. Similarly, Bai et al. (2018) have prepared an N-succinyl hydroxybutyl chitosan (NSHBC) pH/temperature-dual sensitive hydrogel as a sustained drug delivery system. Compared to the hydroxybutyl chitosan (HBC) hydrogel, significant impacts on the biodegradability and protein delivery capability were shown by the NSHBC hydrogels, which displayed rapid and complete release of BSA in simulated intestinal conditions while minimal degradation and quite low BSA release in simulated gastric conditions. As such, these hydrogels demonstrated ideal behaviour for enabling high bioavailability of orally delivered drugs to the small intestine.

Chitosan-based hydrogels as drug carrier for different routes of administration are being also investigated. Table 6.2 gives a great overview about other reports on chitosan-based hydrogels as drug delivery systems.

Table 6.2 General overview of chitosan-based hydrogels for drug delivery systems

HG formulation	Model drug	Administration route	References
N-Trimethyl chitosan chloride, glycerophosphate (GP), and poly(ethylene)glycol (PEG)	–	Intranasal	Nazar et al. (2011)
Chitosan and hydroxyl propyl methyl cellulose	Dopamine D2 agonist ropinirole	Intranasal	Khan et al. (2010)
Chitosan, glycerophosphate, and salt ($CaCl_2$ or $MgCl_2$)	Exenatide (EXT)	Intranasal	Li et al. (2018)
Glycol chitosan and oxidized alginate	Avastin®	Ocular	Xu et al. (2013)
Carboxymethyl chitosan (CMC) and poloxamer (F127)	Nepafenac (NP)	Ocular	Yu et al. (2017)
Chitosan and gelatin	Timolol maleate	Ocular	El-Feky et al. (2018)
Carboxymethyl chitosan (CMCS) and alginate	–	Oral	Lv et al. (2018)
Methoxy poly (ethylene glycol)-grafted carboxymethyl chitosan (mPEG-g-CMC) and alginate	BSA	Oral	Yang et al. (2013)
Chitosan and glycerophosphate disodium (GP)	Venlafaxinehydrochloride (VH)	Subcutaneous	Peng et al. (2013)
Cefuroxime-conjugated chitosan	Cefuroxime	Subcutaneous	Pawar et al. (2019)

6.6 Mechanisms of Drug Release

In the past few years, hydrogels have been widely utilized as delivery systems for a variety of therapeutic agents ranging from low-molecular-weight compounds to macromolecular proteins, peptides, drugs, and so on (Hoare and Kohane 2008; Calo and Khutoryanskiy 2015). The success of these soft materials in this kind of application can be associated with their unique physicochemical properties. For example, the hydrophilic nature of the hydrogels contributes to their swelling in the aqueous environment, while their porous structure permits loading therapeutic agents into the hydrogel matrix as well as controlling the subsequent release process (Hoare and Kohane 2008; Calo and Khutoryanskiy 2015). Generally, the release process occurs at a rate dependent on the diffusion coefficient of the loaded molecule through the hydrogel network. According to Hoare et al., the main benefit of hydrogels for drug delivery application lies on the fact that such materials are able to modulate the pharmacokinetics process because their network can be elaborated to elute the loaded drugs in a slow manner (Hoare and Kohane 2008). As result, the local concentration of the drug is maintained high over an extended period (Fig. 6.2), which has several advantages from the clinical viewpoint (Wen et al. 2015). Despite this, hydrogels can be also used for systemic delivery. Another relevant advantage of hydrogels is that they are generally biocompatible materials (Kopecek 2007; Calo and Khutoryanskiy 2015). This property is ascribed to two characteristics: the high water content into the hydrogel network and the physicochemical similarity of the hydrogel to the native extracellular matrix (ECM). Such similarity can be understood in terms of composition (most evident in the carbohydrate-based hydrogels) and mechanical properties (elasticity, toughness, etc.) (Hoare and Kohane 2008). Furthermore, other interesting properties such as biodegradability or dissolution, responsiveness to an environmental stimulus, and bioadhesivity (in certain cases)

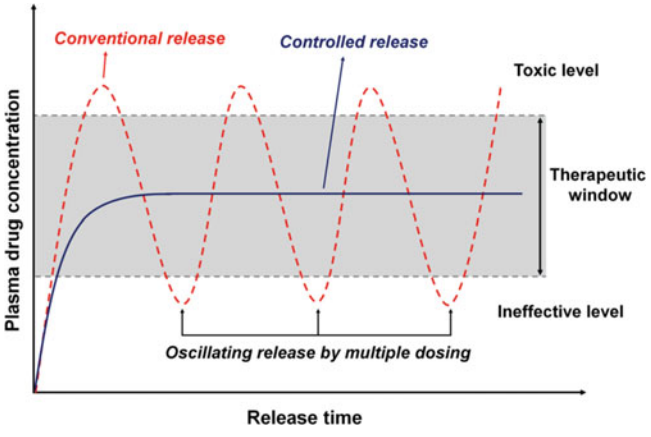

Fig. 6.2 A hypothetical plasma drug concentration profile as function of release time from conventional multiple dosing and controlled delivery

rank hydrogels as efficient delivery systems (Li and Mooney 2016). From a practical viewpoint, the efficiency of a hydrogel as a drug delivery system can be determined from two crucial moments: the drug loading and its further release. The next lines are devoted to highlight and discuss the most relevant aspects related to the mechanism of drug release from hydrogels.

The understanding of basic concepts associated with the drug release process from a hydrogel network requires a careful observation of the water movements through this soft material. In general, the water movement (absorption or desorption) causes the polymeric matrix swelling, solute diffusion, and material degradation (Amsden 1998). These are the main driving forces for drug transport through the hydrogel matrices. For many applications, drug-loaded hydrogels are in the dehydrated state (or glassy state); therefore, the release process encompasses the simultaneous absorption of water by the hydrogel and the desorption of drug via swelling-controlled mechanism (Lee 1985). Ping et al. consider that such swelling and diffusion processes generally do not follow a Fickian diffusion mechanism (Lee and Kim 1991). Physiologist Adolf Fick reported in 1855 a series of laws describing the processes that govern the transport of mass through diffusive means (porous hydrogels, for instance). Specifically, Fickian diffusion refers to the solute transport process in which the polymer relaxation time (t_r) is greater than the solvent characteristic diffusion time (t_d). However, when t_r is equal to t_d, the solute release becomes anomalous or non-Fickian. A non-Fickian release suggests that the polymer relaxation is a slow process, and depending on the relative magnitude of the rate of hydrogel swelling to the rate of drug diffusion, various release profiles may be possible (Rehage et al. 1970). Overall, the mobility of drugs and their rates of diffusion in swollen porous hydrogels are determined by the amount of liquid retained into the hydrogel, the distance between the polymer chains, the flexibility of those chains, and the drug-polymer interaction forces (Lin and Metters 2006; Fu and Kao 2010).

Of course, several other physicochemical aspects have a straight relationship with the drug release mechanisms and kinetics. As noticed in the literature, this seemingly simple diffusional process is affected by multiple complex factors. Depending on the composition of the hydrogel (type of polymer, type of drug, presence or absence of additives, etc.), geometry (shape and size), preparation technique (chemical or physical crosslinking, dual network, etc.), physicochemical properties of the drugs (solubility, stability, etc.), and environmental conditions during the release process (pH, temperature, presence of enzymes, among others), one or more physical and chemical phenomena regulate the drug release (Peppas et al. 2000; Lin and Metters 2006). To describe the mechanism of drug transport through the hydrogel matrices, some nice mathematical models have been formulated and detailed by many researchers. Fu et al. suggest that the purpose of mathematical modelling is to simplify the complex release process and to gain insight into the release mechanisms of a specific material system (Fu and Kao 2010). In contrast, several studies have reported disconnections between the mathematical theories and experimental data since there are multiple driving forces involved in the drug release from a hydrogel (Brazel and Peppas 2000; Caccavo et al. 2015). Further, more sophisticated delivery

systems (e.g. stimuli-triggered delivery systems) are poorly described by the existing mathematical models (Zhang et al. 2013).

The most part of these mathematical models was elaborated considering the rate-limiting step for drug release, and therefore they are categorized as diffusion-controlled, swelling-controlled, and chemically controlled mechanisms (Lin and Metters 2006; Frenning 2011). In general lines, diffusion-controlled is the most applicable mechanism for describing drug release from hydrogels. These models are elaborated taking into account Fick's diffusional law with either constants or variable diffusion coefficients. In this case, drug diffusion is assessed empirically (Amsden 1998). Swelling-controlled release occurs when the drug diffusion rate is higher than the swelling rate. Differently, of the diffusion-controlled mechanism, the modelling of this mechanism considers the boundary conditions at the interface hydrogel-release medium. Finally, the chemically controlled-release mechanism is useful to describe the release process triggered by reactions occurring with the hydrogel matrix (e.g. cleavage of polymer chains via hydrolysis, enzymatic degradation, reactions between the polymer network and the loaded drug) (Rizwan et al. 2017). For example, under certain conditions, the surface or bulk erosion/degradation of hydrogels will affect the rate of drug release. Kim et al. utilized this strategy to develop a chitosan-lysozyme conjugate hydrogel (Kim et al. 2018). According to the authors, the incorporation of lysozyme to chitosan hydrogels accelerated the degradation rate of the crosslinked hydrogel in a dose-dependent manner. These three mechanisms of drug release from hydrogels are illustrated in Fig. 6.3.

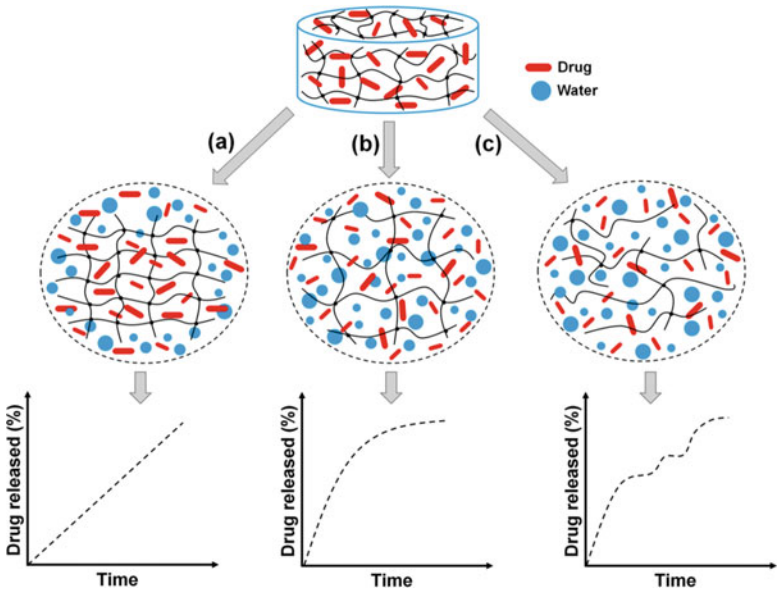

Fig. 6.3 Drug release mechanisms from hydrogels and their respective kinetic profiles. (**a**) Diffusion-controlled, (**b**) swelling-controlled, and (**c**) chemically controlled mechanisms

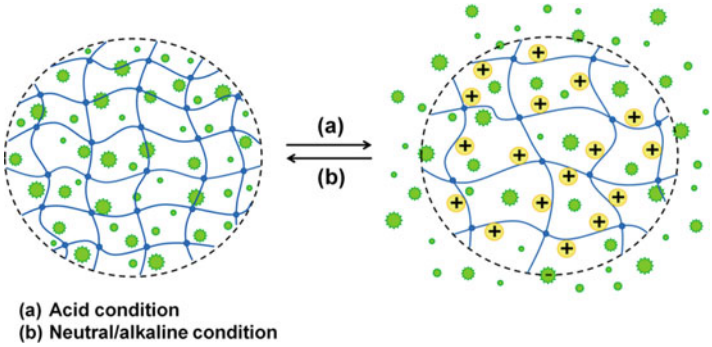

Fig. 6.4 Illustrative scheme of drug release from pH-sensitive chitosan hydrogels

Stimuli-responsive hydrogels (also called as "smart hydrogels") have their mechanism of drug release affected by environmental external stimuli, such as pH, ionic strength, temperature, magnetic or electrical field, ultrasound, etc. (Koetting et al. 2015; Ferreira et al. 2018). This occurs because these external factors affect the hydrogel volume and elasticity in a continuous or discontinuous manner (Ahmed 2015). As consequence, the swelling rate is altered favouring (or not) the drug release process. For example, chitosan-based hydrogels generally are pH-sensitive materials because under acidic conditions, the amino groups available on the chitosan chain become protonated, which increases the repulsive forces within the hydrogel matrix. As a result, the hydrogel network expands favouring the liquid absorption and the diffusion of drugs loaded into these hydrogels (Fig. 6.4) (Aycan and Alemdar 2018; Zhang et al. 2018a). The pH also affects the release profile of physical hydrogels based on chitosan. Overall, this kind of hydrogel is prepared from the polyelectrolyte complexation between chitosan (a polycation) and a polyanion (alginate, chondroitin sulphate, etc.) (Lin et al. 2005; Luo and Wang 2014). Under acidic conditions, these physical hydrogels are degraded allowing the release of loaded drug to the external medium.

The association of chitosan with other responsive polymers is also an interesting strategy to prepare responsive delivery systems. A thermosensitive hydrogel based on chitosan and poly(*N*-isopropylacrylamide) was prepared for ocular delivery of timolol maleate. Poly(*N*-isopropylacrylamide) is a well-known thermosensitive polymer with a thermoreversible phase transition temperature of 32 °C, which is close to human body surface temperature. Above this temperature, the system chitosan-poly(N-isopropylacrylamide) is collapsed due to hydrophobic forces forming a hydrogel network, which controls the release of the loaded drug. Therefore, these external stimuli can trigger the drug release, which is a very interesting approach to design systems for target delivery and controlled release.

6.7 Conclusion and Future Prospects

With the advances in polymer science, the chitosan-based HGs have attracted much attention because of its advantages like low cost, renewability of the resources, biocompatibility, biodegradability, and versatility. Especially, there is a continuous effort in the development of these hydrogels with special focus on biomedical applications.

Here, the basic concepts and properties of hydrogels, the strategies for its preparation and drug loading, some of the most recent works and results regarding its biomedical application, as well as the mechanisms of drug release were summarized. As shown, chitosan-based HG properties can be tuned by employing/investigating different strategies (e.g. previous chemical modification of the polysaccharide) and materials.

This chapter also provides a discussion of future directions that can be expected to extend or advance the biomedical application of chitosan-based hydrogels. A future goal on chitosan-based hydrogels is to get more successful biomedical applications, by developing smart devices which may be capable of both reducing the side effects related to drug administration and delivering cells for potential clinical application in both drug delivery and tissue engineering. With the effort of researchers, we expected to enjoy a definite promising prosperity of chitosan-based HG in the near future.

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Recent Advances in Chitosan-Based Systems for Delivery of Anticancer Drugs

7

Mehmet Polat and Hurriyet Polat

Abstract

Problems in transporting drug molecules to tumor sites in required dose or constitution lead to low efficacy and significant side effects. Shielding the drug molecules in micelles, liposomes, or nanoparticles is a major line of investigation to improve chemotherapeutic treatment. Though compatibility for proper envelopment of the drug and timely release at the tumor site are required of such a carrier, protecting its own physicochemical and morphological integrity during transport is another precondition.

Because of its superior polymerization capability, biocompatibility, pH dependence, and charging characteristics, chitosan has been in the forefront of potential drug carriers. Numerous synthesis routes for chitosan-based nanocarriers have been suggested to the extent that a search of the literature published since 2000 with the keywords "novel + nano + chitosan" *in the title* results in 527 articles, indicating the bewildering quality and quantity of the new information.

This review was carried out not only to peruse this large amount of work on chitosan-based anticancer drug delivery but also to extract manageable patterns from numerous synthesis routes. The main conclusion is that the synthesis methods suggested in literature can be combined into two main routes, and the degree of hydrophobicity of the drug determines which route should be followed.

Keywords

Anticancer · Drug carrier(s) · Chitosan · Nanoparticle(s)

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_7

7.1 Introduction

7.1.1 Cancer and Anticancer Drugs

7.1.1.1 Cancer

Cancer is the uncontrolled breakdown of the systematic mechanisms of cell growth and division to form new cells as required by the body to replace the old or damaged cells. Though it is required for the upkeep of the body, in the long run, this process leads to the development of cells with defective DNA. These cells which divide and grow without control may form growths called tumors. Though many a time the tumors are benign and remain dormant in the body, some are malignant masses of tissue and have the potential to spread into, or invade, nearby tissues. In addition, they can also detach and travel in the body through the blood or the lymph system and form new tumors in other organs removed from the original tumor.

Cancer is the second leading cause of death globally and has been responsible for an estimated 9.6 million deaths in 2018. This translates into the fact that about one in six deaths globally is due to cancer. Approximately 70% of deaths from cancer occur in low- and middle-income countries. Late-stage presentation and inaccessible diagnosis and treatment are common. In 2017, only 26% of low-income countries reported having pathology services generally available in the public sector. More than 90% of high-income countries reported treatment services are available compared to less than 30% of low-income countries. The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in 2010 was estimated at approximately US$ 1.16 trillion (WHO 2018).

The treatment of cancer is complicated and may require combination of various parallel or successive treatments, such as surgery with chemotherapy and/or radiation therapy depending on the nature and degree of cancer and, of course, on the patient. Though it is mainly treated using chemotherapy, radiation therapy, and surgery, there are several treatment methods available (https://www.cancer.gov):

Chemotherapy: It is the treatment of cancer by the use of cytotoxic and other drugs. It is considered a systemic therapy and affects the entire body. It also suffers from treatment-related side effects, off-target effects, and drug resistance limits.

Radiation Therapy: It is a type of cancer treatment which uses high-energy particles or waves, such as X-rays, gamma rays, electron beams, or protons, to destroy or damage cancer cells. Unlike chemotherapy, which usually exposes the whole body to cancer-fighting drugs, radiation therapy is usually a local treatment.

Surgery: It is a procedure where the tumor cells and nearby tissue are removed from the body during an operation. It can be curative, preventive, diagnostic, staging, debulking, palliative, supportive, or restorative surgery.

Immunotherapy: It helps immune system fight cancer by enhancing the body's antitumor immune functions. An immunotherapy approach includes monoclonal antibodies, immune checkpoint blockers, cancer vaccines, and cell-based therapies.

Targeted Therapy: It is a cancer treatment which uses drugs as chemotherapy with the difference that it works by targeting the cancer's specific genes, proteins, or the tissue environment that contributes to cancer growth and survival.

Hormone Therapy: It is usually used for the treatment of breast and prostate cancers which depend on hormones for growth. Hormone therapy acts by disrupting the mechanism of the hormone action and by keeping the hormone away from the hormone receptor cancer cells.

Stem Cell Transplant: Stem cell transplants are procedures that restore blood-forming stem cells in cancer patients who have had theirs destroyed by very high doses of chemotherapy or radiation therapy. Stem cells can function as novel delivery platforms by homing to and targeting both primary and metastatic tumors, secretion of bioactive factors, and immunosuppression.

Precision Medicine: It is an evolving approach to cancer treatment which aims to leverage the pathogenesis of cancer to more precisely target therapy. Precision medicine helps doctors select treatments that are most likely to help patients based on a genetic understanding of their disease.

7.1.1.2 Some Common Anticancer Drugs

Chemotherapy, or the forms of it, is considered the most effective treatment method by targeting cancer cells for termination, thereby stopping the spread or slowing the cancer cell from growing. There are numerous compounds which are commercially available as chemotherapeutic anticancer drugs. A summary of the major classes of these compounds is discussed below.

Alkylating Agents: Alkylating agents are compounds that work by adding an alkyl group to the guanine base of the DNA molecule, preventing the strands of the double helix from linking as they should. This causes breakage of the DNA strands, affecting the ability of the cancer cell to multiply. Eventually, the cancer cell dies. The five traditional categories of alkylating agents are nitrogen mustards (e.g., bendamustine, chlorambucil, cyclophosphamide, ifosfamide, mechlorethamine, melphalan), nitrosoureas (e.g., carmustine, lomustine, streptozocin), alkyl sulfonates (e.g., busulfan), triazines (e.g., dacarbazine, temozolomide), and ethylenimines (e.g., altretamine, thiotepa).

Antibiotics/Antineoplastics: It is an antibiotic compound which inhibits the growth of bacteria (bacteriostatic effect) or destroys them (bactericidal effect). The antibiotic effect can be obtained by different mechanisms which damage the microbial DNA.

Antimetabolites: Antimetabolites are drugs that interfere with one or more enzymes or their reactions that are necessary for DNA synthesis. They affect DNA synthesis by acting as a substitute to the actual metabolites that would be used in the normal metabolism (e.g., antifolates interfere with the use of folic acid). Folic acid antagonist: methotrexate. Pyrimidine antagonist: 5-fluorouracil, floxuridine, cytarabine, capecitabine, and gemcitabine. Purine antagonist: 6-mercaptopurine and 6-thioguanine. Adenosine deaminase inhibitor: cladribine, fludarabine, nelarabine, and pentostatin.

Hormones/Antineoplastics: The activated hormone receptor complexes, binding to specific receptors of chromatin, react with the role of various components of the nucleus, which causes DNA replication and cell division by a series of enzymatic reactions, thus affecting the physiological function of cells. These antineoplastic drugs include aromatase inhibitors, aromatase inactivators, estrogens, antiestrogens, progestins, androgens, anti-androgens, luteinizing hormone agonists, glucocorticoids hormones, adrenal blockers, and others.

Platinum Compounds: Strategies for improving platinum-based anticancer drugs usually involve changes in the neutral spectator ligands, in the nature of the anions (halides vs various carboxylates), and in the oxidation states of the metal (PtII vs PtIV).

Vinca Alkaloids: Vinca alkaloids are obtained from the Madagascar periwinkle plant. There are four major vinca alkaloids in clinical use: vinblastine, vinorelbine, vincristine, and vindesine. These are sometimes called monoterpenoid indole alkaloids in the scientific literature. All vinca alkaloids are administered intravenously. They are eventually metabolized by the liver and excreted. The vinca alkaloids are cytotoxics – they halt the division of cells and cause cell death. The main mechanisms of vinca alkaloid cytotoxicity are due to their interactions with tubulin and disruption of microtubule function, particularly of microtubules comprising the mitotic spindle apparatus, directly causing metaphase arrest. Nevertheless, the vinca alkaloids also have an effect on both nonmalignant and malignant cells in the nonmitotic cell cycle, because microtubules are involved in many nonmitotic functions (Moudi et al. 2013).

Protein Tyrosine Kinase Inhibitors: A protein kinase inhibitor is a type of enzyme inhibitor that can block the action of protein kinases. Protein kinases add a phosphate group to a protein in a process called phosphorylation, which can turn a protein on or off, therefore affecting its level of activity and function.

Antineoplastic Interferons: Antineoplastic interferons are interferons (alpha) that are manufactured using recombinant DNA technology and used therapeutically to treat certain types of cancers and viral infections.

The use of alkylating agent mechlorethamine, a nitrogen mustard, to treat lymphomas in the 1940s and antimetabolite methotrexate to cure a solid tumor in the 1956 was the first in cancer treatment. In 1957, 5-fluorouracil to cure tumor was first of pyrimidine analogs. Since then many anticancer drugs have been developed and used with some success. Numerous studies have focused on plant-derived compounds with curative potential and have been used widely in medicines (Verma et al. 2008; Shi et al. 2006). A review by Nahata (2017) compiles the most promising anticancer agents and lists their major cancer curative potentials. Some of the specific agents discussed by Nahata (2017) are summarized below:

Paclitaxel: Paclitaxel (Taxol by Bristol-Myers Squibb) (Wani et al. 1971; Schiff et al. 1979; Honore et al. 2004) blocks a cell's ability to break down the mitotic spindle during mitosis (cell division). It is given intravenously. It irritates the skin and mucous membranes on contact and is most effective against ovarian

carcinomas and advanced breast carcinomas. *Taxus baccata* and *Taxus brevifolia* are members of the yew family (Taxaceae). It is not water soluble.

Docetaxel: Rhone-Poulenc Rorer has trademarked Docetaxel as Taxotere. Like paclitaxel, it prevents the mitotic spindle from being broken down by stabilizing the microtubule bundles, but clinical trials indicate that it is about twice as effective as paclitaxel in doing so. Docetaxel, which is also given intravenously, is being tested on carcinomas of the bladder, cervix, lung, and ovaries, on malign antimelanoma and on non-Hodgkin's lymphoma. It's water solubility is threefold higher than paclitaxel.

Beta-lapachone and Lapachol: It is a quinone derived from lapachol (a naphthoquinone), which can be isolated from the lapacho tree (*Tabebuia avellanedae*), a member of the catalpa family (Bignoniaceae). ß-Lapachone inhibits DNA topoisomerase I. Beta-lapachone keeps the chromosomes wound tight, and so the cell can't make proteins. As a result, the cell stops growing. Because cancer cells grow and reproduce at a much faster rate than normal cells, they are more vulnerable to topoisomerase inhibition than are normal cells. Beta-lapachone is effective against several types of cancer, including lung, breast, colon, and prostate cancers and malignant melanoma. The use of beta-lapachone in humans has been limited due to its toxicity.

Colchicine: It is a water-soluble alkaloid found in the autumn crocus that blocks or suppresses cell division by inhibiting mitosis. Specifically, it inhibits the development of spindles as the nuclei are dividing. Because cancer cells divide much more rapidly than normal cells, cancers are more susceptible to being poisoned by mitotic inhibitors such as colchicine, paclitaxel, and the vinca alkaloids, vincristine, and vinblastine.

Natural Anticancer Agents: Besides curing cancer, the synthetic drugs also harm the normal cells of the body and are producing severe side effects that are not only long living but may pose threat to human's life and are more toxic to body. Therefore there has been numerous work to test the anticlastogenic, antimutagenic, and anticarcinogenic activity of natural plants and herbs which have been traditionally known to have anti-inflammatory, antifungal, antiallergenic, anthelminthic, and other biological curing properties. Though the list can be extended much further, some of these natural plants and herbs standing out in cancer research have been summarized below:

Ganoderma lucidum (reishi mushroom): Ganoderma lucidum is a natural medicine that is widely used and recommended by Asian physicians and naturopaths for its supporting effects on the immune system. Laboratory research and a handful of preclinical trials have suggested that *Ganoderma lucidum* carries promising anticancer and immunomodulatory properties. However, there is no systematic review that has been conducted to evaluate the actual benefits of *Ganoderma lucidum* in cancer treatment (Gao et al. 2004; Nahata 2017, Nahata et al. 2011, 2012a, b, 2013; Chi et al. 2013).

Sphaeranthus indicus (Compositae): Also known as East Indian globe thistle, this herb is found mostly in southern India. It has been demonstrated to have remarkable anti-allergic effects in vitro in preventing mast cell degranulation.

In vitro studies with cancer cell lines demonstrated 80–100% anti-proliferative effect, which competed with many reference drugs used in cancer therapy. However, all of these studies are highly preliminary (Nahata et al. 2011, 2013).

Radix sophorae: This herb is the root of *Sophora flavescens* Ait. and has been used to treat abscess, edema, dysentery, eczema, ulcers, skin burns, skin itch, and atopic dermatitis in traditional medicine for thousands of years. It has also been shown to have antitumor effects (Cheung et al. 2007).

Punica granatum: This is a pomegranate extract rich in polyphenols and has demonstrated antiproliferative, antimetastatic, and anti-invasive effects on various cancer cells line in vitro as well as in vivo animal model or human clinical trial (Lanksy et al. 2007).

Betulinic acid: Betulinic acid is a pentacyclic triterpenoid of plant origin that is widely distributed in the plant kingdom throughout the world. For example, considerable amounts of betulinic acid are available in the outer bark of a variety of tree species, e.g., white-barked birch trees. It has been shown to have a range of biological effects including potent antitumor activity (Pisha et al. 1995).

Turmeric: This herb is a spice grown in many Asian countries and is also known as Indian saffron, jiang huang, haridra, and haldi. It belongs to the ginger family and is a main ingredient of curry powder. The main active ingredient in turmeric is curcumin or diferuloyl methane. Laboratory studies have shown curcumin has anticancer effects on cancer cells (Yasmin et al. 1998; Gupta et al. 2010; Andriani et al. 2015)

Glinus lotoides: This is used as a dietary vegetable and medicinal plant in Asia and Africa. The seed of *Glinus lotoides* has been shown to have antitumor, antifungal, and anthelminthic activity which has been attributed to its saponin and flavonoid content (Kavimani et al. 1999).

Andrographis paniculata: Andrographis paniculata belongs to the family Acanthaceae or Kalmegh and is commonly known as "king of bitters." It is extensively used as home remedy for various diseases in Indian traditional system as well as in tribal system in India for multiple clinical applications. It has been tested to have anticlastogenic, antimutagenic, and anticarcinogenic properties (Kumar et al. 2002).

β-Hydroxyisovaleryl-shikonin: This compound which is isolated from the roots of the plant *Lithospermum radix* has been shown to have inhibitory ability on the proliferation of various human cancer cells, notably the lung and cervical cancers (Masuda et al. 2004).

Saussurea lappa: This plant which has been long used in certain systems of alternative medicine, including Ayurveda and traditional Chinese medicine, is also known as snow lotus. It has been shown to have an effect on asthma, inflammatory diseases, ulcers, and stomach problems in Korea, China, and Japan. Several studies have suggested that it also has anticancer effects in neuroblastoma, lung cancer, hepatocellular carcinoma, gastric cancer, and prostate cancer (Ming et al. 2003; Tian et al. 2017).

Litchi fruit pericarp extract: Litchi is a nonclimacteric subtropical fruit that, once harvested, loses its red pericarp color because of browning reactions probably

involving polyphenols. Litchi fruit pericarp (LFP) extract contains significant amounts of polyphenolic compounds and exhibits powerful antioxidant activity against fat oxidation in vitro and has been shown to have anticancer activity (Wang et al. 2006).

Lignans from stem wood of Cedrus deodara: The lignan mixture of this plant comprising lignans from stem wood of *Cedrus deodara* consisting of wikstromal, matairesinol, and dibenzylbutyrolactone has been shown to demonstrate in vitro cytotoxicity against human cancer cell lines (Singh et al. 2007).

Pine needles: Pine needles (*Pinus densiflora* Siebold et Zuccarini) have long been used as a traditional health-promoting medicinal food. It has been shown that pine needle oil could induce DNA damage in a dose-dependent manner and has potential anticancer effects and antioxidant, antimutagenic, and antitumor activities (Kwak et al. 2006).

Polyalthia longifolia: Polyalthia longifolia is a lofty evergreen tree found in India and Sri Lanka. It has been shown that the methanolic extract from the leaves of *Polyalthia longifolia* has significant anticancer potential (Verma et al. 2008).

Ashwagandha: This plant is a popular Ayurvedic herb used in Indian traditional home medicine and has been shown to have anti-inflammatory effects in addition to relaxing the central nervous system in animals. Other research suggest that Ashwagandha extract and its purified component withanone are selective in killing of cancer cells (Widodo et al. 2007).

7.1.2 Problems Associated in Using Anticancer Drugs

Use of anticancer drugs depends on several factors: (1) the type and location of the cancer, (2) its severity, (3) the type of therapy (surgery/radiation), and (4) the side effects associated with the drug. Though some can be taken orally or can be injected intramuscularly or intrathecally (within the spinal cord), most anticancer drugs are administered intravenously.

The chemotherapeutic treatment is complicated in that the anticancer drugs are generally toxic and they cannot differentiate normal cells from the cancer cells. This leads to harming of the normal cells causing serious side effects, some of which are life-threatening. Some commonly encountered side effects include low blood counts, tiredness, mouth soreness, nausea, vomiting, loss of appetite, constipation or diarrhea, hair loss, skin changes or reactions, pain or nerve changes, and changes in fertility and sexuality. In rare instances prolonged use of anticancer drugs can also lead to the development of secondary cancers. One way of encountering this problem and reducing the side effects of the anticancer drugs is application of multidrug therapy on the patient. This method is based on the nanoscale drug co-delivery systems, which loads at least two anticancer drugs with different physicochemical and pharmacological properties into a combined delivery system. Different types of anticancer drugs exert their effects in a certain part of the cell cycle (e.g., cell growth phase, cell division phase, resting phase). Thus, while one drug

may be used to stop the growth of cancer cells in a certain phase, another agent may work at a different phase. Nano-drug co-delivery systems are said to synergistically inhibit the growth of the tumor compared with the free drugs. Qi et al. (2017) highlighted the current state of co-delivery nanoparticles and the most commonly used nanomaterial. They discussed challenges and strategies and prospect future development.

However, the very wide spectrum of the observed side effects clearly indicates that the real problem in drug-based cancer treatment lies in the targeting stage of the drugs, in other words successful transport and delivery of the drugs in the body. It seems that they do not reach the targeted cancer cell and they do not arrive there in one piece. The result is the spread of a cytotoxic agent into the body in free form, hence the side effects. This is the main reason for the fact that though there have been numerous compounds which have been demonstrated to be very successful in destroying or killing the cancer cells in laboratories, they have been short of showing progressive improvement when applied in the human bodies. Hence, the targeted drug delivery and controlled drug release stand out as one of the most important and promising avenues in increasing the efficiency of the anticancer drugs, which in laboratory show considerable success, while reducing the side effects significantly. To summarize, there are two main impediments on the way of cancer treatment agent no matter how exceptional it is in reaching the tumor area with maximum efficacy and destroying the cancer cells: the first is the lack of success in preventing the agent from interacting the healthy non-cancer cells, especially in free form (side effect prevention), and the second is the problems in proper arrival (to the right address in desired form) of the drug into tumor site. Therefore, drug carriers (vehicles) have become the essential tools by which one can deliver drugs into tumor cells in the desired form and concentration with minimum drug leakage into normal cells. This will be the subject of the following paragraphs.

7.1.3 Common Drug Carrier Systems for Anticancer Drugs

As explained in the above paragraphs, the major problems encountered in conventional chemotherapy are poor bioavailability, high-dose requirements, hostile side effects, and low therapeutic records.

Most chemotherapeutic drugs have low solubility in water, hence in body fluids. The poor water solubility of hydrophobic anticancer drugs creates difficulties in loading and delivery and limits their overall therapeutic efficacy, hence their clinical use, unless the solubility is increased by some modification or the drug is enveloped in a soluble environment. The remaining hydrophilic anticancer agents which are freely soluble in bloodstream also face complexities in treatment because of their interactions with the blood components such as proteins.

Therefore, enveloping the anticancer agents whether they are hydrophobic or hydrophilic in nature has become one of the most sought-after preparation methods for increasing the effectiveness of chemotherapeutic treatment. To start with these anticancer drug carriers must offer nontoxicity, good biodegradation, or

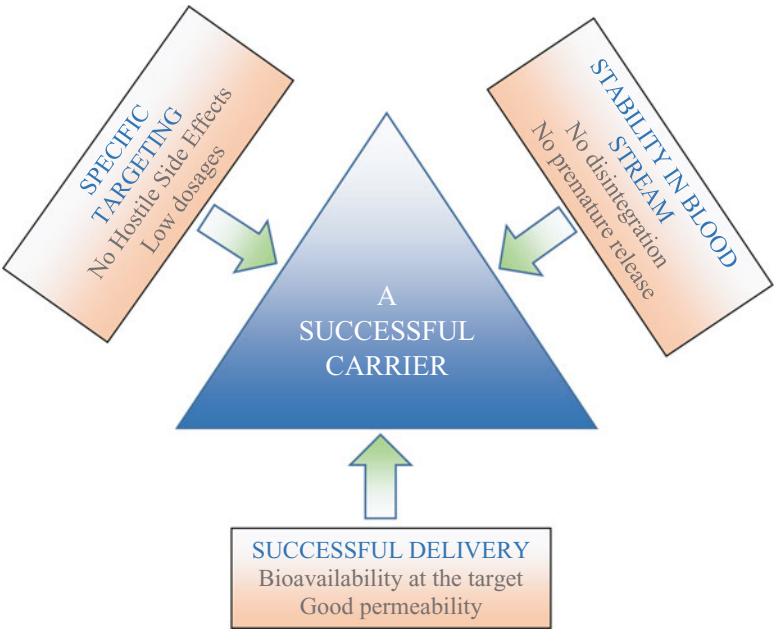

Fig. 7.1 A schematic of the general requirements expected from a successful drug carrier

bioavailability characteristics. In addition, the carrier must also fulfill the following requirements:

- The correct chemical properties to be able to dissolve and envelop the drug in its structure
- Sufficient stability in the circulation system to protect the drug during transport
- Proper attributes to deliver the drug once it reaches the target cells

The problems associated with a carrier if it does not satisfy the above requirements are summarized schematically in Fig. 7.1. Therefore, numerous research has been carried out in the literature for addressing these problems and developing successful drug delivery vehicles for anticancer drugs to the desired sites of therapeutic action with minimal adverse side effects. Many types of materials with different structural characteristics as popular delivery vehicles for chemotherapeutic agents-anti-cancer drugs that have emerged from these studies are summarized below:

Polymers: Polymeric nanoparticles are solid, biocompatible, and biodegradable systems. They have easy structural modification and allow wanted properties to be built into the nanoparticle. Polymeric nanoparticles can be prepared from synthetic polymers, e.g., poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(lactic-co-glycolic acid), *N*-(2-hydroxypropyl)-methacrylamide copolymer

(HPMA), and poly(styrene-maleic anhydride) copolymer, or from natural polymers, such as gelatin, dextran, guar gum, chitosan, and collagen (Hartwell et al. 1971; Cragg et al. 1993; Kumar et al. 2000; Newman et al. 2003; Shi et al. 2006; Park et al. 2008; Parveen and Sahoo 2008).

Lipids: Liposomes are small, spherical, self-closed structures with at least one concentric lipid bilayer and an encapsulated aqueous phase in the center. They have biocompatible-biodegradable nature and unique ability to encapsulate hydrophilic agents (hydrophilic drugs, DNA, RNA, etc.) in their inner aqueous core and hydrophobic drugs within the lamellae, which makes them versatile therapeutic carriers (Hande et al. 1998; Chabner and Lango 2001; Mo et al. 2014; Dong et al. 2014).

Inorganic Carriers: Inorganic nanocarriers have great advantages, such as large surface area, good drug loading capacity, bioavailability, low toxic side effects, controlled drug release, and their tolerance toward organic solvents (most of them). Quantum dots, carbon nanotubes, layered double hydroxides, mesoporous silica, and magnetic nanoparticles are commonly used in cancer treatment in various ways (Wani et al. 1971; Bianco et al. 2008; Zrazhevskiy, et al. 2010; Kairdolf et al. 2013; Li et al. 2015).

Polymeric Hydrogels: Hydrogels are three-dimensional polymeric and hydrophilic networks that can absorb large amounts of water. The key success of hydrogel development is in situ gelation. The gelation process is time and concentration dependent and can be triggered by an external stimulus, such as pH, temperature, or light (Schiff et al. 1979; Peppas et al. 2000; Lin and Metters 2006; Tomme et al. 2008; Qi et al. 2015; Ghosh et al. 2015).

Micelles: Micelles are spherical and amphiphilic colloids formed by self-assembly of amphiphilic block copolymers in an aqueous solution, resulting in a hydrophobic core and a hydrophilic shell. They can be formed spontaneously under certain concentrations (critical micelle concentration (CMC)) and temperatures. The hydrophobic core serves as a reservoir for hydrophobic drugs, whereas the hydrophilic shell stabilizes the hydrophobic core and renders both polymer and hydrophobic drugs water soluble (Park et al. 2008; Deshayes et al. 2013; Shi et al. 2014; Jin et al. 2016; Gilbreth et al. 2016; Kumari et al. 2017).

Protein-Based Nanocarriers: Albumin-based nanocarriers have high binding capacity for various drugs and they are nontoxic, non-immunogenic, biocompatible, and biodegradable, and have a long half-life in circulating plasma. Albumin has functional groups as amino and carboxylic groups to easily bind targeting ligands and other surface modifications (Dreis et al. 2007; Hawkins et al. 2008; Zhao et al. 2010; Elzoghby et al. 2012).

Note that the reference list is much longer and should be taken only as an example for the specific vehicle since they have been more exhaustively summarized in excellent review papers recently (Senapati et al. 2018; Zhu and Liao 2015; Dong et al. 2019).

As response to the characteristics required of a drug carrier summarized in Fig. 7.1, the research has focused on developing nanoscale alternative delivery systems such as micelles, polymeric nanoparticles, and liposomes.

Compared with the direct administration of bare chemo-drugs, drug encapsulation in a carrier offers a number of advantages, such as protection from degradation in the bloodstream, better drug solubility, enhanced drug stability, targeted drug delivery, decreased toxic side effects, and improved pharmacokinetic and pharmacodynamics drug properties. To date, an impressive library of various drug delivery vehicles has been developed with varying sizes, architectures, and surface physicochemical properties with targeting strategies. There are excellent review papers (Senapati et al. 2018; Qi et al. 2017; Zhu and Liao 2015) that summarize some examples of drug delivery systems that have either been approved or are in clinical or preclinical development stages. These structures are expected to encapsulate the hydrophobic or hydrophilic anticancer agents for minimizing the side effects due to disintegration during intravenous delivery and improving the therapeutic efficacy through the enhanced availability, permeability, and retention at the target.

Amphiphilic block copolymers which contain chemically tethered hydrophilic and hydrophobic segments have been used extensively for the purpose of delivering therapeutic compounds with low water solubility in body fluids. In aqueous solutions, these polymers associate into nano-sized core/shell structures called micelles above a critical concentration (the critical micelle concentration or CMC). The hydrophobic core section of a micelle serves as a reservoir for the hydrophobic drug molecules, whereas the hydrophilic shell (corona) provides water solubility. Once stabilized inside a micelle's core, the probability of the drug molecules to avoid premature degradation and ingestion before reaching the target tissues is expected to increase significantly (Gaucher et al. 2005; Sachs-Barrable et al. 2007; Plapied et al. 2011; Hunter et al. 2012; Ensign et al. 2012; Maeda et al. 2013; Xu et al. 2013; Talelli et al. 2008). Nevertheless, efficiency of the micelles as drug carriers is lower than desired. Interaction of the micelles with the native blood plasma components is one obvious obstacle. If present, such interactions may alter the conformation, size, and surface properties of the carrier and negatively influence both the drug holding capacity and activity at the target site. The most potent binding partners in blood are albumin, immunoglobulins, fibrinogen, apolipoproteins, and complement cascade proteins. Therefore, there have been lots of studies on the stability of polymeric micelles in aqueous solutions (including body fluid), and these studies have been summarized by some excellent reviews addressing these issues (Owen et al. 2012; Shi et al. 2017; Zhou et al. 2016).

In recent years, there have been several studies on the fixation of anticancer drugs in the core of polymeric micelles to increase their intravenous stability (Kabanov et al. 2002; Batrakova and Kabanov 2008). Very recent findings by the authors of this paper demonstrate that dilution of the micellar nanocarriers must also be taken into account in devising drug carriers from polymeric surfactant aggregates (Polat et al. 2019). The most obvious solution to this would be encapsulation of the micelles by a third phase as suggested by the same authors in their previous work (Cihan et al. 2017). In that study, we designed and developed spherical chitosan

nanoshells which enveloped the micelles of a polymeric surfactant whose cores provided the necessary solvation characteristics and safeguarded delivery of strongly lipophilic drug.

In summary, nanoparticles have immense potential as drug-delivery carriers due to their unique physicochemical properties whether they directly accommodate the drug molecules or envelop other nanoscale structures which contain the drug molecules (such as micelles; see Cihan et al. 2017). These particles have the potential to improve the pharmacological and therapeutic properties of the anticancer drugs by controlling release rates and targeted delivery process, which eliminate the limitations of conventional anticancer treatment methods. A wide variety of inorganic or organic materials such as silica and polystyrene have been employed to synthesize the nanoparticle-based drug carriers. However, among these materials, natural polymers stand out as the most promising agents due to their biodegradability, biocompatibility, and favorable physicochemical responses. The following section gives a summary account of such materials as nanoparticle feedstocks and will describe why chitosan emerges as one of the most promising natural polymers as an anticancer drug carrier.

7.2 Chitosan as an Anticancer Drug Carrier

7.2.1 Characteristic Properties of Chitosan

Natural polymers such as cellulose, starch, chitosan, carrageenan, alginates, etc. are among the preferred materials for drug delivery applications due to their chemically inert, nontoxic, biocompatible, and biodegradable structures and availability. There are several studies on the use of these materials for enveloping the anticancer drugs for developing efficient chemotherapeutic treatment systems (Toti and Aminabhavi 2004; Kaur et al. 2013; Gao et al. 2013; Mazumder et al. 2018; George et al. 2019; Singh and Singh 2019). Among these materials, chitosan has been receiving special attention due to its superior characteristics such as (i) high drug-carrying capacity, (ii) multifunctionality, (iii) prolonged circulation ability, and (iv) favorable targeting and penetrability of the cell membranes because of the primary amine groups in its structure. Moreover, molecular weight and molecular fraction of glucosamine units in the chitosan structure influence the solubility and antimicrobial and biological activity (Tikhonov et al. 2006). Because of these properties, chitosan has received an immense attention in the literature. A search of the papers in Web of Science with the word "chitosan" *in the title alone* results in 33,502 published journal articles between the years 2000 and 2019 (Fig. 7.2a). It can be seen that the interest in chitosan has grown drastically in the last 20 years.

Chitosan is natural polysaccharide containing β-(1-4)-D-glucosamine and N-acetyl-β-(1-4)-D-glucosamine units. It is produced by deacetylation of chitin. Deacetylation process includes treatment of chitin with aqueous NaOH at 110–115 °C for several hours without oxygen. When the deacetylation degree is over 50% the product is termed "chitosan" (Chang et al. 1997). Dissimilar to

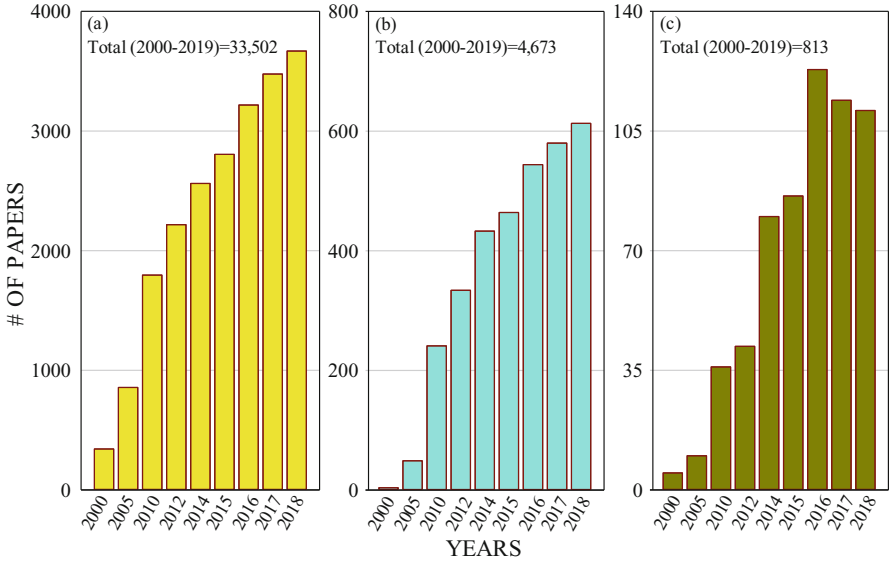

Fig. 7.2 The trend in the number of articles published in respected journals with keywords (**a**) *chitosan∗*, (**b**) *chitosan + (nanoparticle∗ or nano-particle∗ or nano-particle∗)*, and (**c**) *chitosan + (tumor∗ or cancer∗) in the title* in Web of Science between 2000 and 2019

cellulose, chitosan contains hydroxyl groups, acetylamine, or free amino groups that provide many unique properties. Also these amino groups and hydroxyl groups present in its structure provide flexible sites for managing the type and degree of modification for the purposes of the requirements of the end user. It is found that mucoadhesion of chitosan increases with an increasing deacetylation degree and decreases with an increase in the cross-linking (George et al. 2006). These groups are responsible for its outstanding properties such as its cationic nature, pH sensitivity, in situ gelation ability, antimicrobial activity, and permeability which have important implications for targeted drug delivery and controlled drug release. All these features make chitosan nanocomposites ideal candidates for applications such as biomedical scaffolds but also for the delivery of macromolecular therapeutics, like protein and peptides.

Chitosan is soluble in weakly acidic solutions (formic acid, acetic acid, hydrochloric acid, etc.) depending on the number of its amino groups but insoluble in water and alkaline solutions (Krajewska 2004). Because of this it can be used as a pH-dependent material and must be regarded if any system consists of chitosan. It has been observed that protonation of chitosan in different acidic environments depends on pH and pK value of the acid. Though most of the polysaccharides have been observed to have neutral or negatively charged surface in acidic media, chitosan molecules are charged positively when it is dissolved in acidic environment since the amino groups ($-NH_2$) of the glucosamine become protonated ($-NH_3^+$).

Chitosan is widely used in oral delivery relying on its mucoadhesive property. Because of the negative charge of mucosal surfaces, strong mucoadhesive force

occurs between such surfaces and chitosan. It is found that mucoadhesion of chitosan increases with an increasing deacetylation degree and decreases with an increase in the cross-linking (George et al. 2006). In addition, thiolation and trimethylation increase mucoadhesion of chitosan. These properties of chitosan make it a preferred material for controlled release of orally delivered drugs.

Because of all these reasons outlined in above paragraphs, various forms of chitosan materials such as beads, films, microspheres, nanoparticles, nanofibers, hydrogels, and nanocomposites have been developed and tested as drug delivery devices and applications. The recent review papers by Prabaharan (2015), Elgadir (2015), Ali et al. (2018), Pella et al. (2018), and Naskar et al. (2019) report the vast literature available on chitosan-based materials in drug delivery applications. Some representative pictures of chitosan beads, foams, and sheets synthesized in the authors' laboratories are presented in Fig 7.3.

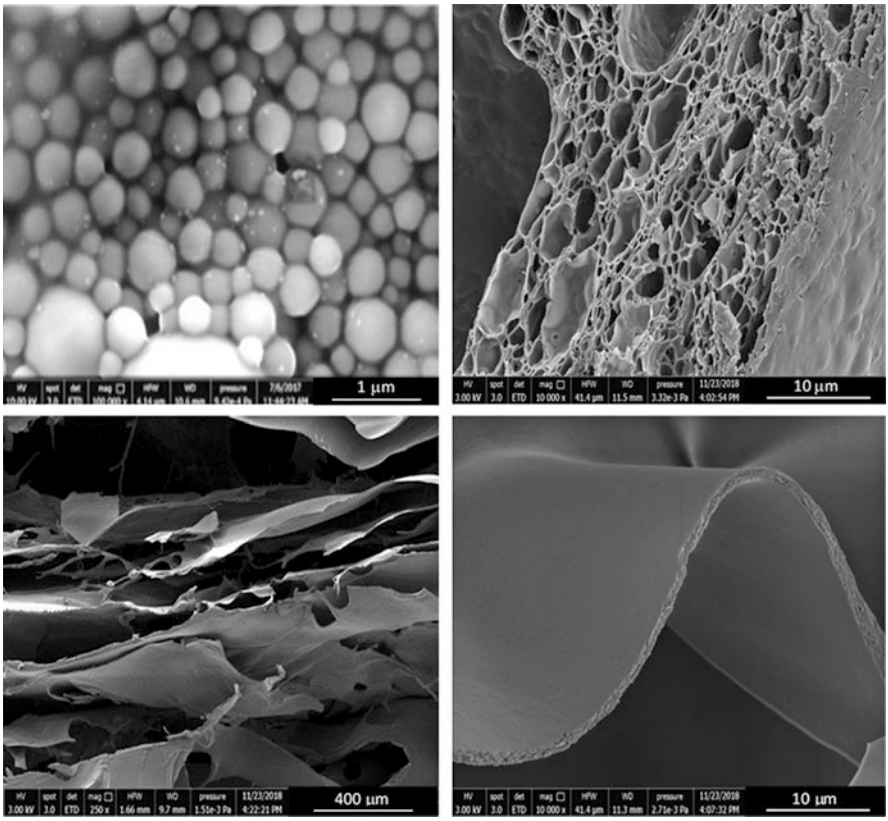

Fig. 7.3 Different forms of chitosan nanostructures synthesized in the authors' laboratories: *nanoparticles (top left), nanofoam (top right), microsheets (bottom left), porous microfilms (bottom right)*

7.2.2 Preparation of Drug-Loaded Chitosan Nanoparticles

Complications always exist in the synthesis of the chitosan nanoparticles even in the absence of any drug in the structure due to the difficulties in controlling the size, morphology, and integrity of the synthesized particles which makes the design of chitosan carriers a state of art. Despite these difficulties, and most probably because of them, the literature on the synthesis of chitosan nanoparticles is immense. Searching the papers in Web of Science with the word "chitosan" and "nanoparticle" *in the title alone* results in 4,473 published journal articles between the years 2000 and 2019 (Fig. 7.2b). It can been seen that there is a similar growth trend in using chitosan to synthesize nanoparticles in the last 20 years to that observed with the articles dealing with chitosan alone.

Various synthesis methods of chitosan nanoparticles are summarized by Grenha (2012), Vyas et al. (2016), Naskar et al. (2019) and Shanmuganathan et al. (2019). The grouping of the methods suggested by these authors is presented below without change in the terminology in order to make comparison with the literature easier. However, it should be stressed that many of the methods proposed are actually variation of one of the few main routes to creating chitosan nanoparticles. Therefore, we have added our specific comments where more need to be said or where certain misunderstandings to be corrected. Therefore, a reordering and generalizing of the grouping of these methods will also be presented at the end paragraphs of this section in Fig. 7.4 based on our comments in this sections.

Ionic Gelation/Polyelectrolyte Complexation: This is the most straightforward of the methods employed in the literature. It is based on contacting the cationic chitosan molecules dissolved in an aqueous phase with a negatively charged cross-linking agent slowly to allow complex formation, polymerization, and precipitation of chitosan by electrostatic forces (Kawashima et al. 1985a, b; Fernandez-Urrusuno et al. 1999; Pan et al. 2002; Ahmad et al. 2012; Aydin et al. 2012; Rampino et al. 2013; Motwani et al. 2008; Nanjwade et al. 2010; Meng et al. 2011; Alam et al. 2012; de Campos et al. 2001; Wu et al. 2005; Bhattarai et al. 2006; Pawar et al. 2013; Xue et al. 2015a; Gao et al. 2016; Andriani et al. 2015).

Nevertheless, in our experience, control of this system to manufacture particles of desired size and morphology is somewhat difficult, and if applied directly, the method usually results in precipitation of a chitosan phase in an uncontrolled manner. This is the main reason for introducing separate components or phases (such as creating some form of an emulsion system as mentioned in the following paragraphs) for controlling polymerization reactions to certain sizes and shapes.

However, as we have seen in our studies, introduction of the micelles into the system to present nano-sized nucleation sites for the chitosan polymerizing may allow creation of particles of desired size and morphology. Such approach also lets a more proper use of the micelles (see the reverse micellization method below for the misleading use of the micelle term).

Modified Ionic Gelation with Radical Polymerization: In this approach, a polymeric acid such as polyacrylic acid or polymethacrylic acid is added to the aqueous chitosan solution. Polymerization takes place due to the interaction between the

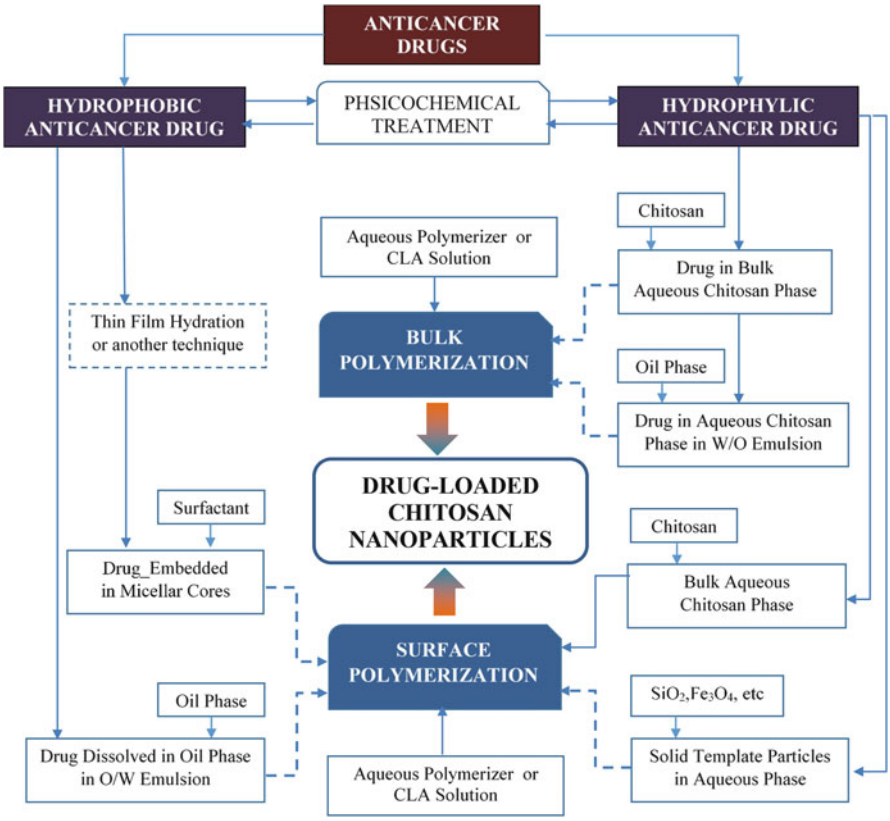

Fig. 7.4 A combination of various synthesis methods for drug-loaded chitosan nanoparticles. *Only one of the inputs shown by the broken lines is added to the system depending on the synthesis procedure*

cation of chitosan and the anionic polymeric acid. In some cases, the acid added in monomeric form is polymerized by use of a polymerization agent such as potassium persulfate to initiate polymerization of the acid in chitosan solution leading to precipitation of chitosan nanoparticles (Hu et al. 2002; Sajeesh and Sharma 2005, 2006). Nevertheless, as the name implies, this is a variation of the ionic gelation method with the difference that instead of a more traditional cross-linking agent such as TPP, a monomeric or polymeric acid is employed.

Desolvation: This method is also a straightforward approach to chitosan polymerization in that it simply is based on decreasing the solubility of chitosan in an aqueous solution by addition of a precipitation agent rather than a cross-linking agent. The precipitating agents could be electrolytes such as sodium sulfate or solvents such as acetone in the presence of some stabilizing agents if necessary. In some cases, a polymerizing agent can also be added to create a more compact and

sturdy chitosan phase (Jiang et al. 2018). However, since precipitation takes place due to desolubilization of chitosan, controlling the size, morphology, and stability of the synthesized particles could be a challenge as was the case with the ionic gelation/polymerization method discussed in the previous paragraph.

Emulsification Cross-Linking: This is one of the main methods in creating chitosan nanoparticles. In this method, a W/O emulsion is created by introducing aqueous chitosan solution into an oil phase to create dispersed chitosan solution droplets which act as micro- or nano-reactors. Then, a polymerization agent such as glutaraldehyde is added into the emulsion and is expected to diffuse into the dispersed aqueous chitosan droplets initiating the polymerization within the confined volume of the droplets by cross-linking through the aldehyde groups of the polymerizer and the amino groups of the chitosan molecules to form nanoparticles. A suitable stabilizing surfactant is usually employed to keep the aqueous phase dispersed in oil (Ohya et al. 1994; Grenha 2012; Yuan et al. 2010). In this case, the drug should be dissolved in the chitosan solution previously by use of a suitable method if the drug molecules is to be incorporated into the chitosan nanoparticles.

W/O Emulsion Droplet Coalescence: This is a variation of the emulsification cross-linking method above with the difference that instead of a single W/O emulsion into which the polymerizer is added in aqueous phase directly, two W/O emulsions are prepared separately in hydrophobic organic phases. Of the two W/O emulsions, one includes an aqueous solution of chitosan with or without the dissolved drug molecules, and the other may contain a strongly aqueous solution of a base such as sodium hydroxide or a cross-linking agent for initiating chitosan precipitation. When the two systems are mixed under high shear conditions, the droplets from the two W/O emulsions collide randomly and coalesce, creating droplet-size reactors where drug-loaded chitosan solution is precipitated by the base, or the polymerizer, in the form of nanoparticles. The use of a stabilizer to ensure emulsification through surface tension and viscosity reduction followed by steric or electrostatic stabilization and high-speed homogenization conditions may also be necessary (Reddy et al. 2013; Tokumitsu et al. 1999).

Emulsion Solvent Diffusion: In this method, a hydrophilic drug is initially dissolved in an organic phase with the help of a solvent. The drug-containing organic phase is then dispersed in chitosan solution with the help of a stabilizer. Under high-pressure evaporation conditions, the organic phase evaporates, while the solvent diffuses into the aqueous phase. While the diffusion of the solvent into aqueous solution brings about polymerization of chitosan, evaporation of the organic phase precipitates the drug leading to drug-loaded chitosan nanoparticles (El-Shabouri et al. 2002; Niwa et al. 1993; Anto et al. 2011). The same procedure can be used to load hydrophobic drugs into the organic hydrophobic phase directly without the use of any solvent. However, precipitation of chitosan may be achieved by addition of a separate cross-linking agent slowly into the emulsion phase if this is the case.

Reverse Micellization: A surfactant is dissolved in an organic phase or a combination of organic phases initially. Then, aqueous chitosan solution is slowly added to the organic phase creating a W/O emulsion. During this phase, the surfactant molecules transfer to the oil-water interface and stabilize the droplets. Introduction

of the polymerization agent to the emulsion system allows the cross-linking agent to slowly diffuse into the emulsion droplet which acts as micro reactors to precipitate chitosan (Banerjee et al. 2002; Mitra et al. 2001; Kafshgari et al. 2012).

It should be noted that the use of the reverse micelle term in the literature is erroneous and misleading in this case since the emulsion droplets stabilized this way by the surfactant (polar heads looking into the droplet while the hydrocarbon chains extending into continuous organic phase) are not true micelles. They are simply aqueous droplets stabilized by the surfactant molecules in an organic hydrophobic phase. Introduction of the drug into this system could be made by dissolving the drug in the aqueous phase by employing proper solvation routes which would lead to drug-loaded chitosan particles after polymerization.

Nanoprecipitation: This is another variation of the emulsification methods with the difference that chitosan is dissolved in a suitable organic solvent instead of an aqueous phase. The organic phase is then gradually added into another phase which is not miscible with it and in which chitosan is insoluble (such as ethanol). The diffusion of the chitosan molecules from the organic phase into the dispersed phase creates the precipitated nanoparticles. The formed particles can be kept dispersed by adding a stabilizing surfactant into the dispersing phase (Fessi et al. 1989; Bilati et al. 2005; Luque-Alcaraz et al. 2016).

Spray-Drying: This is a rather classical method of particle formation where aqueous chitosan solution is atomized using a spray dryer. Then small droplets obtained via atomizer are mixed with a drying gas to evaporate the liquid phase to obtain chitosan nanoparticles (Ngan et al. 2014; Mehrotra et al. 2010; Liu et al. 2018)

It can be observed from the description of the methods in the above paragraphs that all the methods described can actually be combined into two main categories (Fig. 7.4) which can be best expressed as:

- Methods where chitosan polymerization is achieved in bulk solution
- Methods where chitosan polymerization is achieved on the surface of some template material such as micelles, oil droplets, or solid particles

It can be seen that either bulk polymerization or surface polymerization can be employed to create drug-loaded chitosan nanoparticles depending on the degree of hydrophobicity of the drug.

Bulk Polymerization: This method is employed for hydrophilic drug molecules unless drug molecules are intentionally imparted hydrophobicity by a suitable physicochemical treatment such as grafting by a specific end group or by a surfactant. In bulk polymerization, the hydrophilic drug is dissolved in an aqueous chitosan solution initially. Polymerization can be carried out by direct addition of the polymerization agent into this solution. In this case, the polymerization agent and chitosan diffuse into each other in bulk and precipitation takes place. But, controlling the size and morphology of the precipitated particles is a challenge.

To achieve better control, the chitosan solution containing the dissolved drug is first used in creating a W/O emulsion such that the drug is within the dispersed

micron or nano-sized droplets of the aqueous chitosan phase such that each droplet acts as a micron or nano-sized reactor for the polymerization reactions. Introduction of the aqueous polymerizer solution to initiate chitosan precipitation can be made by direct addition, which creates a W/O emulsion of the polymerizer solution in bulk, or by dispersing the polymerizer solution in an oil phase before creating the W/O emulsion of the polymerizer separately. In either case, the precipitation reactions occur when the droplets from both W/O emulsions, one containing the polymerizer and the other chitosan and the drug, collide and coalesce, leading to a slower and a better controlled synthesis step.

Surface Polymerization: This method is employed (when chitosan polymerization is achieved on the surface of micelles and oil droplets not solid particles) for hydrophobic drug molecules unless drug molecules are intentionally made hydrophilic by grafting by a specific end group or by a surfactant. In surface polymerization, the hydrophobic drug is dissolved either in the micellar core of a polymeric or directly in an oil phase. In either case, the micellar solution or the oil phase containing the drug molecules is dispersed in an aqueous chitosan solution. The micelles and the drug-containing oil droplet act both as nucleation sites and soft templates on the surface of which polymerization of chitosan takes place upon introduction of the polymerizer. In some cases, chitosan is precipitated on the surface of solid particles (such as silica, calcium ferric, iron oxide, etc.) together with the drug in order to create particles with magnetic properties or with a better strength.

Figure 7.5 presents some recent results from our laboratory where we have prepared micelles of the polymeric surfactant Pluronic P-123 to dissolve a strongly hydrophobic drug probucol which were then camouflaged with chitosan using surface polymerization. Dissolving the drug in the micelle structure has been carried out by thin-film hydration in this case (Cihan et al. 2017). In some cases, however, a physicochemical treatment can be applied to modify the drug to impart a hydrophilic nature instead of using micelles as stated above.

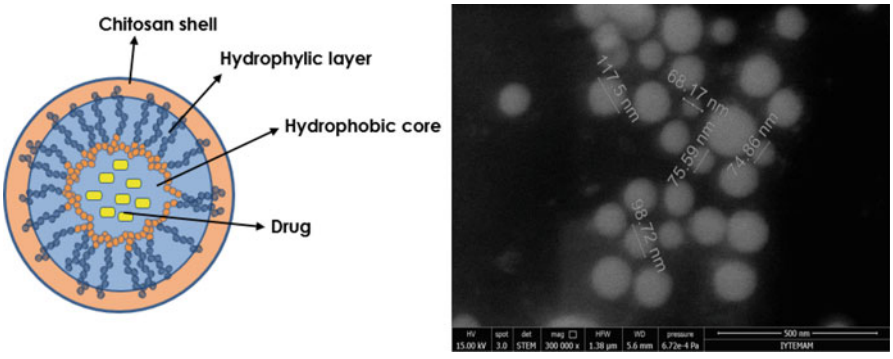

Fig. 7.5 A schematic view (left) of the chitosan nanoshells which camouflage Pluronic P-123 micelles in whose cores a strongly hydrophobic drug probucol is dissolved and the SEM pictures of the nanoshells (right)

In addition, a variation of the bulk polymerization method, which is named as self-assembly in literature, has been suggested by various researches (Yang et al. 2014; Petrov et al. 2008; Mu et al. 2019a, b). The main aspect of this method is that the dissolved chitosan molecules in acidic aqueous phase are made hydrophobic by attaching selected monomers (e.g., acid-anhydrides of benzoic or valeric acids) onto the amine groups. This is similar to decreasing the degree of solvation of the chitosan molecules, in this case brought about by making the molecules hydrophobic in an aqueous solution, leading to creation of chitosan aggregates (or chitosan micelles) into which hydrophobic drug molecules are incorporated.

7.2.3 Characterization of Chitosan Nanoparticles

Nanoscale materials may present different properties than from their bulk counterparts, as their high surface-to-volume ratio results in an exponential increase of the reactivity at the molecular level. In this section, we describe various surface and structure characterization methods which are widely employed to characterize nanostructures such as the chitosan nanoparticles we deal in this paper. While these techniques can be employed exclusive for the study of a particular property, they can be used in combination in most cases to describe a better picture of the system under analysis. The techniques which are employed most commonly will be discussed in detail in the following paragraphs. An excellent summary of the techniques has been carried out by Mourdikoudis et al. (2018). A schematic view of the characterization techniques is presented in Fig. 7.6 below.

Scanning Electron Microscopy: SEM is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample. The electron beam is scanned in a raster scan pattern, and the position of the beam is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. SEM may be used to determine the surface morphologies and structural integrity of micro- and/or nano-sized particles. A representative SEM photograph of nano-sized silica particles manufactured in our laboratories using Stöber synthesis is presented in the top left section of Fig. 7.7.

Scanning Transmisson Electron Microscopy: STEM is a type of transmission electron microscope (TEM). In STEM, the electron beam is focused to a fine spot (0.05–0.2 nm) unlike the conventional transmission electron microscope and then scanned over the sample in a raster illumination system constructed in a way that at each point sample is illuminated with the beam parallel to the optical axis. Scanning transmission electron microscopy (STEM) analysis can be used for the investigation of surface and morphology of chitosan and micelle-embedded chitosan nanoparticles. Again a representative STEM photograph of the drug-loaded Pluronic P-123 micelles prepared in our laboratories using thin-film hydration method is presented in the bottom left section of Fig. 7.7.

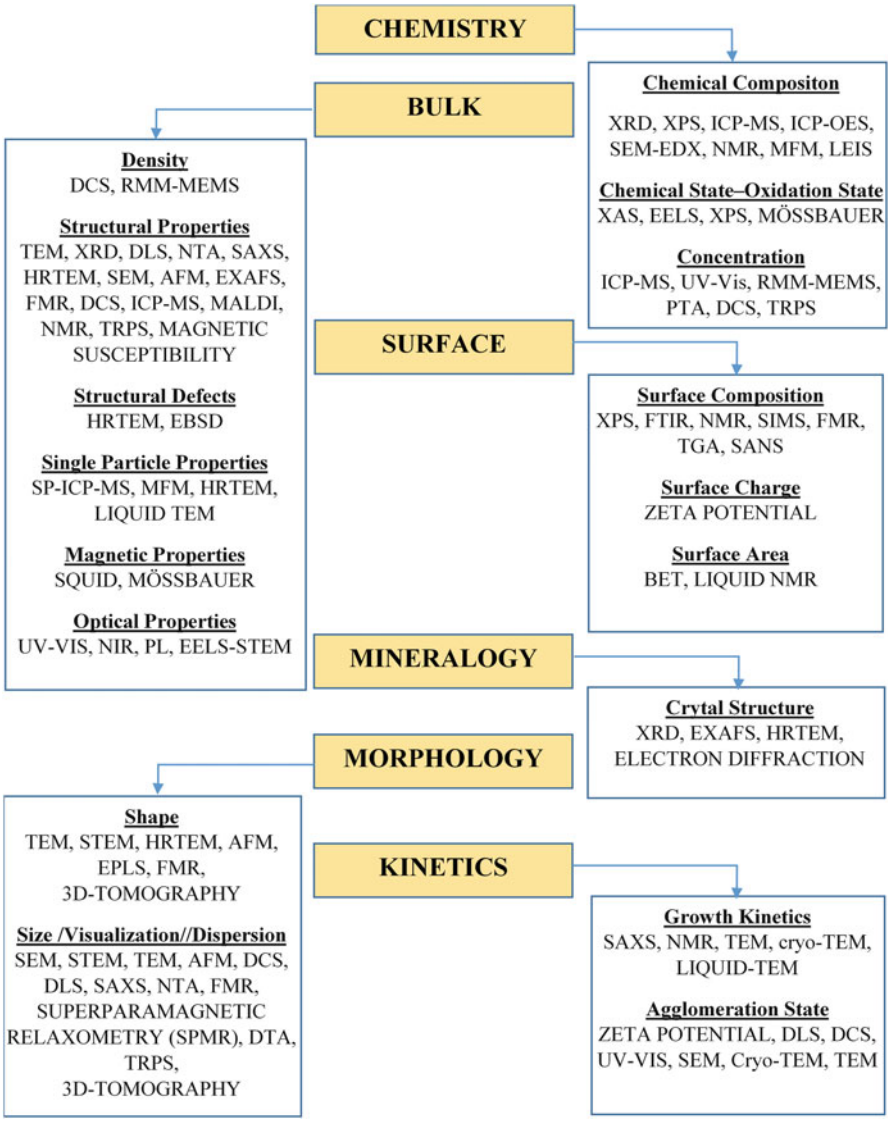

Fig. 7.6 Nanoparticle characterization methods

Transmission Electron Microscopy: TEM is a very powerful tool for material science. A high-energy beam of electrons is shone through a very thin sample, and the interactions between the electrons and the atoms can be used to observe features such as the crystal structure and features in the structure like dislocations and grain boundaries. TEM can reveal the finest details of internal structure – in some cases as small as individual atoms. TEM can be used for the investigation of surface and

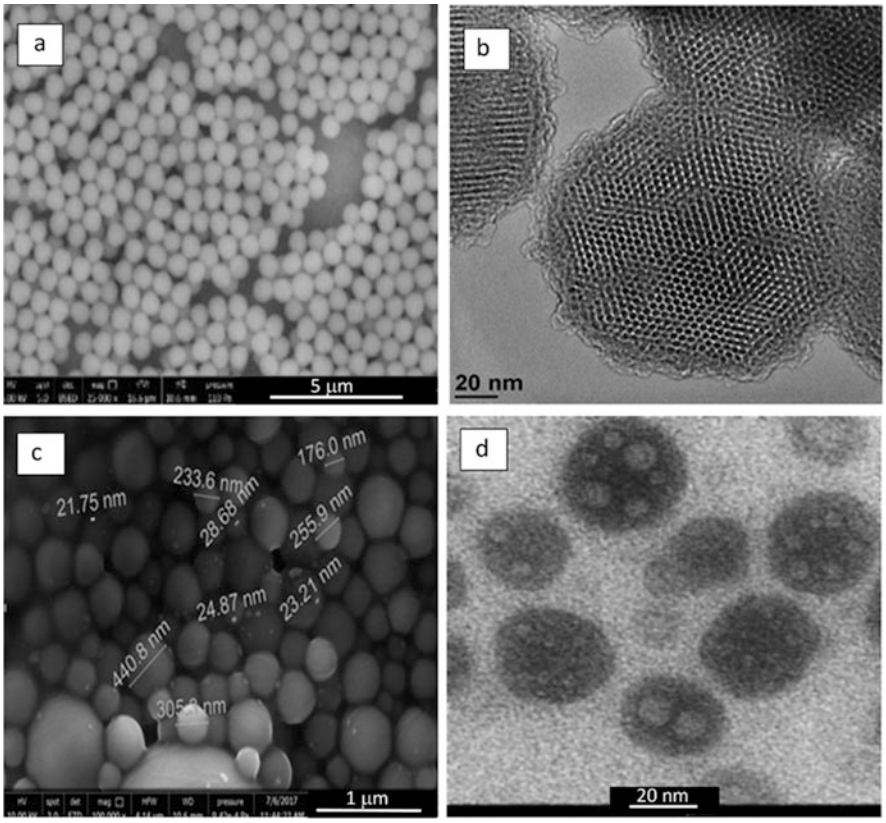

Fig. 7.7 Some examples of the SEM, STEM, and TEM pictures of the nanoparticles synthesized in the authors' laboratories
Mono-sized mesoporous silica particles; photographs obtained with SEM (a) and TEM (b)
Drug-loaded Pluronic P-123 micelles; photographs obtained with STEM (c) and TEM (d)

structure of chitosan and drug-embedded chitosan nanoparticles. TEM has priority on imaging samples with a great magnification rather than SEM and other electron microscope techniques, so it makes TEM the best way to determine morphological properties of nanoparticles and drug-loaded micelles in samples. Two representative TEM photographs of the silica particles and drug-loaded Pluronic micelles are presented in the top and bottom right sections of Fig. 7.7.

Dynamic Light Scattering: Size measurements of chitosan nanoparticles can also be obtained using DLS methods. Size of particles which is measured by the method is inversely proportional to angle seen after the particles scatter light. These particles pass through a focused laser beam during the laser diffraction measurement. A series of photosensitive detectors are used to get the angular intensity of scattered light. Particle size is calculated by using the map of scattering intensity versus angle. Particles are moving because of Brownian motion which is due to random collision

with the molecules of the liquid that surrounds the particle. Stokes-Einstein equation defines the relationship between size of particle and its speed due to Brownian motion.

Fourier Transform Infrared Spectroscopy: FTIR is used in the qualitative analysis of drug-loaded chitosan nanoparticles. The presence or absence of functional groups is investigated. FTIR spectroscopy is based on vibrational energy as a consequence of radiation absorption on atoms. Vibrational energy leads to determine functional groups and bonds in compounds.

Small-Angle X-Ray Scattering: SAXS is a nondestructive method for investigating nanostructures in liquids and solids. In a SAXS experiment, an X-ray beam is aimed at a nanostructured sample (for example proteins, macromolecules, or nanoparticle dispersions). The beam is scattered following its interaction with the electrons of the sample and collected by a detector. The detected scattering pattern is characteristic for the nanostructures of the sample and can be used to determine important structural parameters such as particle size, shape, internal structure, porosity, and arrangement (orientation). Small-angle X-ray scattering (SAXS) ideally complements microscopic methods (AFM, TEM) since it provides representative structural information about a large sample area.

Nuclear Magnetic Resonance Spectroscopy: NMR or nuclear magnetic resonance spectroscopy is a technique used to determine a compound's unique structure. It identifies the carbon-hydrogen framework of an organic compound. Using this method and other instrumental methods including infrared and mass spectrometry, scientists are able to determine the entire structure of a molecule. Even though there are many other spectrometers including C-NMR and N-NMR, hydrogen (H-NMR) was the first and is the most common atom used in nuclear magnetic resonance spectroscopy. The atomic nucleus is a spinning charged particle, and it generates a magnetic field. Without an external applied magnetic field, the nuclear spins are random and spin in random directions. But, when an external magnetic field is present, the nuclei align themselves either with or against the field of the external magnet.

7.3 Modification of Chitosan Nanoparticles for Anticancer Therapies

Multitudinous work exists in the literature on the use of chitosan-based nanoparticles as anticancer drug carriers, and the number has been increasing at a greater rate in the last 20 years. Searching the papers in the Web of Science with the word "chitosan + cancer or tumor" *in the title* results in 813 published journal articles between the years 2000 and 2019 (see Fig. 7.2c). It can been seen that there is a growth trend in the last 20 years in employing chitosan in anticancer research in one form or another. Majority of this work is about synthesizing chitosan nanoparticles for use in anticancer therapy by one of the routes described in Sect. 7.2.2 and summarized in Fig. 7.4. The literature reviewed in the following paragraphs deal specifically with modifying the chitosan nanoparticles for more efficient tumor-targeted anticancer

drug delivery applications. The following paragraphs contain a concise classification of these latter studies.

Chemical Modification: Chitosan is insoluble in water except acidic media and in other organic solvents due to the presence of amine groups in its structure. Various chemically modified chitosan derivatives have been produced through modification of the amine or hydroxyl functional groups to increase its solubility, which is extremely important in controlling the interaction of the polymer with the drugs (Casettari et al. 2012; Kurita et al. 2006; Ahsan et al. 2018; Shukla et al. 2013; Gorochovceva et al. 2004; Luckachan et al. 2006; Hua et al. 2008; Tang et al. 2007; Radhakumary et al. 2007; Sutirman et al. 2018; Shariatinia et al. 2019).

Rahimi et al. (2019) prepared chitosan-quinoline nanoparticles as hydrophobic anticancer drug nanocarriers using 2-chloro-3-formylquinoline and 3-formylquinolin-2(1H)-one as nontoxic modifying agents via Q/W nanoemulsion technique. Their characterization using FTIR, UV-vis spectrophotometry, XRD, SEM, AFM, and DLS techniques demonstrated that the drug-loaded chitosan-quinoline nanoparticles have a regular nanorod shape and monolithic structure with a particle size range of between 141 and 1745 nm and a zeta potential range between −2.4 and −14.1 mV. They found that the loading capacity and encapsulation efficiency with the hydrophobic anticancer drug quercetin were between 4.8–9.6% and 65.8–77%, respectively.

Zhang et al. (2019) studied the utilization of the marker GX1 to fabricate a multifunctional vascular targeting of docetaxel-loaded nanoparticles with N-deoxycholic acid glycol chitosan as the carrier and GX1-PEG-deoxycholic acid conjugate as the targeting ligand for gastric cancer therapy. They found that in vitro drug release tests showed sustained and pH-dependent release. In vivo delivery of marker/carrier/drug composite nanoparticles inhibited tumor growth in mice at a rate of 67.05%.

Razmi et al. (2013) studied platinum-based anticancer drugs that have limited applications due to their severe toxicity. They described a drug delivery system comprising a platinum complex (bipyridine morpholine dithiocarbamate Pt (II) nitrate) within nanoparticles composed of casein and chitosan. They studied the effect of pH using UV-vis spectrometry, dynamic light scattering (DLS), and scanning electron microscopy (SEM). They observed that the optimum pH for complex formation is between the pI of casein (5.3) and the pKa of chitosan (6.5), and there is an enhancement in the cytotoxicity and cellular uptake of platinum by its entrapment in casein-chitosan nanovehicles. Their findings suggest that this drug delivery system enables drugs to be thermodynamically stable in aqueous solutions and is potentially useful for targeted oral delivery applications.

Sutar et al. (2018) introduced a chitosan-polylactic acid-drug conjugate, and its iron transport protein (transferring) receptor targeted polyelectrolyte complex nanoparticles, encapsulating free drug to increase its potency and specificity. The model drug was strongly hydrophobic curcumin and incorporated in the system in both conjugated and encapsulated form. Chitosan-polylactic acid-curcumin copolymer was characterized by 1 H NMR, FTIR, UV-visible spectrometry, differential scanning calorimetry, and zeta potential measurements. The nanoparticles

demonstrated high curcumin loading over 92% with extended periods for release (60% and 85% at pH 7.4 and 5, respectively, even after 8 days). It was concluded that curcumin-loaded transferrin-chitosan-polylactic acid-curcumin nanoparticles may provide an efficient and targeted delivery for cancer treatment.

In some other studies, chitosan was modified by grafting polymer chains onto the surface of magnetic chitosan (Cesano et al. 2015; Kloster et al. 2015; Liu et al. 2009; Suzariana Samuri et al. 2016; Roveimiab et al. 2012; Jiang et al. 2012; Bagheri et al. 2015; Niu et al. 2014). In these studies, three types of grafting methods "grafting from," "grafting through," and "grafting onto" were employed. The "grafting onto" method is based on using pre-synthesized polymer chains. Among these three methods, "grafting from" (Hua et al. 2008; Jiang et al. 2012) can be performed using controlled radical polymerization techniques (Hojjati et al. 2008). Atom transfer radical polymerization (Renggli et al. 2017; Lanzalaco et al. 2017), reversible addition-fragmentation chain transfer (Hua et al. 2008; Cao et al. 2015; Yamamoto et al. 2014; Hosseinzadeh et al. 2018), and living free radical nitroxide-mediated polymerization (Hua et al. 2008; Ballard et al. 2017; Nicolás et al. 2013) are among the controlled radical polymerization techniques (Yamamoto et al. 2014). Hosseinzadeh et al. (2019) prepared a unique stimuli-responsive hydrogel nanocomposite via surface reversible addition fragmentation chain transfer copolymerization of acrylic acid and N-isopropyl acrylamide onto chitosan and subsequent in situ synthesis of magnetic Fe_3O_4 nanoparticles for anticancer drug doxorubicin delivery. The maximum of doxorubicin loading efficiency of nanocomposite was 89%, and 82% of total doxorubicin was released from the hydrogel within 2 days. In this study, temperature and pH responsiveness of the nanocomposite were demonstrated, and they suggested that the chitosan-based nanocomposite may be utilized as a promising drug carrier for controlled and sustained release of anticancer drugs.

Another chemical modification technique is the use of carboxymethyl moieties. The water solubility of carboxymethyl chitosan at various pH environments is governed by the carboxymethylation degree. Shariatinia (2018) has reviewed the literature on carboxymethyl chitosan and its properties and biomedical applications. The most recent applications of carboxymethyl chitosan derivatives with antimicrobial, anticancer, antitumor, antioxidant, and antifungal biological activities in various areas like wound healing, tissue engineering, drug/enzyme delivery, bio-imaging, and cosmetics have been discussed in their review.

Tana et al. (2013) also synthesized a glycol chitosan-carboxymethyl-cyclodextrins (G-chitosan-CM-dextrins) for delivering different hydrophobic anticancer drugs. They showed that the three anticancer drugs (5-fluorouracil, doxorubicin, and vinblastine) could be successfully loaded into the cavities of the covalently linked CM-dextrins. pH-sensitive release of doxorubicin has been observed and suggested that different drugs should be released in different ways.

Coating on Nanoparticles: Parsian et al. (2016) studied the targeted delivery of the hydrophilic gemcitabine to increase its cellular uptake and efficacy. For this purpose, chitosan-coated iron oxide nanoparticles have been synthesized by coprecipitation that encapsulates gemcitabine as described in Fig. 7.4. They

optimized the loading of gemcitabine as 30 μM with the highest drug release as 65% at pH 4.2, while it was 8% at pH 7.2. This is desired since pH of tumor tissue and endosomers is acidic. They tested the cellular uptake and targetability of these nanoparticles on MCF-7 breast cancer cell lines and indicated the increased efficacy of gemcitabine when loaded onto nanoparticles. Kamaraj et al. (2018) have developed curcumin-loaded hybrid nanoparticles of vanillin-chitosan coated with paramagnetic calcium ferrite nanoparticles using ionic gelation method. The vanillin-chitosan nanoparticles were functionally modified by the Schiff base reaction to enhance the hydrophobic drug encapsulation efficiency. Calcium ferrite nano particles were added to the system to improve the biocompatibility. The maximum encapsulation efficiency obtained was 98.3% under the conditions of 0.1, 0.75, and 1.0 for the drug to chitosan-vanillin, CFNP to chitosan-vanillin, and TPP to chitosan-vanillin ratios, respectively. They executed the curcumin release at various pH, initial drug loading concentrations, and magnetic fields and predicted the drug release mechanism by fitting the experimental kinetic data with various drug release models. The cytotoxicity test of nanocarriers is performed against MCF-7 breast cancer cell line to check the anticancer property of the hybrid nanocarrier with the curcumin drug. Chen et al. (2019) created composite structures by cross-linking chitosan onto drug-loaded mesoporous silica nanoparticles through disulfide bonds. This created a thin film of chitosan which led to site-specific and timely drug delivery. The system was also able to trigger drug release by the changes in such factors which are common to cancer cells. They suggested that this surface chemical modification strategy promises a powerful approach constructing smart drug delivery systems for efficient and safe chemotherapy.

Sasirekha et al. (2019) investigated the use of a mesoporous, biodegradable nanomaterial obtained from the natural silica found in the diatom species Amphora subtropica for drug delivery applications. The cultures of this material were cleaned and chemically treated to obtain Amphora frustules (exoskeleton) followed by surface functionalization with chitosan. Results of their experiments demonstrated high drug loading, strong luminescence, and biodegradable and biocompatible nature of the doxorubicin tethered diatom.

Rao et al. (2018) proposed in situ preparation method of Au NPs (hexagonal and rod-shaped structures) in the lumen as well as the surface cage of biocompatible halloysite nanotubes using curcumin as anticancer drug and subsequently coating with bioadhesive chitosan. The anticancer potential of halloysite nanotube hybrid nanoparticles on MCF-7 cancer cells was studied and showed efficient anticancer activity under intracellular tumor cell environment (pH 5.5) than extracellular conditions (pH 7.4). They suggested that the developed halloysite nanotube hybrid nanoparticles consisting of Au nanoparticles (NIR-responsive property) and pH-responsive curcumin release could make it suitable for cancer cell-targeted drug delivery.

Self-Assembled Amphiphilic Chitosan Nanoparticles: A review on the recent progresses in the design and fabrication of chitosan-based self-assembled nanomaterials and their applications in the delivery of different therapeutic agents was done by Yang et al. (2014). In a recent study, Petrov et al. (2008) produced a

drug delivery system by encapsulating quercetin into pH-sensitive self-assembled amphiphilic chitosan nanoparticles. Up to 83% of quercetin was entrapped by the nanoparticles. They found that the payload release is larger at an acidic pH of 5.0 than at the physiological pH of 7.4. They further revealed that quercetin maintains its metabolism inhibition against MCF-7 cells after encapsulation and that nanoparticles accumulate on the cell surface.

Mu et al. (2019a, b) synthesized quercetin-chitosan conjugate for oral delivery of doxorubicin to improve its oral bioavailability by increasing its water solubility, opening tight junction, and bypassing the P glycoprotein. The prepared quercetin-chitosan self-assembled into micelles which could encapsulate doxorubicin with high encapsulation rate, small particle size (137 nm), and strong zeta potential (+16.2 mV). Quercetin-chitosan-doxorubicin micelles displayed sustained-release profile in gastrointestinal simulation fluid (pH 1.2/pH 7.4). Quercetin-chitosan micelles could promote cellular uptake of doxorubicin, which was 2.2-fold higher than that of free doxorubicin. They showed that quercetin-chitosan micelles are promising vehicles for the oral delivery of insoluble anticancer drugs.

Chitosan-Mediated Co-delivery: Different anticancer drugs affect different parts of the cell and should be used together in chemotherapy. Nanoparticle systems which allow the simultaneous use of multiple drugs are called the co-delivery systems and synergistically inhibit the tumor growth. Qi et al. (2017) highlighted the current state of co-delivery nanoparticles and the most commonly employed nanomaterials. They discussed challenges and strategies and prospect future development. Afkham et al. (2018) designed chitosan-mediated co-delivery nanoparticles for the efficient encapsulation of the anticancer drugs SN38 (7-Ethyl-10-hydroxy-camptothecin) and snail-specific small interfering RNAs (siRNA). They found that chitosan nanoparticles encapsulating SN38 and snail-specific siRNA may represent huge potential as an effective anticancer drug delivery system for the treatment of prostate cancer.

There are also studies which employ chitosan nanoparticles for immunotherapy of cancers. The use of chitosan nanoparticles in this field is summarized in a review paper by Naskar et al., (2019) where various types of chitosan nanoparticles have been listed. The paper discusses the attempts made to form new therapeutic approaches that can restore immune competence in cancer patients (Li et al. 2011; Zhao et al. 2011a, b; Xue et al. 2015b; Garg et al. 2016; Jesus et al. 2017; Yaguchi et al. 2011).

7.4 Drug Release Studies with Chitosan Nanoparticles

The most important feature of chitosan-based nanoparticles/nanoshells is their pH-dependent release behavior. Chitosan structures degrade at the acidic pH environment of the cancer cells and release the contained drug. The pH-dependent release of drug with a minimal release at the physiological pH of 7.4 makes the chitosan carriers as one of the most promising candidates for chemotherapeutic cancer treatment with reduced side effects.

Qiu et al. (2013) studied the release profiles of doxorubicin from self-assembled chitosan nanoparticles. The self-assembled phytosterol-fructose-chitosan nanoparticles have been synthesized from water-soluble fructose-chitosan solutions. The procedure consisted of forming fructose-chitosan polymeric material by adding fructose to chitosan-acetic solution and polymerization by sodium borohydride as a first step. Then, phytosterol hemisuccinate was coupled to fructose-chitosan for self-assembly by succinyl linkages with phytosterols as hydrophobic moieties. In the final step doxorubicin was physically entrapped inside the self-assembled nanoparticles by the dialysis method. A slow sustained release of doxorubicin over a 48 h period was observed, and the release rate in phosphate buffered saline solution at pH 7.4 was much slower than that in pH 5.5 and pH 6.5.

Wang et al. (2015) prepared chitosan-modified polylactic acid nanoparticles as carriers for encapsulation of docetaxel by anti-solvent precipitation method. They characterized the polylactic acid/chitosan nanoparticles by SEM, DLS, FTIR, and XPS. The particles were 250 nm in size and had a zeta potential value of +53.9 mV. They showed that docetaxel releases from the prepared polylactic acid/chitosan nanoparticles with 40% initial burst release in 5 h and 70% cumulative release within 24 h. The release of docetaxel from the polylactic acid nanoparticles, on the other hand, was 65% in 5 h. The study suggested that polylactic acid/chitosan nanoparticles prolong drug release while decreasing the initial burst release.

Sun et al. (2017) studied the sustained-release properties of the biodegradable nano-drug delivery systems to improve the residence time of the chemotherapeutic agent in the body. The 5-fluorouracil-loaded chitosan nanoparticles have been prepared in their study. They found that when the mass ratio of 5-fluorouracil and chitosan was 1:1, the maximum drug loading of nanoparticles was around 20% with an encapsulation efficiency of around 44%. The mean size of the particles was 284 nm and the measured zeta potential was 45.3 mV. The prepared nanoparticles had both burst-release and sustained-release phases in vitro release studies.

Karimi et al. (2018) developed κ-carrageenan-cross-linked magnetic chitosan with different molecular weights as pH-responsive carriers for controlled release of the hydrophobic anticancer drug sunitinib. They found that drug encapsulation efficiency and release performance were influenced by the size of magnetic nanoparticles. Encapsulation efficiencies of sunitinib by low, medium, and high molecular weights of magnetic chitosan carriers were found to be 62.4, 69.6, and 78.4%, respectively. The in vitro sunitinib release from magnetic chitosan/κ-carrageenan carriers has shown to be pH-dependent and followed a Fickian release mechanism. They showed that sunitinib was efficiently released from magnetic carriers into the environment under acidic pH and the release rate was size- and molecular weight-dependent.

These sample drug release studies demonstrate that the use of chitosan nanoparticles as drug carriers presents a decisively positive contribution. In many cases, however, the research tends to employ chitosan together with other materials to create a synergy for delivery purposes. However, it is important to note that one should be careful about not trading away the outstanding properties of this

biopolymer (such as its pH-dependent degradation and positive charge) when it is employed with other materials to design a composite drug carrier.

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Chitosan-Based Systems for Gene Delivery

8

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Abstract

Conventional methods for gene delivery with non-viral or viral delivery carriers are beset with various disadvantages such as immune reactions, low transfection efficiency, and toxicity. Innumerable methods are under investigation to help improve the development of biodegradable polymers with low cytotoxicity, transcellular transport ability, favorable physicochemical properties, ease of modification by targeting ligands, and high transfection efficiency. Chitosan is a biodegradable polymer that has attained a lot of attention as a gene delivery vector due to its ease of modification, high transfection efficiency, and exceptional biocompatibility. Chitosan being cationic in nature can form polyelectrolyte complexes with negatively charged DNA allowing nucleic acid condensation along with protection from nucleases, which is widely beneficial in gene therapies. Moreover, factors such as pH, degree of acetylation, N/P ratio, and surface modifications can be suitably investigated to improve transfection efficiency of chitosan-based vectors. Various chitosan-based gene delivery systems developed in the past decade including chitosan-based polyplexes, nanoparticles, and DNA vaccines have been discussed in this chapter. The goal of this book chapter is to review recent advancements in gene therapy with major focus on chitosan and its applications as a gene delivery vector.

Keywords

Non-viral vectors · Gene therapy · DNA vaccine · Chitosan-based nanoparticles

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_8

8.1 Introduction

8.1.1 Introduction to Gene Therapy

Genes are sequence of nucleotides in DNA and RNA within the genome that contain instructions to code for their respective proteins. These instructions have been a paramount source of information for understanding life on earth as well as for the development of therapeutics based on its core structure. Gene therapy is a relatively new branch in medical science with huge therapeutic potential for treating a disorder at its genetic root. It includes a set of practices, which help in delivering genetic material into specific cells using vectors to temper expression or suppression of biosynthesis of certain proteins leading to genetic alteration for the betterment of a patient. However, the success of gene therapy majorly depends on the vector's or vehicle's ability to selectively and efficiently deliver a gene to the target site with minimal or no side effects. A number of adverse events have been reported over the past two decades related to vectors which focused attention to associated risks before therapeutic benefits (Hacein-Bey-Abina et al. 2008; Raper et al. 2003; Marshall 1999). Although viruses are very efficient in transducing cells, they remain one of the major concerns in gene therapy owing to their serious adverse effects and limitations including carcinogenesis, immunogenicity, broad tropism, limited nucleic acid packaging capacity, and cost-effectiveness (Yin et al. 2014). Consequently, there is an increased interest in development and use of non-viral gene delivery systems.

Over the past few decades, there is extensive effort in development of non-viral systems for safe and efficient delivery of therapeutic genetic material. Non-viral vectors have the advantage of having a favorable safety profile and low immunogenicity but are often associated with low transfection efficiencies (Kong et al. 2005). Subsequently, massive interest in chitosan-based non-viral gene delivery systems has been developed due to their versatility, good safety profile, and favorable properties as gene carriers. This chapter will evaluate and summarize chitosan as a non-viral gene delivery vector along with its physicochemical properties, scope of chemical modification, and mechanism of gene delivery. Various currently investigated chitosan-based systems will be scrutinized and presented with the limitations and challenges associated with such delivery systems. Finally, a comprehensive review of recent applications of such delivery systems will be presented along with some comments on further approaches and future perspectives of chitosan-based gene therapy.

8.1.2 Various Gene Delivery Systems and Methods

The major goal of gene therapy is to correct the defective gene via administration of a genetic material. There are various types of gene delivery systems which can be used to specifically upregulate or downregulate a gene(s). In 1989, the first gene transfer into humans took place using tumor-infiltrating lymphocytes modified by a

retroviral gene (Rosenberg et al. 1990). Shortly after in 1990, gene therapy was performed by transferring adenosine deaminase (ADA) gene into the T cells of two children suffering from severe combined immunodeficiency (SCID) defect (Blaese et al. 1995). Broadly, there are two categories for gene therapy: germline therapy and somatic gene therapy. Although germline therapy is forbidden due to ethical reason, it has great potential (Sugarman 2015; Nielsen 1997). For now, this field has been limited to somatic cells and there is noteworthy development in the field with growing technological advancements. It involves transfer of nucleic acids (transgene) in the cells to repair, replace, and silence a dysfunctional gene for therapeutic outcomes. Owing to the large size, hydrophilic nature, susceptibility to enzymatic degradation, and poor cellular uptake, delivery of such transgenes is often mediated via a carrier or vector (Sharma and Singh 2017). An ideal gene delivery system should have the following features: (A) It should not cause any immune reactions, (B) it should be able to penetrate biological membranes and have long duration of expression, (C) it should be specific and stable with an adequate carrying capacity, and (D) it should be easy to manufacture and store. There are various types of viral and non-viral gene delivery systems but no system till date possesses all ideal characteristics. Each of the methods has their own advantages and disadvantages which are covered in Table 8.1.

8.1.3 Viral Vectors vs Non-viral Vectors for Gene Therapy

Early advancements in the field of gene therapy recognized the potential of mammalian viruses as proficient gene delivery vehicles for the treatment of genetic disorders (Mann et al. 1983). Initial clinical studies in gene therapy for the treatment of adenosine deaminase-severe combined immunodeficiency defect (ADA-SCID) employ retroviral vectors based on murine leukemia virus (MLV) (Aiuti et al. 2009). In 1996, human immunodeficiency virus (HIV) was first fabricated into a lentiviral-based gene delivery vector capable of effective delivery to mitotic and nondividing cells (Naldini et al. 1996). Since then many other lentiviruses have been converted into vectors. These vectors had been utilized in clinical trials for the treatment of thalassemia, Wiskott-Aldrich syndrome, and leukemia with positive results (Brentjens et al. 2013; Kalos et al. 2011; Aiuti et al. 2013; Cavazzana-Calvo et al. 2010). However, biodistribution properties of these vectors and deactivation by serum components make their use as in vivo gene delivery vehicles a challenging task (DePolo et al. 2000). On the other hand, adeno-associated virus (AAV)-based vectors containing non-enveloped nonpathogenic parvovirus with icosahedral capsid have shown promising potential for in vivo gene delivery (Daya and Berns 2008). The recombinant versions of AAV can lead to stable expression in dividing and nondividing cells for years (Flotte 2004). These recombinant vectors were initially employed in clinical trials related to cystic fibrosis, rheumatoid arthritis, and hemophilia (Moss et al. 2007; Mease et al. 2009; Manno et al. 2006). In November 2012, the European Medicines Agency approved the use of alipogene tiparvovec (Glybera) under special circumstances. Glybera is an intramuscular injection containing

Table 8.1 Advantages and disadvantages of various gene delivery systems and methods

Viral vector	Advantages	Disadvantages	References
Adenovirus	Efficient in transducing different types of dividing and nondividing cells ex vivo as well as in vivo	Inflammatory immune toxicity with multiple administration	Danthinne and Imperiale (2000) and Liu and Muruve (2003)
		Remains episomal with transient expression	
		Packaging cell line required with no targeting ability	
Retrovirus	Incorporates into cellular genome with long-term expression	Packaging cell line required with no targeting ability	Kamimura et al. (2011)
	Large insert size (9–12 kb)	Need dividing cells for transfection	
		Poor transduction ability	
Lentivirus	Efficient in transducing dividing and nondividing cells with long-term expression	Random integration into genome	Naldini et al. (1996)
		Limited insert size of 8 kb	
		Safety issues: HIV origin	
Adeno-associated virus	Long-term expression with a wide range of hosts	Packaging cell line required with no targeting ability	Bett et al. (1994) and Hermonat and Muzyczka (1984)
	Absence of viral genes	Limited insert size of 5 kb	
		Potential mutations	
Herpes simplex virus	Efficient in transducing in vivo with large insert size (40–50 kb)	Toxic to cells	Epstein 2009 and Burton et al. (2002)
	Efficient in CNS	No genome integration with transient expression	
		Packaging cell line required with no targeting ability	

(continued)

Table 8.1 (continued)

Viral vector	Advantages	Disadvantages	References
Vaccinia virus or poxvirus	Efficient in transducing different types of dividing and nondividing cells with high expression level	Immune toxicity with multiple administration	Ratko et al. (2003) and Roth and Cristiano (1997)
	Large insert size (>25 kb)	No genome integration with transient expression	
Non-viral vectors			
Plasmid DNA direct delivery	Safety, simplicity	Poor efficiency	(Olins et al. (1967), Herweijer and Wolff (2003), Laemmli (1975), and Hickman et al. (1994)
Gene gun	Efficient compared to needles	Restricted to targeted area	O'Brien and Lummis (2002), Al-dosari and Gao (2009), Valsalakumari et al. (2013), and Romano et al. (2000)
		Invasive procedures required for internal organs	
Electroporation	High efficiency	Restricted to targeted area with tissue damage	Drunen Littel-van den Hurk and Hannaman (2010) and Heller et al. (2005)
		Invasive procedures required for internal organs	
Sonoporation	Site specific	Poor efficiency with tissue damage	Al-dosari and Gao (2009)
Magnetofection	Site specific	Poor efficiency	Scherer et al. (2002)
Cationic lipid and polymer systems	High efficiency in vitro	Low transfection in vivo	Roth and Cristiano (1997), Al-dosari and Gao (2009), Valsalakumari et al. (2013), Romano et al. (2000), and Nishikawa and Huang (2001)
	Simple fabrication and storage	Short-term expression	
	Good safety profile	Difficulty in targeting	
	No immunogenicity		
	No restriction on insert size		

AAV1-based vector that delivers cDNA for human lipoprotein lipase. In clinical trials, Glybera showed promising safety profile with long-term gene expression and protein activity (Stroes et al. 2008; Carpentier et al. 2012). Besides these vectors, adenovirus, herpes simplex virus (HSV), and vaccinia virus (poxvirus) were also

used in a large number of clinical trials (Scallan et al. 2013; Hill et al. 2010; Smaill et al. 2013). The common feature of these viral vectors is that they do not integrate into host genome. Therefore, the risks related to insertional mutation are not associated with these vectors. Vaccines based on adenoviral vectors have also been developed against malaria, tuberculosis, and influenza. Attempts have been in effect regarding the development of HIV vaccination, but so far they have been unsuccessful (Buchbinder et al. 2008).

Viral vectors have shown promising results with clinical success over the past several decades but these vectors are also associated with severe side effects and toxicities in many clinical trials. There are also several drawbacks associated with these vector like potential for insertional mutagenesis and fatal acute inflammatory responses (Thomas et al. 2003). The major hazard associated with these vectors is that they cause delayed cellular or humoral immunological responses. Multiple clinical trials have been reported leading to fatal consequences due to the serious adverse reaction of virus-based vectors (Thomas et al. 2003; Wilson 2009). This led the motivated researchers to focus on the development of non-viral gene delivery vectors.

Non-viral gene delivery vectors are majorly associated with natural or synthetic compounds which have low toxicity and immunogenicity compared to viral vectors. Moreover, these vectors are readily available and cost-effective, contain high nucleic acid packaging capacity, and can be modified with ligands to target particular cell types in the body. These vectors are comparatively easy to produce and can be administered multiple times. Although non-viral vectors are associated with less efficacy and short-lived gene expression compared to their viral counterparts, recent advancements in physical methods are making non-viral gene delivery clinically relevant. Physical methods enable DNA entry into the cells by making short-lived penetration into the cell membrane. The easiest physical method for transferring DNA is by direct injection into the tissue. This method has been used in the skin, heart, liver, and tumor (Hickman et al. 1994; Habib et al. 1996; Park et al. 2001; Choate and Khavari 1997). Gene gun or microprojectile gene transfer is another method initially developed for gene delivery in plants (Klein et al. 1987). Since then, gene gun has been developed to transfer gene into mammalian cells in vitro *and* in vivo (O'Brien and Lummis 2002). Its applications have been directed towards muscle, liver, and surgically exposed organs (Yang et al. 1990). This method is majorly used for vaccination and immune therapy (Cassaday et al. 2007; Drape et al. 2006). Electroporation is another method for gene delivery which works by making pores into the cell membrane via electric pulses. Efficiency of this method is majorly determined by intensity, duration, and frequency of the pulse (Heller et al. 2005). This method was also tested for vaccination and for tumor treatment in clinics. (Drunen Littel-van den Hurk and Hannaman 2010). Sonoporation, similar to electroporation, uses ultrasound to form temporary pores in the cell membrane. This technique was developed in the 1990s (Bao et al. 1998; Miller and Song 2003). Similar to electroporation, the efficiency of this method is also determined by intensity, duration, and frequency of the pulse (Huber et al. 1999). This technique had been validated in various parts of the body like the cornea, brain, kidney,

pancreas, liver, muscle, and heart (Bekeredjian et al. 2005; Shen et al. 2008; Chen et al. 2006; Hou et al. 2005; Hynynen et al. 2003; Sonoda et al. 2006). Compared to other methods, sonoporation is still far from being developed for clinical purposes.

Chemical non-viral vector system is another type of gene delivery system majorly composed of nanomeric polycations complexed with negatively charged nucleic acid via electrostatic forces (Su et al. 2012). The chemical vectors generally have the following abilities: (A) protection of condensed gene from nucleases, (B) targeting potential toward specific cells, (C) disintegration from genetic material when in cytosol, and (D) controlling release of therapeutic gene at target site (Jin et al. 2014). These cationic particles can be directed to a specific group of cells by covalently attaching cell-targeting ligands to them. These ligands can be in the form of peptides, antibodies, carbohydrates, vitamins, and proteins (Hood et al. 2002; Zhang et al. 2004a, b; Chiu et al. 2004; Wolschek et al. 2002; Kim et al. 2004; Khalil 2006). Cationic lipids are one of the most widely explored non-viral gene delivery systems. In 1987, Felgner and colleagues established lipid-based gene transfer (Felgner et al. 1987). Currently hundreds of lipids have been investigated for gene transfection. Most of these lipids are amphiphilic in nature, and their transfection efficiency is majorly based on their structure and charge ratio with genetic material (Wasungu and Hoekstra 2006). Generally, the size of these lipid particles is <200 nm in diameter (Wheeler et al. 1999). In 1980, it was first shown that lipid particles called liposomes can encapsulate and deliver DNA to monkey hepatocytes (Fraley et al. 1980). In subsequent studies, a higher level of transfection was obtained and delivery of DNA to other cell lines was achieved (Felgner et al. 1987). In 2014, lipid-based formulation (DMRIE-DOPE) with DNA plasmid was unable to pass phase 3 clinical trial for treatment of advanced metastatic melanoma (Hersey and Gallagher 2014). However, different types of lipid-based formulations are investigated in clinical trials for the treatment of various diseases (Alton et al. 2013; Lu et al. 2012). Similar to cationic lipids, cationic polymers are also used as non-viral gene delivery vectors. They are an attractive alternative due to their immense chemical diversity and their potential for modifications. Homopolypeptide of lysine, called poly-L-lysine (PLL), is known to condense DNA since the 1960s (Olins et al. 1967; Laemmli 1975). PLL potential was investigated clinically for the treatment of cystic fibrosis (Konstan et al. 2004). Another early example is polyethylenimine (PEI), which is one of the most studied polymeric DNA vectors. PEI potential for gene therapy was discovered in 1995 in vitro as well as in vivo (Boussif et al. 1995). PEI-DNA polyplexes had shown transfection ability in lungs and tumors (Coll et al. 1999; Goula et al. 1998). In humans, PEI has been investigated for gene delivery in various types of cancers like bladder cancer, ovarian cancer, and pancreatic cancer (https://clinicaltrials.gov/ct2/show/NCT00826150; https://clinicaltrials.gov/ct2/show/NCT00595088; https://clinicaltrials.gov/ct2/show/NCT00711997). Although PEI is known for its substantial cytotoxicity, a number of modifications have been investigated for improving its biocompatibility and stability (Breunig et al. 2007; Petersen et al. 2002; Nguyen et al. 2000; Lv et al. 2006). Due to substantial efficacy and biocompatibility issues with PLL and PEI, other polymers are currently evaluated for gene delivery.

One such carbohydrate-based polymer called chitosan has been long investigated as a promising vector for gene delivery. Chitosan was first reported as gene delivery vector in 1995 (Mumper et al. 1995). In 1998, chitosan was demonstrated to be used as a potential gene delivery vector in vivo in mucosal epithelia (MacLaughlin et al. 1998). Subsequently, there were several studies demonstrating the potential use of chitosan and its derivatives as gene delivery vectors using DNA, siRNA, and miRNA (McKiernan et al. 2013; Vauthier et al. 2013; Mao et al. 2010; Harish Prashanth and Tharanathan 2007; Katas and Alpar 2006; Liu et al. 2005; Mansouri et al. 2004).

8.2 Chitosan for Gene Delivery

Chitin, a structural component in crustacean exoskeleton, is the second most abundant biomolecule in nature (Motiei et al. 2017; Tharanathan and Kittur 2003). It is very similar in structure to cellulose and has a significant role in biochemical cycling of nitrogen and carbon in aquatic ecosystem (Vollenweider 2000; Cauchie 2002). Chitosan, which is deacetylated chitin, gathered interest around the globe for application as a gene delivery vector. Chitosan consists of unprecedented qualities like cationic charge, high biocompatibility, ease of chemical modification, and low immunogenicity (Lee et al. 2005; Shu and Zhu 2002). It is virtually harmless to animals and humans (Rao and Sharma 1997; Aspden et al. 1997). The first scientific report of chitosan dates back to 1859 (Struszczyk 2002). Currently, it is widely researched in many fields due to its unique properties like easy modification of amine groups, availability of different chain lengths, biocompatibility, stability, wound healing properties, and effectiveness in cellular adhesion and proliferation (Diebold et al. 2007; Kean and Thanou 2010). Chemical and biological properties of chitosan can differ among chitosan polymers which can be attributed to different processes used for its extraction from chitin, thereby affecting their chain length and degree of deacetylation. Although chitosan has some unique properties, its shortcomings are related to its solubility issues at physiological pH and relatively low transfection ability (Lai and Lin 2009; Kato et al. 2003). However, ease of chemical alteration of the chitosan structure helps obtain the desired properties.

8.2.1 Physicochemical Properties of Chitosan

Chitosan is part of a polymer family of randomly distributed d-glucosamine (deacetylated units) and N-acetyl-d-glucosamine (acetylated units) linked by β-(1,4)-glycosidic bonds (Onishi and Machida 1999). A chitosan polymer consists of a polysaccharide backbone which degrades before melting and therefore lacks thermoplastic properties which are typical of polymers with extensive hydrogen bonding (Lizardi-Mendoza et al. 2016). Chitosan molecules in solid state are generally organized in an ordered crystalline manner. It has two polymorphs: hydrous and anhydrous crystal form (Okuyama et al. 2000; Ogawa et al. 2004).

Chitosan is cationic in nature at acidic pH (pH < 6) owing to the protonation of its basic amino groups in polymer chain (pKa = 6–6.5) (Wu et al. 2002). This property of chitosan allows it to easily solubilize in dilute acidic solutions but insoluble in most of organic solvents as well as in aqueous solutions at basic and neutral pH (Mao et al. 2010). Solubility of this polymer not only depends on the degree of deacetylation but also on the chain length. Low molecular weight chitosan is easily soluble as compared to high molecular weight polymer chain (Kritchenkov et al. 2017). Chitosan is also a very good viscosity enhancer under acidic conditions (Nilsen-Nygaard et al. 2015). The viscosity of a chitosan solution is affected by pH, molecular weight, concentration, temperature, degree of deacetylation, and ionic strength. The viscosity of a solution decreases with increase in temperature (El-Hefian et al. 2010). It also depends on the acid used to solubilize chitosan. Additionally, the gel formation property of chitosan with anionic hydrocolloids is used for controlled drug delivery. Furthermore, high charge density on chitosan makes it antimicrobial in nature (Zheng and Zhu 2003; Muzzarelli et al. 1990).

The reactivity of chitosan is mainly based on the presence of free amino and hydroxyl groups on its polymer backbone. The primary amine groups majorly undergo formation of Schiff base or N-acylation reactions. The presence of a positive charge on a chitosan backbone allows it to form polyelectrolyte complexes with negatively charged phosphate groups in nucleic acids to form polyplexes. Also the positive nature of chitosan as micelles helps in its interaction with negatively charged cell membrane resulting in its easier internalization via endocytosis. Additionally, at neutral or basic pH, it has been reported to bind with nucleic acids via hydrogen bonding and hydrophobic interactions (Messai et al. 2005). Degree of deacetylation, molecular weight, charge ratio of nucleic acid to polymer, and pH are some of the factors which can influence the efficiency of transfection using chitosan-based delivery systems (Köping-Höggård et al. 2001; Kiang et al. 2004; Romøren et al. 2003). It was seen that intracellularly low molecular weight chitosan releases DNA easily, whereas large molecular weight chitosan protects DNA more efficiently. Hence, a reasonable balance should be achieved to obtain high transfection efficiency (Lavertu et al. 2006). Also it was noted that a smaller chain length chitosan require higher nitrogen to phosphate ratio (N:P) for stable polyplex formation. At low N:P, smaller chain length chitosan form unstable complexes with nucleic acids yielding low transfection efficacy (Lavertu et al. 2006).

8.2.2 Modifications of Chitosan

Ease of chemical modification of chitosan led to its widespread suitability in various fields. The major purpose of modification is to improve specific functional properties and solubility at physiological pH, basic pH, or in organic solvents. The presence of primary amine and hydroxyl groups makes it possible to achieve the desired properties for the polymer like solubility, viscosity, charge density, hydrophobicity, and targeting ability. Amino groups of chitosan are more reactive to chemical modification; therefore, they require protection if hydroxyl groups are intended to

be modified (Lizardi-Mendoza et al. 2016). Across the globe, scientists have achieved a vast array of modifications to achieve a chitosan polymer with unique properties. Functional groups of chitosan can undergo reactions like acetylation, etherification, quaternization, esterification, phosphorylation, and carboxymethylation, among others. Carboxymethylation is one of the most common modifications on chitosan which helps in the formation of its water-soluble derivatives (Xu et al. 2010). Carboxymethylated derivatives of chitosan attracted great interest from fields like gene delivery, biosensors, biological imaging, and food science. Increased solubility of chitosan with carboxymethyl modification is largely due to the presence of either excess positive or negative charges. Moreover, quaternization of more than 25% amine groups on a chitosan polymer chain allows it to be soluble at all pH (Xu et al. 2010).

Furthermore, scientists have modified chitosan to attach various ligands to achieve targeting ability to specific cellular locations in vivo. Chitosan derivatives having functional molecules on its surface can potentially enhance its ability to target a specific organ (Park et al. 2010). Ligands including but not limited to galactose, transferrin, mannose, and folate have been studied to target chitosan-based delivery systems to their respective receptors (Issa et al. 2006; Kadiyala et al. 2010; Gao et al. 2003; Jiang et al. 2008; Corbet et al. 2016; Jin et al. 2016). It was reported that introduction of thiol groups helps in increasing the stability of chitosan-based polyplexes (Breunig et al. 2008; Varkouhi et al. 2010). Additionally, thiol modification also helps in the release of nucleic acid inside the cell. Modification of chitosan with thioglycolic acid or 2-iminothiolate has also been reported to improve the transfection efficiency as well as for efficient intracellular release (Martien et al. 2007; Lee et al. 2007). Several peptides and amino acids have also been studied to aid in targeted delivery and cell penetration (Layek et al. 2015a; Sharma et al. 2013). Conjugation of chitosan with arginine and with nonpolar amino acids has also been investigated to improve the overall hydrophilicity or hydrophobicity of the complex, respectively. Such conjugation also facilitates a higher level of penetration inside cells alongside improved transfection (Gao et al. 2008; Malhotra et al. 2013). Thus, fine-tuning chitosan with chemical modifications can lead to new and biocompatible non-viral gene delivery systems with a promising wide array of therapeutic benefits.

8.2.3 Mechanism of Chitosan-Based Gene Delivery Systems

The transfer of genetic material from the extracellular environment to an intracellular organelle (nucleus for pDNA and shRNA or cytoplasm for siRNA and miRNA) is the major goal of gene delivery. The mechanism of gene delivery is divided into five stages as follows: extracellular protection, biological membrane, endosomal system, nucleus, and transcription and translational processes (Pichon et al. 2010). The presence of DNase in the extracellular environment is additionally one of the major barriers to gene delivery. Due to the presence of DNase, DNA gets degraded spontaneously upon intravenous, intramuscular, or mucosal administration (Mumper et al. 1996; Glasspool-Malone and Malone 1999; Kawabata et al. 1995).

Moreover, the negative charge of both DNA and cell membrane results in very low uptake of DNA by the cell owing to poor association between them. Therefore, DNA is generally complexed with a molecule like chitosan to enhance internalization, to protect it from enzymatic degradation, and to keep it stable during internalization process. It was shown that cellular uptake did not appear to be a rate-limiting step for chitosan particles having a positive charge and size less than 200 nm in diameter (Thibault et al. 2010; Corsi et al. 2003). Many groups around the globe conjugated ligands to chitosan nanoparticles to target receptors/transporters specific to cells in order to increase cellular uptake via endocytosis (Peng et al. 2011). The endocytosis pathways include phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and micropinocytosis (Sahay et al. 2010). Chitosan-DNA polyplex after internalization into endosomes needs to release the gene into the cytoplasm for transfection process. The transfection efficiency of chitosan is believed to be dependent on its release from the endosome into the cytoplasm via the proton sponge effect (Mao et al. 2001). Poor endo-lysosomalytic proton sponge effect, long duration for uncoupling chitosan-gene complex, and limited membrane perturbing ability explain the low transfection ability of chitosan (Köping-Höggård et al. 2001).

Additionally, the membrane perturbation ability of chitosan-based systems is believed to be associated with its degree of protonation (Fang et al. 2001). It was seen that incorporation of poly(propyl acrylic acid) (PPAA), a pH-sensitive polymer, with a chitosan-DNA complex enhances its transfection efficiency due to improved membrane disruption ability (JONES et al. 2003). Similar results were observed when uronic acid and PEI were combined with chitosan in separate studies showing improved transfection efficiency (Tae et al. 2005; Kim et al. 2003). Increasing osmolarity and rupturing of endosome by chitosan degradation products is another method of endosomal escape (Regnström et al. 2006). Endosomal escape is believed to be the major rate-limiting step in chitosan-related transfection (Thibault et al. 2010; Raftery et al. 2013). Once the pDNA reaches the cytoplasm, various types of DNA-binding proteins make a large complex with the plasmid (Badding et al. 2013). The transcription factors complexed with plasmids interact with importin-β and various other proteins that connect the complex with kinesin and dynein for their movement beside microtubules towards the nucleus (Badding et al. 2012). The pDNA then enters the nucleus via nuclear pore complex (Dean 1997; Dowty et al. 1995).

8.2.4 Biocompatibility, Biodegradability, and Stability of Chitosan-Based Systems

Chitosan is considered to be biocompatible with approval for dietary applications in Italy, Japan, and Finland (Illum 1998). It has LD_{50} of 16 g/kg in mice (oral test) which is equivalent of salt and sugars (Roller and Covill 1999; Coma et al. 2003). Chitosan has been approved for its use in wound dressings by the FDA (Wedmore et al. 2006). A group in Sweden compared the toxicity profile of chitosan and PEI at

genetic level (Regnström et al. 2006). Cyclooxygenase 1 and 2 (COX-1 and COX-2) are enzymes involved in inflammatory processes. The Chung group found that the expression of both cyclooxygenases was increased with the transfection process through PEI, whereas it was downregulated in case of chitosan (Xu et al. 2010). Moreover, heme-oxygenase-1 (HO-1) gene, induced by oxidative stress and inflammatory cytokines, is upregulated by PEI but not chitosan (Chung et al. 2012). Chitosan degradation product, N-acetylglucosamine, has shown anti-inflammatory properties and can be responsible for this effect. Chitosan cyto-compatibility has been tested and proven numerous times with various types of cells (Chatelet et al. 2001). It was shown that the degree of deacetylation (DDA) affects cell attachment (Xu et al. 2010). It was reported that the higher the DDA, the more the cellular attachment in case of keratinocytes and Schwann cells (Chatelet et al. 2001; Cao et al. 2005; Revi et al. 2014). Inside the body, chitosan can be broken down by enzymes which can cleave glucosamine to N-acetyl-glucosamine, glucosamine to glucosamine, and N-acetyl-glucosamine to N-acetyl-glucosamine bonds. Earlier chitosan was thought to be degraded via lysozymes and bacterial enzymes in the colon, but recently eight chitinases have been found in humans with some suggested to attain enzymatic activities (Funkhouser and Aronson 2007; Kean and Thanou 2011). The magnitude and degree of biodegradation of chitosan in vivo depend on its DDA (Xu et al. 1996; Yang et al. 2007). Interestingly, various proteases were found to be capable of degrading chitosan with leucine aminopeptidase being most efficient (Rao and Sharma 1997). Chitosan degradation after intravenous administration has been hardly reported in literature. In one of the few studies published, chitosan oligosaccharides resulted in upregulation of lysozyme activity in rabbit's blood (Hirano et al. 1991).

Stability of the formulation is also one of the major parameters for efficient therapeutic effects. Although chitosan-based systems gained much attention over the last few decades, very few studies have looked into the long-term stability of these systems (Harding 2010; Jones et al. 1996; Kam et al. 1999; Knapczyk 1992). Lyophilized chitosan-DNA complexes were able to maintain their transfection efficacy in excess of 4 weeks (Patel et al. 2016; Jayakumar et al. 2010). However, chitosan-DNA complex stored in PBS remained stable for only a couple of hours (Leong et al. 1998). There are various factors which affect the stability of a formulation. These factors can be divided into two broad categories: (A) internal factors (such as purity level, moisture content, DDA) and (B) external factors (such as humidity and temperature). The molecular weight of a chitosan polymer was also found to affect its thermal stability (Mucha and Pawlak 2002). Heat generated during compression in table processing can affect the molecular weight distribution of chitosan in a tablet (Buys et al. 2013). Several studies have shown that the DDA of chitosan can affect its hydrolytic degradation and thermal behavior (Mucha and Pawlak 2002; Wanjun et al. 2005; López et al. 2008). It was found that the DDA and the rate of acidic hydrolysis of a chitosan polymer are inversely correlated (Varum et al. 2001). In addition environmental temperature also has an effect on chitosan degradation. Studies show that chitosan solution stored at room temperature or higher leads to increased rate of degradation of chitosan chains, whereas no

significant chain loss was observed at 5 °C (Varum et al. 2001; Nguyen et al. 2008). For long-term storage of chitosan nanoparticles, lyophilization is a well-established method. Lyophilized chitosan-lecithin nanoparticles after seven months of storage did not show any alteration in their physicochemical properties and were able to re-disperse easily without any aggregates (Hafner et al. 2011). Nevertheless, lyophilization can damage unmodified and, in some cases, modified chitosan polymer chains (Schuetz et al. 2008). Despite great potential, long-term stability of chitosan can pose a substantial drawback in its pharmaceutical applications and requires keen investigation.

8.3 Chitosan-Based Gene Delivery Systems

Non-viral vectors, such as polycation complexes and liposomes, have been emerging as an efficient tool in recent years for effective delivery of genes, due to their low immunogenicity, modification under control conditions, and ease of production, as compared to viral vectors. Chitosan and chitosan-based derivative formulations have been used and studied in recent years for safe and potential delivery of nucleic acids for a variety of applications including cancer, diabetes, arthritis, influenza, and hepatitis.

8.3.1 Chitosan-Based Nanoparticles

Chitosan and its derivatives have been studied as delivery systems in gene therapy. As discussed earlier, due to its positive charge, it is able to form electrostatic complexes with DNA. The transfection efficiency of chitosan-based nanoparticle depends on several factors including size and charge, chemical structure of chitosan, interaction between polyplexes and cells, and the cell types. Chitosan-based nanoparticles have been studied in the fields of cancer, bone repair, rheumatoid arthritis, and infectious diseases (Table 8.2). Salva and coworkers demonstrated the suppressive effect on the vascular endothelial growth factor gene (VEGF) expression and tumor volume, when chitosan siRNA nanoparticles were injected in a rat breast cancer model, thereby targeting the VEGF gene (Şalva et al. 2012). In another study done by Howard et al., downregulation of TNF-α expression was demonstrated in rheumatoid arthritis (Howard et al. 2009). They injected chitosan-siTNF-α nanoparticles intraperitoneally in collagen-induced arthritic mice model and demonstrated downregulation of TNF-α-induced inflammation, thereby causing reduction in joint swelling. A summary of chitosan-based nanoparticles is listed in Table 8.2.

Table 8.2 Studies using chitosan-based gene delivery in animal models

Formulation	Chitosan	Gene	Disease	Route of administration	Key outcomes	References
TAT-g-CS nanoparticles	MW 11.3 kDa	siRNA	Breast cancer	Intratumoral	Good biocompatibility, downregulate the expression of target genes, inhibit proliferation and metastasis of 4 T1-Luc tumor cells	Yang et al. (2013)
NCS-DNA E7 nanoparticles	MW 360 kDa	HPV-16 E7	Cervical cancer	Intramuscular	Induced strongest E7-specific CD8 + T-cell activation and proliferation, stimulated interferon (IFN)-γ and interleukin (IL)-4 production to promote antitumor immunocytotoxic response resulting in reduced tumor size	Tahamtan et al. (2014)
Man-CS-Phe/ DNA polyplexes	MW 50 kDa	pHBsAg	Hepatitis B	Intradermal	Multifold increase in anti-HBsAg titers observed up to six weeks; increased lymphocyte proliferation as well as increased interferon (IFN)-γ and interleukin (IL)-4 production	Layek et al. (2015b)
CS-pDNA microparticles	MW 1400 kDa	pnlacF	Ampicillin resistance	Oral	Chitosan DNA microparticles protected encapsulated pDNA from nuclease degradation, transport of pDNA to Peyer's patches through M cells, demonstrated higher level of gene expression in stomach and small intestine of mice	Guliyeva et al. (2006)
PEG-CS-PLR polyplexes	MW 50–150 kDa	siSVN, siGFP, siRFP	Lung cancer	Intratumoral	Increased cellular delivery of siRNA, low cytotoxicity and increased serum stability, reduced overexpression of genes in tumor tissue in vivo	Noh et al. (2010)
pDNA/ N-acyl LMWC Polyplexes	MW 50 kDa	pUMVC3-mIL4, pUMVC3-mIL10	Type 1A diabetes	Intramuscular	Demonstrated significantly high expression of interleukin (IL)-4 and interleukin (IL)-10 production in mice, reduced levels of blood glucose, TNF-α, and IFN-γ	Mandke and Singh (2012)

CS-pDNA nanoparticles	MW 71.3 kDa	pCAGGS	Influenza	Intramuscular	Demonstrated high antibody titer in mice serum, increased HI antibody titer in mice serum, stimulated specific B lymphocytes, increased humoral immunity and antibody titers	Zhao et al. (2011)
CS-pDNA chitoplexes	Low viscous	pCAGGS-MCS	Influenza	Intranasal	High levels of ion channel protein (M2)- and nucleoprotein (NP)-specific serum IgG and IgA antibodies in mice serum	Sawaengsak et al. (2014)
CS-pDNA nanoparticles	MW 9.3–270 kDa	pVax1-4sFGF-2, pVax1-PDGF-BB	Growth factor therapy	Intramuscular, subcutaneous	High expression of PDPF-BB and FGF-2 with specific antibodies in mice serum, more efficient delivery of plasmids by SC than IM injections	Jean et al. (2009)
Psi-tCS nanoparticles	MW 250 kDa	siRNA	Rheumatoid arthritis	Intravenous	Rapid cellular uptake and excellent in vitro TNF-α gene silencing efficacy, increased accumulation in arthritis joints of mice, IV injections demonstrated inhibition of inflammation and bone erosion in mice model	Lee et al. (2014)

8.3.2 Chitosan-Based DNA Vaccines

There is a need of an excellent delivery vehicle for efficient cellular and humoral responses induced by genetic immunization which would ultimately help in improved exhibition of transgene products to antigen-presenting cells (APCs) (Gurunathan et al. 2000). Chitosan and its derivatives have been studied to deliver DNA vaccines, thereby improving cell uptake of plasmids into cells with effective membrane permeability (Dodane et al. 1999; McNeela et al. 2000). The route of administration for a DNA vaccine is the most important decision to its clinical application that can be through systemic delivery or directly to a targeted tissue. The common choice of route of administration for mucosal vaccination is either oral or intranasal because it can generate a local immune response. Roy and group used chitosan pDNA nanoparticle against peanut allergen gene, Arah2, for oral immunization (Roy et al. 1999). They demonstrated that immunized mice showed reduction in the levels of IgE, a plasma histamine associated with the decrease in allergen-induced anaphylaxis. Several published reports demonstrated that chitosan pDNA nanoparticles may directly route to the mucosal membrane and transfect the APCs directly when given intranasally. A study demonstrated the use of chitosan-pDNA nanoparticles intranasally in Balb/c mice for immunizing against hepatitis B virus (Khatri et al. 2008). They showed that nanoparticles expressed the surface antigen of hepatitis B virus, thereby demonstrating generation of humoral and cellular responses in mice. A summary of chitosan-based DNA vaccines is given in Table 8.2.

8.4 Challenges Associated with Chitosan-Based Gene Delivery Systems

Viral vectors have been demonstrated to be efficient gene delivery systems; however, the related safety risks and unexpected adverse effects have limited their applications. Accordingly, the interest for non-viral gene vectors has consistently increased (Buschmann et al. 2013). The development of successful gene delivery systems must consider the parameters which influence transfection. Despite the advantages already described of chitosan as a non-viral gene delivery vector, the application of this system faces significant limitations. The factors as well as the limitations involved in the transfection efficiency of chitosan as a gene delivery vector will be discussed in the following sections.

8.4.1 Factors Affecting Chitosan as a Gene Delivery Vector

The effectiveness of gene delivery vectors reflects their ability to protect the genetic material from environment degradation, together with delivery of the nucleotides in sufficient amount to the target cells to produce a therapeutic effect (Ibraheem et al. 2014). The development of suitable nontoxic, biocompatible, biodegradable, and

effective gene delivery systems must overcome not only the physical and biological barriers but also take into consideration the physicochemical properties of the vector (Naldini 2015).

Post administration, the contact of the genetic material with the existent serum nucleases contributes to their rapid degradation. The high molecular weight and anionic nature of nucleic acids hinder their interaction with the cell membrane restricting their internalization into cells (Gonçalves and Paiva 2017). As a result, the internalization of such molecules into the cell is limited through the endocytic pathway. Intracellularly, before reaching the target site, the therapeutic genes have to overcome enzymatic degradation inside the endolysosomes. In general, gene vectors intend to enhance the transfer of exogenous nucleic acids into cells and obtain the desired therapeutic effect (Kumar et al. 2016; Collins and Thrasher 2015). Chitosan as a gene delivery vector should provide stability to vector-cargo complexes, protecting them from extracellular and intracellular degradation, delivering the cargo into the cells while also allowing for dissociation of the complexes to enable transfection (Whinnery and Kirsch 2016). The chitosan degree of deacetylation (DD), polymer length, and ratio of chitosan to nucleic acid (N/P ratio) exert significant influence on the polymer's physicochemical properties and biological performance, thereafter reflecting on the transfection extent (Buschmann et al. 2013).

The molecular weight of chitosan has a critical influence on particle size, stability, and dissociation of chitosan/nucleic acid complexes. Additionally, it also impacts the internalization of complexes into cells and the dissociation of nucleic acids from the complexes, thereafter influencing the transfection efficiency (Lavertu et al. 2006; Huang et al. 2005; Nimesh et al. 2010). In general, it has been reported that as the molecular weight of chitosan decreases, the particle size of the complexes also decreases (Nimesh et al. 2010). However, a higher complex size was observed on studies with chitosan of 2–17 kDa. It was suggested that probably the lower short chains were not able to condense the nucleic acids and form discrete and stable complexes (Ragelle et al. 2013). Therefore, the selection of chitosan of appropriate molecular weight might determine the transfection efficiency.

The effect of chitosan DD, which refers to charge density, i.e., the number of protonated amines (NH_2), also plays an important role on transfection efficiency. High DD results in increased positive charges, influencing chitosan properties such as solubility, crystallinity, and degradation (Kiang et al. 2004). Additionally, chitosan DD also determines the nucleic acid-chitosan binding affinity and stability of the complex, which, as a consequence, affects the release of nucleic acid from the complex, cellular uptake, and transfection efficacy. It has been reported that higher chitosan DD generated more stable DNA complexes and consequently was more capable of protecting the chitosan-DNA complexes from degradation due to DNase and serum components, which correlated well with efficient transfection (Kiang et al. 2004; Huang et al. 2005). After analyzing the effect of combinations of molecular weight and DD of chitosan on transfection level, Lavertu et al. (2006) observed maximum transgene expression not only when lowering the MW and increasing the DD but also when increasing the MW and decreasing the DD. Physicochemical studies also suggested the dependence on MW and DD in

modulating the binding affinity of chitosan-DNA complex to produce efficient transfection (Strand et al. 2005; Ma et al. 2009), emphasizing the role of complex stability and balance between complex binding strength and transfection efficiency.

It has been well established that the ratio between chitosan nitrogen (N) and DNA phosphate (P) (N/P ratio) is important to achieve stability and effective transfection. Systematic studies have provided evidences that an effective N/P ratio of chitosan gene vectors usually involves N/P of at least 3–5 and sometimes up to 30 (Mao et al. 2010; Lu et al. 2014; Alameh et al. 2018; Hao et al. 2009). The excess of chitosan in the polyplex may lead to a higher osmotic pressure in the endosomes favoring the transfection (Buschmann et al. 2013). A fine balance of N/P ratio can be correlated to complex stability. Lower ratios will produce unstable complexes, while high ratios will result in overly stable complexes, and both cases would compromise transfection.

The cationic charge of chitosan is pH-dependent and directly affects properties of chitosan, such as solubility and aggregation, interaction with DNA, and polyplex dissociation. Considering that chitosan pKa is 6.5, a high degree of protonation is expected at pH 5.5, while a lower degree of protonation is observed at physiological pH (Mao et al. 2010; Ma et al. 2009; Chang et al. 2011). Hence, stable and condensed polyplexes can be prepared at lower pH due to the presence of a higher amount of protonated amines. Based on literature, a good balance between chitosan and DNA association/dissociation can be achieved at a pH range of 6.5–7.0, enabling successful transfection (Strand et al. 2005; Agirre et al. 2015). Enhancement of transfection of chitosan gene delivery systems has been observed in the presence of serum (Mao et al. 2001). The reasons behind the increase in transfection due to serum concentration are still not clearly explained.

8.4.2 Limitations of Chitosan-Based Gene Delivery Systems

Several extracellular and intracellular barriers limit the efficiency of gene transfer by non-viral methods. Depending on the route of administration, the delivery system faces diverse anatomical barriers starting from epithelial and endothelial cells to extracellular matrix to access the target site (Zhang et al. 2012; Nayerossadat et al. 2012). Simultaneously, the gene delivery vector needs to be capable of escaping the phagocytes and extracellular nucleases present at systemic circulation. Overpassing these, another critical step is to cross the cell membrane which commonly involves multiple mechanisms such as endocytosis, pinocytosis, or phagocytosis or even passive transport (Al-dosari and Gao 2009). Intracellularly, upon uptake by endosomes, the delivery system can be subjected to enzymatic degradation. Chitosan-based delivery systems have demonstrated an ability to escape endosomes, and the mechanisms involved are discussed in previous sections. Upon their release from endosomes, the presence of enzymes and proteins in the cytoplasm makes the traffic to the nucleus very critical. The nuclear envelope represents an additional barrier. Finally, the last limitations comprise the release of nucleic acid from the

complex and cellular transfection (Mansouri et al. 2004; Buschmann et al. 2013; Ragelle et al. 2013).

Chitosan without structural chemical modification has limited applications as gene delivery systems (Ji et al. 2014; Jiang et al. 2014). The low charge density under physiological pH leads to low solubility, aggregation, and poor stability of the unmodified chitosan-based form. Consequently, it leads to a compromised buffering capacity, and the weak proton sponge effect does not contribute to an endosomal escape mechanism resulting in a low endosomolytic effect (Sashiwa and Aiba 2004; Zhang et al. 2010; Cheung et al. 2015). Several strategies have been implemented to overcome these limitations that chitosan imposes, such as modification of the chitosan structure, conjugation, grafting, copolymerization, or encapsulation into nanoparticles (Whinnery and Kirsch 2016). This process includes a careful choice of a specific chitosan considering its molecular weight, degree of deacetylation, chemical modifications, and the nature of the eventual substituents to suit the physicochemical properties. Thus, it enables extensive possibilities for chitosan derivatives and the management of product properties for the desired function. Targeted chitosan-based gene delivery has been designed to address a very important obstacle in the development of these types of formulation and the lack of cell specificity (Corbet et al. 2016; Rudzinski et al. 2016; Kwon et al. 2013; Yhee et al. 2015; Ragelle et al. 2015; Van Woensel et al. 2016). Despite progress in the pharmaceutical field, chitosan-based gene therapy needs to improve the therapeutic effectiveness to achieve clinical significance.

8.4.3 Comparison of Chitosan with Other Gene Delivery Systems

Ideal gene delivery systems should be able to condense nucleic acids, overpass extracellular and intracellular barriers, confer sufficient protection to nucleic acid against enzymatic degradation, and dissociate the nucleic acid from the polyplex at the target site to allow efficient transfection (Hardee et al. 2017; Shi et al. 2017). Various types of non-viral vectors with a basis in nanotechnology have been explored for gene therapy. Based on the nature of the nanomaterial, they can be organized into inorganic (proteins and peptides), lipid-based, and polymer-based vectors. This section presents a brief comparison of chitosan-based gene delivery systems with other non-viral gene delivery systems.

The potential of inorganic nanoparticles has received much attention as gene delivery systems that exhibit unique physicochemical properties providing advantages compared to traditional lipid-based vectors, such as high surface area per unit volume, ability to translate physical properties of the metal core (i.e., optical and magnetic) to the delivery vehicle, and multifunctional capabilities. Carbon nanotubes, quantum dots, and gold and magnetic nanoparticles are examples of inorganic nanoparticles used in gene delivery (Chen et al. 2016; Riley and Vermerris 2017; Dizaj et al. 2014). Carbon nanotubes possess unique structure and properties providing efficient delivery abilities, capability to hold a high aspect ratio, and the capacity to translocate through cellular membrane. However, concerns regarding the

toxicity of carbon nanotubes need to be addressed. Adequate control of physicochemical characteristics can result in lower toxicity and high transfection of this type of nanoparticles, as suggested by Munk et al.'s (2017) study using carboxylic multiwalled carbon nanotubes. The latter showed efficient transfection efficiency in primary fibroblast cells at 50 μg/ml. On the other hand, Cifuentes-Rios et al. (2017) showed the influence of physicochemical properties, such as rigidity and length of carbon nanotubes, on the transfection ability and biodistribution. Quantum dots were first described as nanoscale semiconductor crystals for biological imaging application (Matea et al. 2017). Graphene quantum dots produced by Ghafary et al. (Matea et al. 2017) demonstrated versatile features of the nanocarrier for gene delivery and nuclear targeting. Scalable production and the possibility of functionalization with specific ligands together with controllable cytotoxicity and biodistribution have made gold nanoparticles attractive gene vectors. Several studies have shown that a wide range of nanoparticles can be conjugated to this noble metal and yield in effective platforms for gene delivery applications (Gupta et al. 2018; Sharma et al. 2011; Mendes et al. 2017; Ding et al. 2014). Magnetic nanoparticles not only propose to enhance transfection but also the targetability of the therapeutic gene to specific tissues or location in the body (Majidi et al. 2016). However, similar to a majority of nanodelivery systems, the clinical applications of magnetic nanoparticles are still under development mainly due to toxicity concerns and therapeutic efficiency. Characterization of transfection properties of magnetic-based carriers has shown that these approaches have potential applications as gene delivery systems (Müller et al. 2016; Voronina et al. 2016; Wang et al. 2014).

The characteristics of protein and peptides featuring good biocompatibility, biodegradability, low toxicity, and delivery properties have attracted them as gene delivery systems. They cover a huge class of molecules with diverse structure, nature, and properties (Chen et al. 2016; Riley and Vermerris 2017). Gelatin is the most used protein for this purpose. Although gelatin is biodegradable, biocompatible, cheap, and easily modified, scaling up the production brings manufacturing and toxicity issues, which limit the application (Riley and Vermerris 2017). Albumin can assist other gene delivery systems enhancing the transfection efficiency (Look et al. 2015). Due to versatile applications, silk-based materials have emerged as potential gene vectors. Genetic engineering enables the design and functionalization of silk-based vectors providing a customized vector with adjustable properties (Deptuch and Dams-Kozlowska 2017). In vitro characterization performed by Numata el al. (2009) and Yigit et al. (2014) has shown the potential of silk as a gene delivery system. Cell-penetrating peptides (CPP) are a diverse class of short peptides (5–30 amino acids) able to translocate the cell membrane and transport cargo into cells with no interaction with specific receptors. Since their discovery, the studies concentrated in a few peptides such as Tat, pAntp transportan, pVec, and MAP (Milletti 2012; De Figueiredo et al. 2014; Bashyal et al. 2016). Considering the high delivery efficiency, Tat and derivatives have been extensively characterized and explored together with other delivery systems as a potential gene carrier (Malhotra et al. 2013; Yamano et al. 2011, 2014; Qin et al. 2011; Simon et al. 2009). Although Tat is

the still widely used CPP, the discovery of new CPP recently reflects the interest for new and efficient delivery systems. Clinical studies have been performed to identify potential CPP-derived vectors, but the lack of tissue specificity and short duration of action have hindered their progression as gene delivery vectors in clinical application (Guidotti et al. 2017).

A variety of lipid-based nanoparticles have been studied for gene delivery purposes. Liposomes have been the most used nanoparticles as gene delivery vectors, which can be composed of a variety of phospholipids and a surface modified with ligands including peptides, antibodies, carbohydrates, small molecules, and polyethylene glycol (PEG) (Zylberberg et al. 2017). Several studies have reported efficient in vitro and in vivo transfection using liposomes for diverse purposes (Sau et al. 2018; dos Santos Rodrigues et al. 2018; Zhang et al. 2018). Many clinical trials of gene therapy using non-viral vectors are ongoing worldwide although none of these approaches have so far been approved by the FDA. Some examples of current nanoparticle-based gene therapy studies under clinical evaluation can be found in Table 8.3. Lipid-based nanoparticles such as liposomes appear in most clinical trials among non-viral gene delivery systems. Polyethylenimine (PEI) is considered the gold standard effective transfection reagent. High gene transfection has been suggested to be a result of the strong ability to condense DNA/RNA and buffering capability. However, significant toxicity issues have limited their application in clinical trials (Hu et al. 2016; Jayant et al. 2016). Studies with modified PEI-based formulations have shown efficient gene transfection with low cytotoxicity (Song et al. 2018; Sadeghpour et al. 2018; Ryu et al. 2018). The efficacy of polymeric gene delivery vectors, however, is still moderate, and clinical applications of these vectors remain limited. Therefore, further improvements of polymeric carriers are necessary (Chen et al. 2016; Riley and Vermerris 2017).

8.5 Commercial and Investigational Applications of Chitosan-Based Gene Therapy

Currently, no chitosan-based commercial transfection reagents are available and chitosan is not under investigation as a gene delivery vector in any clinical trial in the United States. A search for "chitosan gene delivery" or "chitosan gene delivery vector" in clinicaltrials.gov did not yield any result, while a search for "gene delivery virus vector" showed 22 active studies in the United States. This clearly indicates that utilization of chitosan as a gene delivery vector in the clinics may be far into the future. As aforementioned, gene delivery using chitosan is mostly through direct complexation with nucleic acids or in combination with polyelectrolytes, lipids, and polymers to form composite nanostructures (Rodriguez et al. 2013). Gene delivery using these particles has mostly been used for treatment of diseases, tissue engineering, and bioimaging among others (Ahmad et al. 2018). Since describing all the myriad investigational applications of chitosan for gene therapy is beyond the scope of this chapter, we are limiting the section to few very recent but notable applications.

Table 8.3 Nanoparticle-based gene therapy under clinical evaluation

Delivery system	Product	Sponsor	Disease	Administration	Phase	Status	Gov identifier
Cationic lip	SGT-53+ TMZ	SynerGene therapeutics	Recurrent glioblastoma	IV	2	Recruiting	NCT02340156
Cationic lip	SGT-53 + nab-Pac + gem	SynerGene therapeutics	Metastatic pancreatic cancer	IV	2	Recruiting	NCT02340117
Spherical gold NP	NU-0129	Northwestern University	Gliosarcoma	IV	1	Active, not recruiting	NCT03020017
DOTAP: Chol lip	TUSC2 + Erl + Dex + dip	Genprex	Lung cancer	IV	1/2	Active, not recruiting	NCT01455389
Albumin-stabilized NP	Gem + Pac NP + Sel	Barbara Ann Karmanos Cancer institute	Pancreatic cancer	IV	1/2	Active, not recruiting	NCT02178436
DOPC lip	siRNA-EphA2-DOPC	M.D. Anderson Cancer center	Advanced cancers	IV	1	Recruiting	NCT01591356

Lip liposome, *TMZ* temozolomide, *nab-Pac* nab-paclitaxel, *Gem* gemcitabine, *Erl* erlotinib, *Dex* dexamethasone, *Dip* diphenhydramine, *Pac NP* paclitaxel albumin-stabilized nanoparticles, *Sel* selinexor

In one of the latest studies, investigators used hyaluronic acid/chitosan particles encapsulating plasmid DNA encoding cytokine response modifier A to treat osteoarthritis (Zhou et al. 2018). In a rat knee osteoarthritis model, the investigators demonstrated significant chondroprotection mediated by the nanoparticles against early osteoarthritis through interleukin-1β inhibition. Along the same lines, Deng and coworkers formulated a potential treatment strategy for osteoarthritis by incorporating plasmid DNA encoding interleukin-1 receptor antagonist gene in chitosan/hyaluronic-acid-based nanoparticle (Deng et al. 2018). The formulation increased interleukin-1 receptor antagonist levels and decreased inflammatory effects mediated by interleukin-1β in primary synoviocytes. Shi et al. used folate-polyethylene glycol-chitosan-diethylamine nanocomposites carrying siRNA against tumor necrosis factor-α (TNF-α) to treat rheumatoid arthritis (Shi et al. 2018). In a mouse model of collagen antibody-induced arthritis, the nanocarrier significantly attenuated inflammation, improved clinical outcome, and decreased TNF-α concentrations in target tissues along with articular cartilage damage and loss of bone. Chitosan-based hydrogels have been utilized for tissue engineering such as osteogenesis in rats (Wu et al. 2018). Incorporation of stromal cell-derived factor-1α and antimiR-138 in chitosan/β-sodium glycerol phosphate hydrogels led to sustained release of antimiR-138 and enhanced calvarial bone regeneration significantly in rats. Chitosan-mangafodipir nanoparticles have been developed for gene delivery to the brain via the intranasal route (Sanchez-Ramos et al. 2018). Our recent work using brain-targeted nanoparticles incorporating plasmid green fluorescent protein (pGFP) in chitosan nanoparticles and further encapsulated in cationic liposomes showed excellent transfection in the brain. The system was further successfully utilized for performing imaging and biodistribution studies in mice (dos Santos Rodrigues et al. 2018). Another recent study has also utilized liposome-encapsulated chitosan carriers for improving gene delivery. The lipid-encapsulated carriers afforded better protection to the gene cargo, decreased cellular toxicity, and improved transfection by twofold compared to non-lipid-encapsulated chitosan nanoparticles (Baghdan et al. 2018). In vivo efficacy was demonstrated through efficient transfection of encapsulated pGFP in chorioallantoic membrane without damage to the surrounding vasculature. Wang and coworkers conjugated a cell-penetrating peptide transactivator of transcription (Tat) to chitosan cationic micelles to formulate a nucleus-targeted gene delivery vector (Wang et al. 2018). Using the formulation, plasmid p53 and doxorubicin was co-delivered to the nucleus that resulted in high apoptosis in cancer cells in vitro. In a different study, polyplexes comprising chitosan conjugated to RGD (Arg-Gly-Asp) motif and polyethyleneimine were prepared to target tumor cells overexpressing integrins $\alpha_v\beta_3$ (Kim et al. 2017). The formulation demonstrated significant inhibition of tumor growth in a mouse xenograft prostate cancer model that was mediated by silencing of BCL2 mRNA. Recently, Zai and group used a chitosan-metformin conjugate accompanied with PEGylated phospholipid and cell-penetrating peptide, penetratin, to form self-assembling stable complexes with plasmid interleukin-22 (Zai et al. 2019). The formulation predominantly accumulated in the liver and improved insulin sensitivity, metabolic syndrome, and assuaged hepatic steatosis in mice fed with a high-fat

diet, thereby showing promise as a novel strategy for the treatment of nonalcoholic fatty liver disease. Chitosan conjugated to gonadotrophin-releasing hormone (GnRH) was also recently investigated as a gene delivery vector to spermatogonia cells that overexpress GnRH receptors. GnRH-modified chitosan showed higher specificity and pGFP transfection in the cells compared to unmodified chitosan (Boonthum et al. 2018). Tahamtan and coworkers used chitosan nanoparticles to co-deliver plasmids encoding human papilloma virus (HPV16-E7) and interleukin-12 to mice to investigate if the formulation afforded protection against challenge with TC-1 cells that express HPV16-E6 and HPV16-E7 oncoproteins. (Tahamtan et al. 2018). The authors noted higher antitumor effect, E7-specific lymphocyte proliferation index, and cytotoxic T lymphocyte activity along with increased interleukin-4 and interferon-γ (IFN-γ) levels with a corresponding lower interleukin-10 level compared to chitosan or interleukin-12-alone treatment, demonstrating promise as a DNA vaccine formulation against HPV.

Our group earlier demonstrated that grafting hydrophobic moieties such as hydrophobic amino acids or fatty acids can improve cellular uptake and transfection. Such modifications lead to formation of amphiphilic polymers that can self-assemble to form micellar structures (Sharma and Singh 2017). Conjugation of alanine, valine, leucine, or isoleucine to chitosan significantly improved transfection efficiency of pGFP in HEK-293 cells in 48 h, compared to unmodified chitosan (Layek and Singh 2013). Additionally, the amino acid grafted chitosan polyplexes exhibited excellent compatibility with blood and no cellular toxicity. In a separate study, we showed that intradermal injection of plasmid DNA encoding hepatitis B surface antigen in phenylalanine and mannose-grafted chitosan generated higher hepatitis B surface antigen-specific antibody response in Balb/c mice compared to formulation controls, demonstrating the feasibility of application of the formulation as a DNA vaccine against hepatitis B (Layek et al. 2015b). Similarly, conjugation of palmitic acid to 50 kDa chitosan was found to increase transfection efficiency of plasmid β-galactosidase in HEK-293 cells (Sharma and Singh 2017). It is postulated that hydrophobic modification improves cellular uptake through enhanced cell surface adsorption and causes efficient intracellular dissociation of gene load from the polyplex due to less tightly bound chitosan-gene complex, compared to unmodified chitosan, resulting in superior transfection of the gene cargo (Sharma and Singh 2017). When N-acylated (N-oleyl or N-linoleoyl) grafted low molecular weight chitosan (6.5 kDa) was encapsulated with plasmids encoding interleukin-4 and interleukin-10 and injected intramuscularly to streptozotocin-induced diabetic mice, a high expression of the respective interleukins was obtained along with reduced plasma blood glucose, TNF-α, and IFN-γ levels (Mandke and Singh 2012). The work demonstrated potential application of the formulation in the prevention of autoimmune diabetes.

8.6 Conclusions and Future Perspectives

Chitosan-based systems are very useful means of safe and effective gene delivery and development of such carriers will hugely benefit the field of gene therapy. This chapter highlights the advances made in chitosan-based gene delivery with emphasis on its physicochemical properties, chemical modifications, delivery mechanisms and systems, as well as the challenges associated with gene delivery using chitosan. The polymer exhibits excellent biocompatibility and ease of chemical modification, which effectively overcome the inherent limitations of virus-based gene delivery systems. However, further explorations into appropriate backbone modification, chain lengths, N/P ratio, and degree of deacetylation are needed to significantly enhance stabilization of encapsulated gene and transfection efficiency of these systems. Additionally, to accelerate development and establish chitosan as a safe and effective gene delivery vector, these systems need to be meticulously evaluated in numerous preclinical studies in both small and large animals before translation to humans. Particularly, judicious formulation design to improve in vivo stability, escape from the reticuloendothelial system, targetability, cell surface interaction, penetration, endosomal escape followed by nuclear trafficking, and sustained gene transcription are needed for successful clinical applications. This will require a thorough understanding of the intracellular trafficking processes, gene expression processes, and the in vivo environment faced by the formulation which can initially vary based on the route of administration.

In summary, we can say that chitosan is a promising gene delivery vector that has significantly advanced the field of gene therapy. The tremendous scientific advancements that have already been made using these carriers will inevitably stimulate further comprehensive investigations and successful development of these systems into clinical products.

Acknowledgments We would like to acknowledge the funding support from the National Institutes of Health (NIH) grant # RO1AG051574.

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Chitosan-Based Interpenetrating Polymer Networks: Drug Delivery Application

9

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Abstract

Multicomponent drug delivery systems have found several potential diagnostic and therapeutic applications. Among these the interpenetrating polymeric network (IPN) has gained great attention in the last decades, which involves a blend of two or more polymers in a network with at least one of the systems synthesized in the presence of the other. The development of IPN is attractive because they provide free volume space for the easy encapsulation of drugs in the three-dimensional network structure which are obtained by the cross-linking of two or more polymer networks. This review focuses on the IPNs based on chitosan for drug delivery and biomedical applications. Chitosan is a natural, biodegradable, nontoxic, mucoadhesive, and biocompatible polymer, which has found diverse pharmaceutical applications. Chitosan-based IPNs have garnered immense attention as a vehicle for oral drug delivery. This review summarizes IPNs based on chitosan and other polysaccharides and also IPNs based on chitosan and different synthetic polymers. The influence of the second network on the stimuli responsiveness of the "smart" chitosan-based IPNs is also discussed based on the most recent publications in the field.

Keywords

Chitosan · Interpenetrating polymer network · Hydrogels · Drug delivery · Controlled release

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_9

9.1 Introduction

An interpenetrating polymer network (IPN) is defined as a blend of two or more polymers in a network with at least one of the systems synthesized in the presence of the other (Myung et al. 2008). This results in a formation of a physically cross-linked network when polymer chains of the second system are entangled with or penetrate the network formed by the first polymer. Each individual network retains its individual properties so synergistic improvements in properties like strength or toughness can be seen (Sperling 1994). An IPN can be distinguished from the polymer blend in the way that an IPN swells but does not dissolve in solvents and creep and flow are suppressed (Sperling 2005). They are also different from graft copolymers and polymer complex that involve either chemical bonds and/or low degree of cross-linking. From this point of view only, IPN can be generally named "polymer alloys" through which polymer blends can be made chemically compatible to achieve the desired phase morphology (Work et al. 2004). IPN can share the properties of both the polymers and produce synergistic effect(s) from the component polymers such as when chitosan as a natural polymer is interpenetrated with poly(vinyl alcohol) as a synthetic polymer, the resultant IPN is expected to have a better capability for the controlled release of drugs. Due to the permanent interlocking of the network segments, thermodynamic incompatibility can be overcome as the reacting ingredients are blended thoroughly at the time of synthesis. They can enhance mechanical strength and biological acceptability which can be acquired from the synergistic properties of both natural and synthetic polymers (Wu et al. 2007; Sperling 1977). The most ideal IPNs are heterogeneous systems comprised of one rubbery phase and one glassy phase which produce a synergistic effect yielding either high impact strength or reinforcement, both of which are dependent on phase continuity (Jain et al. 2011; Bhardwaj et al. 2012).

Natural polymers have biocompatible, biodegradable, nonimmunogenic, and nontoxic properties making them suitable candidates for biomedical applications. Among them, chitosan (CS) is the linear cationic polysaccharide composed of β-(1,4)-2-amino-2-deoxy-D-glucopyranose and β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose units (Jana et al. 2013a). The positive facets of excellent biocompatibility and admirable biodegradability with ecological safety and low toxicity with versatile biological activities such as antimicrobial activity and low immunogenicity have provided ample opportunities for further development (Jayakumar et al. 2007; Rinaudo 2008; Mourya and Inamdar 2008; Kurita 2006). Chitosan is a derived polysaccharide polymeric material found abundantly in nature that is amenable to easy IPN preparation, and in the recent years, it has rightly generated great interest as a polymeric vehicle for drug delivery. Taking these into account, this review aims to give an overview on the preparation and applications of chitosan-based semi- and full IPNs according to the most recent publications in the field. The main strategies of chitosan IPNs, their relevant properties, and biomedical applications will be presented. The suitability of chitosan-based IPNs as potential oral delivery vehicle stems from their capability of imbibing a large amount of body fluid without solubilization, potential for encapsulation of a large amount of drugs, and adaptability when combined with specific responsive polymer(s).

9.2 Classification of IPNs

According to the chemistry of preparation, IPNs can be classified as follows: (i) Simultaneous IPN, as the name suggests, starts with a mix of both monomers and both polymers. These are polymerized simultaneously, but by different routes, such as chain and step polymerization. Thus, while reacting, the two polymers do not interfere with each other, but merely dilute each other. Of course, the rates of the two reactions need not be equal. If one polymer or the other reacts faster, different morphologies may result, with concomitant different physical and mechanical behavior patterns (Wang and Liu 2013). Further, (ii) sequential IPN is typically performed by swelling of a single-network hydrogel into a solution containing the mixture of monomer, initiator, and activator, with or without a cross-linker. If a cross-linker is present, full-IPN results, while in the absence of a cross-linker, a network having linear polymers embedded within the first network is formed (semi-IPN) (Fig. 9.2) (Jain et al. 2011; Bhardwaj et al. 2012; Chivukula et al. 2006; Hoare and Kohane 2008). When a linear polymer, either synthetic or biopolymer, is entrapped in a matrix, forming thus a semi-IPN hydrogel, full IPN can be prepared after that by a selective cross-linking of the linear polymer chains (Dragan et al. 2012a; Yin et al. 2007a).

By their structure, IPN hydrogels can be classified into (i) IPNs, formed by two networks ideally juxtaposed, with many entanglements and physical interactions between them; (ii) homo-IPNs, which are a special case of IPN, where the two polymers which form the independent networks have the same structure; (iii) semi- or pseudo-IPNs, in which one component has a linear instead of a network structure; (iv) gradient IPNs, in which the overall composition and/or cross-link density of the material varies from location to location on the macroscopic scale (one way of preparing these materials involves partial swelling of the first polymer network by the second monomer mix, followed by rapid polymerization before diffusional equilibrium takes place); and (v) thermoplastic IPNs, which utilize physical cross-links rather than chemical cross-links. Thus, the materials may be made to flow at elevated temperatures. As such, they are hybrids between polymer blends and IPNs. Such cross-links may involve block copolymers, ionomers, and/or semicrystallinity. Mechanically enhanced IPN hydrogels as "double networks" have attracted attention due to their potential as biomaterials, mainly as a replacement of natural cartilage (Gong et al. 2003; Haque et al. 2012; Li et al. 2013). The particular feature of this new type of IPN hydrogels, characterized by high resistance to wear and high fracture strength, consists of the preparation first of a densely cross-linked ionic hydrogel, the second network being a neutral loosely cross-linked network (Gong et al. 2003; Haque et al. 2012).

9.3 Advancement of Chitosan as IPN Component

Nowadays, chitosan (CS) has attracted numerous scientists due to its outstanding biological properties like biodegradability, biocompatibility, and antibacterial activity. Because of the high content of amino and hydroxyl functional groups, CS has

also drawn attention as a biosorbent showing high potential for the adsorption of proteins, dyes, and metal ions (Jana et al. 2011).

Chitosan has generated considerable interest as a bioadhesive material. The mucoadhesive properties of chitosan have been illustrated by its ability to adhere to porcine gastric mucosa in vitro, and hence it could be useful for site-specific drug delivery. Ionic interactions between positively charged amino groups in chitosan and the negatively charged mucus gel layer in addition to adhesion by hydration has been suggested as an important mechanism of action. The interactions are strong at acidic and slightly acidic pH levels, at which the charge density of chitosan is high. The increase in molecular weight of chitosan results in stronger adhesion. Due to the presence of these properties, there are several chitosan-based IPNs reported in literature prepared with the combination of other natural, semisynthetic, or synthetic polymers (Crini and Badot 2008; Wan Ngah et al. 2011).

Chitosan IPNs have several properties which make it a suitable vehicle for drug delivery. Chitosan properties can be modified by formation of IPN in combination with other polymers. Chitosan IPNs are prepared by the cross-linking of chitosan and additional polymers. The use of a bifunctional agent can block amino groups and turn chitosan structures more inert and resistant. IPN properties such as porosity, elasticity, degree of swelling, and responsive behavior to a stimulus can be tuned by the appropriate choice of network-forming polymers and suitable cross-linking agent and its proportion. Among the various factors, selection of polymers primarily depends on intention of IPN application. As a cross-linker for CS, glutaraldehyde is usually used due to the fast formation of a Schiff base between the $–NH_2$ groups of CS and aldehyde groups of GA. However, GA is highly toxic in nature, and therefore, pharmaceutical scientists normally do not recommend its use in the synthesis of IPN for the purpose of drug delivery. Recently, a new natural cross-linking agent, genipin, has been successfully used in the preparation of CS-based IPN hydrogels (Khurma and Nand 2008; Muzzarelli 2009). This IPN technique has the advantage of specifically selecting polymers that can complement the deficiencies of one another. Based on the target application, cross-linking density can be adjusted in IPNs; however, it is more difficult to encapsulate a wide variety of therapeutic agents, especially sensitive biomolecules. Various IPNs composed of CS and other polymers have been recently designed and investigated for their biomedical applications, the most relevant being presented in the next sections.

9.4 Chitosan-Based IPNs as Drug Delivery Systems

Development of suitable carrier systems for delivery of active pharmaceuticals always remains a major challenge. New technological advances have brought many innovative drug delivery systems. A variety of approaches have been investigated for the controlled release of drugs and their targeting to selective sites. Chitosan-based IPN drug delivery systems are designed to deliver drugs at a particular rate with minimum fluctuation for a desired period of time. Currently, several approaches are being pursued for improved delivery of therapeutic products

like sheets, films, hydrogel, calcifiable matrix, sponges, tablets, capsules, transdermal patches, microspheres, and nanoparticles. But in previous years, researchers are mostly concerned with chitosan-based IPN hydrogels, microparticles, and films. Therefore, these three IPN-based drug delivery systems are discussed here in detail.

9.4.1 Hydrogel

In recent decades, hydrogels have been extensively used as a smart biomaterial in many biomedical applications such as drug delivery and tissue engineering because of their excellent physical and chemical properties. Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of retaining large amounts of water, or biological fluids, characterized by a soft and rubbery consistence, being thus similar with living tissues (Hoffman 2002; Peppas et al. 2000). Various IPN hydrogels composed of chitosan and synthetic polymers have been recently designed and investigated for their biomedical applications. Numerous investigations were performed to prepare IPN hydrogels composed of CS, or its derivatives, and other polysaccharides, or their derivatives, mainly in order to design novel and more efficient drug release systems. Thus, cellulose (Cai and Kim 2009) and its derivatives (Angadi et al. 2010; Rokhade et al. 2007) have been first blended with CS followed by selective cross-linking of CS with glutaraldehyde (GA).

Guo et al. (2007) synthesized thermo- and pH-responsive semi-IPN polyampholyte hydrogels by using carboxymethyl chitosan and poly (N-isopropylacrylamide) with N,N′-methylene-bis-acrylamide (BIS) as the cross-linking agent. It was found that the semi-IPN hydrogels demonstrated a pH- and temperature-responsive nature of the materials, and it also showed good reversibility. The study on the release of coenzyme A (CoA) showed that within 24 h, the cumulative release ratio of CoA was 22.6% in pH 2.1 solution and 89.1% in pH 7.4 solution at 37 °C, respectively. The release rate of CoA was higher at 37 °C than 25 °C in a pH 7.4 buffer solution. An increased release rate of CoA was observed with the content of carboxymethylchitosan increasing in the hydrogel at 25 °C in pH 7.4 solution (Guo and Gao 2007).

Another pH- and thermo-responsive IPN hydrogels were prepared by El-Sherbiny et al. (2005) for controlled drug delivery studies. The IPN hydrogels were obtained in mild aqueous acid media by irradiation of solutions of N-acryloylglycine (NAGly) mixed with chitosan, in the presence of glutaraldehyde as a cross-linking agent and using 2,2-dimethoxy-2-phenyl acetophenone as photoinitiator. These hydrogels were subjected to equilibrium swelling studies at different temperatures (25 °C, 37 °C, and 45 °C) in buffer solutions of pH 2.1 and 7.4 (similar to that of gastric and intestinal fluids, respectively). 5-Fluorouracil (5-FU) was entrapped in the hydrogels, and drug release studies were also carried out at 37 °C in buffer solutions at pH 2.1 and 7.4 (El-Sherbiny et al. 2005).

IPN hydrogels composed of poly(ethylene glycol) macromer (PEGM) and chitosan were synthesized by UV irradiation in a mild aqueous media. The IPN hydrogels exhibited an equilibrium water content (EWC) in the range of 86–94%.

The results from DSC measurements indicate that the melting endotherms of PEGM, within the hydrogels, decreased in intensities and shifted to lower temperatures compared with a linear PEGM. This was due to the decrease of the crystallinity in the IPN hydrogels with higher contents of PEGM. The electrical response of the IPN hydrogels was also investigated by applying electrical current to the hydrogels immersed in a NaCl solution. The extent of a bending degree of the IPN hydrogel depends on the IPN hydrogel composition and applied electric field strength (Kaewpirom and Boonsang 2006).

Bedel et al. (2011) prepared novel chitosan–poly(N,N′-dimethylaminoethyl methacrylate sulfate) (CS-PDESA) semi-IPN hydrogels for determining antibacterial activity (Bedel et al. 2011). At first, the N,N′-dimethylaminoethyl methacrylate sulfate (DMAEMASA) monomer was synthesized Then, chitosan–poly(DMAEMASA) semi-IPN hydrogels were prepared in chitosan solution in acetic acid at 40°C in the presence of N,N′-methylene-bis-acrylamide as the cross-linker. Ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine were used as initiator and accelerator, respectively. Poly(DMAEMASA) hydrogel was also synthesized at the same conditions and its properties were compared with those of CS-PDESA gels. Antibacterial activities of gels were performed by the inhibition zone method. Equilibrium swelling values of semi-IPN hydrogels were lower than that of poly(DMAEMASA). Poly(DMAEMA) and chitosan showed antibacterial activity, but PDESA did not show any efficient activity.

In another study, thermo-sensitive poly(N-isopropyl acrylamide-co-vinyl pyrrolidone)/chitosan [P(NIPAM-co-NVP)/CS] semi-IPN hydrogels were prepared by free-radical polymerization for sustained release of anionic drug naproxen (NAP). The LCST of hydrogels was adjusted to the vicinity of body temperature by introducing hydrophilic N-vinyl pyrrolidone. The presence of CS in semi-IPN networks improves the swelling behavior and provides a high affinity for anionic drug NAP due to the strong interactions between NAP molecules and CS chains. Release of NAP was suppressed at pH 2.2 and 5.0 and accelerated at pH 7.4 due to the deprotonation of amino groups in CS. Increasing the temperature above LCST, hydrogels showed a continuous release of NAP without burst diffusion due to the shrinkage of PNIPAM restraining the drug release (Li et al. 2012).

Superporous hydrogels containing poly(acrylic acid-co-acrylamide)/O-carboxymethyl chitosan interpenetrating polymer networks (SPH-IPNs) were prepared by Yin et al. (2007a, b) by cross-linking O-carboxymethyl chitosan (O-CMC) with glutaraldehyde (GA) (Yin et al. 2007b). SPH-IPNs possessed both the IPN network and large numbers of pores and the cross-linked O-CMC molecules were located on the peripheries of these pores. The swelling behavior of SPH-IPNs was dependent on the O-CMC content, GA amount, and cross-linking time. Due to the cross-linked O-CMC network, in vitro mucoadhesive force and mechanical properties were greatly improved. An enhanced loading capacity for insulin was also reported by the SPH-IPNs as compared to the nonporous hydrogel.

Biocompatible IPN hydrogels based on chitosan with N-vinylpyrrolidinone (NVP) as well as its copolymer with 2-hydroxymethyl methacrylate (HEMA) were synthesized using the photopolymerization technique without the inclusion of any

photoinitiator or cross-linking agent. It was found that the IPN comprised of chitosan and NVP with a very small amount of N-hydroxymethyl maleimide (HMMI) included exhibited higher swelling abilities and fast drug release rates than the IPN which contained chitosan, NVP, and HEMA. Kinetic studies of water diffusion into these hydrogels and drug release revealed that with the exception of the IPN with HEMA incorporated, the other hydrogels did not adhere to the Fickian diffusion model. These hydrogels were tested for their biocompatibility with human epidermal keratinocyte cells (Ng and Swami 2005).

Rao et al. (2006) developed novel pH-sensitive IPN microgels (MGs) based on chitosan, acrylamide-grafted poly(vinyl alcohol), and hydrolyzed acrylamide-grafted poly(vinyl alcohol) that are cross-linked with glutaraldehyde and used in the controlled release (CR) of cefadroxil (Rao et al. 2006). The blend microgels of chitosan with PVA have shown favorable CR characteristics (the release was more than 10 h) than plain chitosan MGs. Variations in swelling and drug release characteristics of the matrices in different pH media are attributed to morphological changes of the hydrogels.

Semi-interpenetrating polymer network (semi-IPN) hydrogels were prepared by UV irradiation of water-soluble N-carboxylethyl chitosan (CECS) and 2-hydroxyethyl methacrylate (HEMA) aqueous solutions in the presence of Darocur-2959 as photoinitiator. The thermal stability and equilibrium degree of swelling improved obviously with increase of CECS content. Differential scanning calorimetry study indicated that free water content increased with increase of CECS content while bonded water content decreased. Cytotoxicity results suggested that semi-IPN hydrogels had good biocompatibility (Zhou et al. 2009).

Currently, Cui et al. (2014) prepared and characterized IPN hydrogels based on chitosan and gelatin using genipin as the cross-linker. The degree of cross-linking increased with increase genipin concentration. Swelling results revealed that the IPN hydrogels are pH-sensitive, exhibiting reversibility and rather rapid response in swelling to pH changes. It is expected that this IPN hydrogel has potential as a controlled drug delivery system or as alternative sorbents for biomedical and environmental use as pH is altered (Cui et al. 2014).

IPN hydrogels of poly(ethylene glycol) macromer (PEGM) with chitosan in the presence of glutaraldehyde as a cross-linking agent were prepared by UV irradiation in mild aqueous acid media (Leea et al. 2000). Equilibrium water content of these hydrogels was in the range of 74–97%. Photo-cross-linked PEGM segments within the IPNs had considerably reduced crystallinities compared to PEGM itself. All the IPNs revealed two glass transition temperatures (Tg), indicating the presence of phase separation in the IPNs. The tensile strength and elongation at break of IPN hydrogels in the swollen state ranged from 0.06 to 0.18 MPa and 18–48%, respectively.

Dash et al. (2012) synthesized semi-IPN based on chitosan and poly (methacrylamide) under physiological conditions. Free radical polymerization of methacryloylglycylglycine (MAGG) in the presence of chitosan and ethylene glycol dimethacrylate as the cross-linker was performed. Chitosan and poly(MAGG) hydrogels having different compositions and amounts of cross-linker were prepared.

Degradation behavior promoted by lysozyme demonstrated that there was 60%, 68%, and 76% of degradation of the semi-IPN hydrogel with 8%, 4%, and 2% of the cross-linker, respectively, in 28 days. The preliminary investigations of the prepared materials suggested their suitability for applications in tissue engineering and controlled release of bioactive agents (Dash et al. 2012).

In another study, novel granular semi-IPN hydrogels were prepared in situ in an aqueous solution by the free radical grafting and cross-linking reactions among chitosan, acrylic acid (AA), gelatin, and N,N′-methylene-bis-acrylamide for fast and efficient adsorption of Cu^{2+} ion (Wang et al. 2013). The gel strength, adsorption, reuse, and recovery properties of the hydrogels for Cu^{2+} ion were systematically investigated. The results indicate the hydrogel with 2 wt% gelatin has the highest adsorption capacity of 261.08 mg/g with a recovery ratio of 95.2%. And the incorporation of 10 wt% gelatin enhanced the storage modulus by 103.4% and 115.1% and the adsorption rate by 5.67%. Moreover, the adsorption capacity of the hydrogel is still as high as 153.9 mg/g, after five cycles of adsorption–desorption. It was found that the ion-exchange and complexation interactions between the functional groups ($COO-$ and NH_2) of the hydrogels and Cu^{2+} ion are the predominant adsorption mechanisms.

IPN hydrogels composed of poly(vinyl alcohol) (PVA) and chitosan were prepared by UV irradiation. IPN hydrogels exhibited a relatively high swelling ratio in the range of 210–350% at 35 °C. The swelling ratio of PVA/chitosan IPN hydrogels depended on pH and temperature. DSC was used for the quantitative determination of the amounts of freezing and nonfreezing water. Free water contents were found to be in the range of 62.0– 72.3% in pure water, respectively (Kim et al. 2003).

Yang et al. (2013) have recently reported the preparation of novel hydrogels composed of PEG grafted on carboxymethyl chitosan and alginate, and found an improvement of the protein release at pH 7.4, suggesting this composite hydrogel to be promising for protein drug delivery in the intestine (Yang et al. 2013).

Guo and coworkers have reported an interesting approach to obtain thermo- and pH-responsive semi-IPN polyampholyte hydrogels based on carboxymethyl chitosan and poly(dimethylaminoethyl methacrylate) (PDMAEM) (Guo et al. 2007). The semi-IPN hydrogel shrunk most at the isoelectric point (IEP) and swelled when pH deviated from the IEP. In the presence of PDMAEM, which presents a lower critical solution temperature (LCST), the swelling ratio of the composite gel dramatically decreased between 30 and 50 °C, at pH 6.8. The key advantage of this composite hydrogel is that the release rate of coenzyme A could be modulated as a function of temperature, being higher at 50 °C than at 37 and 25 °C, at pH 6.8, making the semi-IPN hydrogel of great promise in pH−/temperature-responsive drug delivery systems.

The synthesis of macroporous ionic composite cryogels consisting of two independently cross-linked and oppositely charged networks was reported by Dragan et al. (2012a). Semi-interpenetrating network cryogels were prepared first by cross-linking polymerization of acrylamide (AAm) with N,N′-methylene-bis-acrylamide (BAAm) in the presence of chitosan (CS), under freezing conditions, with the main

parameters varying such as the cross-linker ratio, pH of the CS solution, and CS molar mass. The generation of an anionic matrix during the formation of the second network led to a high sorption capacity of a model cationic dye, methylene blue (MB), by the full-IPN cryogel, around 750 mg dye/g gel, at 25 °C. The benefits of the full-IPN cryogels include their high reusability and high selectivity in the separation of MB from a mixture with methyl orange (Dragan et al. 2012a).

Two kinds of chitosan-based hydrogels, a cross-linked chitosan reference gel and a chitosan–poly(ethylene oxide) semi-IPN with potential pH-sensitive swelling and drug delivery properties, were characterized by (Khalid et al. 2002). Swelling studies were performed on the two kinds of hydrogels by differential scanning calorimetry (DSC) at pH 1.2 and by the gravimetric method at pH 1.2 and pH 7.2. Both methods lead to similar results. If pH-dependent swelling properties were observed with both hydrogels, they were however improved for the semi-IPN. The amount of bound water in the xerogels could be determined from DSC measurements and a thermogravimetric analysis. The results obtained by both techniques were in good agreement and indicated that the semi-IPN contained more bound water than the reference gel probably due to the presence of the hydrophilic poly(ethylene oxide) chains.

Amoozgar et al. (2012) used blends of photo-cross-linkable 4-azidobenzoic acid-modified chitosan (Az-C) and polyethylene glycol (PEG) as a new in situ-forming bioadhesive for anastomosing and stabilizing the injured nerves. Nerves anastomosed with an Az-C/PEG gel tolerate a higher force than those with fibrin glue prior to failure. A series of ex vivo and in vitro cell experiments indicate the Az-C/PEG gels are compatible with nerve tissues and cells. In addition, Az-C/PEG gels release PEG over a prolonged period, providing sustained delivery of PEG, a potential aid for nerve cell preservation through membrane fusion (Amoozgar et al. 2012).

In another work, a novel IPN hydrogel composed of cross-linked chitosan and poly(acryl acid) was prepared using glutaraldehyde. The model drug, cisplatin, was loaded into the resulting IPN hydrogel. Water absorption of the hydrogel could be switched on and off swiftly by control of pH of the surrounding environment. Therefore, the synthesized hydrogels in this work can be used as a drug delivery system, and the drug release can be controlled by the pH of the solution. The release rate of cisplatin from the hydrogel at pH 7.4 was higher than that at pH 1.2 due to the increased swelling capacity of the hydrogel (Hosseinzadeh 2012).

A method was developed by Zhang et al. (2004) to prepare thermosensitive poly (N-isopropylacrylamide) (PNIPAAm) hydrogels with an IPN structure for the purpose of improving its mechanical properties, response rate to temperature, and sustained release of drugs (Zhang et al. 2004). The mechanical properties of the IPNs were greatly improved when compared with the normal PNIPAAm hydrogel. These IPN hydrogels also exhibited improved intelligent characteristics (e.g., controllable faster response rate) that depended on the composition ratio of the two network components. The effects of a shrinking–reswelling cycle around the LCST on the properties of the IPN hydrogels were examined to determine if these properties would be stable for potential applications. Bovine serum albumin was

chosen as the model protein for examining its release from the IPNs at different temperatures. The release data suggested that an improved controlled release could be achieved by the IPN–PNIPAAm hydrogels without losing their intelligent properties.

Vaghani and Patel (2011) developed a pH-sensitive chitosan/polyvinyl pyrrolidone (PVP)-based controlled drug release system for clarithromycin. The hydrogels were synthesized by cross-linking chitosan and PVP blend with glutaraldehyde to form a semi-IPN. The hydrogels showed more than 97% content of clarithromycin. These hydrogels showed high swelling and mucoadhesion under acidic conditions. The swelling may be due to the protonation of a primary amino group on chitosan. In vitro release study revealed that formulation containing chitosan (2% w/v) and PVP (4% w/v) in the ratio of 21:4 showed complete drug release after 12 h (Vaghani and Patel 2011).

Kim et al. (2006) described an interesting approach for the preparation of semi-IPN composed of CS and poloxamer. Their strategy consists of photo-cross-linking the poloxamer macromer in the presence of CS coupled with freeze-drying to obtain sponge-type hydrogels. These IPN composite hydrogels demonstrated rapid water adsorption, high mechanical strength, and interconnected pores, which recommend them for wound dressing application (Kim et al. 2006).

It was demonstrated that IPN hydrogels are endowed with very fast kinetics for sorption of ionic species like dyes and heavy metal ions compared with the single-network hydrogels. Mechanical properties, swelling kinetics, and reusability of IPN hydrogels in their applications as sorbents have been further improved by conducting the synthesis of the gels under the freezing temperature of the solvent. The presence of reactive hydroxyl (OH) groups at C-3, C-6, and amino (NH_2) groups at C-2 position in chitosan makes it a potential adsorbent for dyes (Crini and Badot 2008).

Maity and Ray (2014) synthesized several semi- and full-IPN hydrogels from chitosan and polymethacrylic acid by changing the polymer/monomer mass ratios and initiator and cross-linker concentrations (Maity and Ray 2014). The hydrogel synthesized with 1 wt% initiator, 1 wt% cross-linker, and 4 wt% chitosan showed high adsorption and removal of Congo red and methyl violet dyes at both low and high water concentration range. The adsorption of these dyes was higher at high-feed dye concentration range. However, adsorption or removal of Congo red was higher than methyl violet for both concentration range. Adsorption data were also observed to fit well to pseudo second-order kinetics in the low concentration range.

Another research paper reported that semi-IPN hydrogels, which are based on polyacrylamide (PAAm) and chitosan (CS), sorbed a higher amount of the anionic dye (Direct Blue 1) than the full-IPN hydrogels. On the other hand, the full-IPN hydrogels sorbed a much higher amount of the cationic dye methylene blue than the semi-IPN hydrogel (Dragan et al. 2012b).

Semi-IPN hydrogel composites for dye adsorption studies were prepared via photopolymerization of poly(ethylene glycol) (PEG) macromer and acrylamide (AAm) monomer in the presence of chitosan (CS) (Zhao et al. 2012). Swelling properties and kinetics of the hydrogel composites were investigated in aqueous

solution and Acid Red 18 (AR 18) solution. The adsorption studies showed that the adsorption capacity for AR 18 increased with the increase of initial dye concentration and chitosan content in the hydrogels, but decreased with the increase of pH and ionic strength of dye solutions. The hydrogel composites could be potentially used as absorbents for anionic dye removal in wastewater treatment process.

Hydrogel networks that combine suitable physical and biomechanical characteristics for tissue engineering scaffolds are in demand nowadays. For example, recently, Gómez-Mascaraque et al. (2014) developed hydrogel networks based on agarose and chitosan using oxidized dextrins as low cytotoxicity cross-linking agents, paying special attention to the study of the influence of the polysaccharide composition and oxidation degree of the dextrins in the final characteristics of the network (Gómez-Mascaraque et al. 2014). The results show that the formation of an interpenetrating or a semi-interpenetrating polymer network was mainly dependent on a minimum agarose content and degree of dextrin oxidation. The analysis of atomic force microscopy images showed the formation of a fibrillar microstructure whose distribution within the cross-linked chitosan depended mainly on the cross-linker. All materials exhibited a viscoelastic behavior typical of gels. The stiffness was strongly influenced by the degree of oxidation of the cross-linker. Cellular response to the hydrogels was studied with cells of different strains, and cell adhesion and proliferation were correlated with the homogeneity of the samples and their elastic properties.

Ionic IPN cryogels (Lozinsky et al. 2003; Jain and Kumar 2009; Artyukhov et al. 2011; Plieva et al. 2005) based on synthetic polymers and biopolymers, like CS and starch, are endowed with very fast swelling rate, a high sorption capacity for ionic species like dyes and heavy metal ions, fast kinetics of sorption, and a high level of reusability, features which recommend them as promising sorbents in the future. Even if it is still a challenging task, the synthesis of ion-imprinted IPN hydrogels constitutes a promising direction in increasing the selectivity of this novel type of sorbents, which is expected to receive much attention in the future.

9.4.2 Microspheres/Microcomposites

Microspheres are another promising class of IPN-based drug delivery systems. Microspheres are free flowing small spherical powder particles made up of natural or synthetic polymers having a diameter in the range of 1 μm to 1000 μm (Lohani and Gangwar 2012). IPN microspheres are considered versatile carriers for controlled-release and drug-targeting applications due to their capability to encapsulate a wide variety of drugs, protection of drugs, increased bioavailability, biocompatibility, patient compliance, biodegradability, and sustained-release characteristics.

pH-sensitive semi-interpenetrating networks (IPNs) based on chitosan (Cs) and acrylamide-grafted hydroxyethylcellulose (AAm-g-HEC) were prepared in the form of microspheres (MPs) by the emulsion cross-linking technique using glutaraldehyde (GA) as a cross-linker. Diclofenac sodium (DS) drug was successfully

encapsulated into IPN microspheres by varying the ratio of Cs and AAm-g-HEC, % drug loading, and amount of GA. DS encapsulation of up to 83% was obtained as measured by UV spectroscopy. MPs with average particle sizes in the range of 188–310 μm were obtained. Diffusion coefficients (D) of water transport through the microspheres were determined using an empirical equation. In vitro release of DS from these matrices has been investigated in pH 1.2 and 7.4 media (Ahmed et al. 2009).

Angadi et al. (2010) prepared glutaraldehyde cross-linked IPN blend microspheres of chitosan (CS) and hydroxyethyl cellulose (HEC) in the form of microspheres (66–82 μm dia) which were investigated for the controlled release (CR) of isoniazid (INH), an antituberculosis drug (Angadi et al. 2010). Fourier transform infrared (FTIR) spectroscopy was used to understand chemical interactions and to assess the IPN structure. Scanning electron microscopy (SEM) was employed to investigate the morphology of microspheres. Drug-loaded microspheres were produced in spherical shapes with encapsulation efficiency ranging from 50% to 66%. Equilibrium swelling measured in pH 7.4 buffer solution as well as in vitro release of drug in pH 1.2 and 7.4 buffer solutions indicated the dependence of drug release on cross-linking as well as the blend composition of the IPN matrix.

Another study describes the formulation and in vitro evaluation of IPN matrix tablets of aceclofenac. IPN microparticles using chitosan and tamarind seed polysaccharide blend were prepared using glutaraldehyde as cross-linker. The drug entrapment efficiency and average particle size of these microparticles were found to be 91.97 ± 1.30% and 498.12 ± 38.67 μm, respectively. These IPN microparticles were characterized by scanning electron microscopy (SEM) (Fig. 9.1) and powder X-ray diffraction (P-XRD) study. These microparticles were compressed with tablet excipients through the direct compression technique. These matrix tablets showed sustained aceclofenac release over 8 h. These matrix tablets

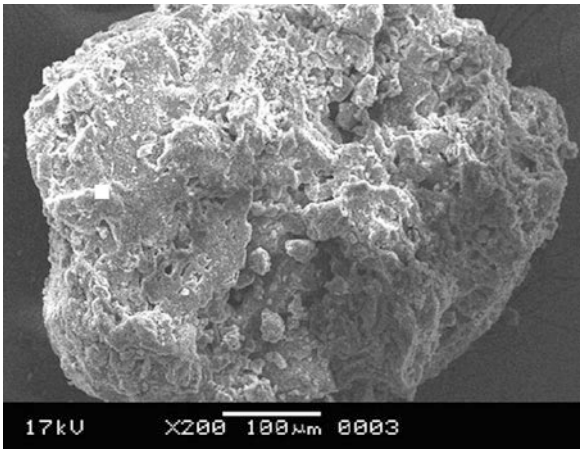

Fig. 9.1 SEM photograph of aceclofenac-loaded chitosan-TSP IPN microparticles. (Reprinted from Jana et al. (2014). Copyright (2014), with permission from Elsevier)

might be helpful to minimize dosing frequency and reduction of various side effects during a prolonged period of treatment (Jana et al. 2014).

Semi-interpenetrating polymer network (IPN) microspheres of acrylamide grafted on dextran (AAm-g-Dex) and chitosan (CS) were prepared through the emulsion cross-linking method using glutaraldehyde (GA) as a cross-linker. The grafting efficiency was found to be 94%. Acyclovir, an antiviral drug with limited water solubility, was successfully encapsulated into IPN microspheres by varying the ratio of AAmg-Dex and CS, % drug loading, and amount of GA. Microspheres with average particle sizes in the range of 265–388 μm were obtained. Acyclovir encapsulation of up to 79.6% was achieved as measured by UV spectroscopy. Both equilibrium and dynamic swelling studies were performed in 0.1 N HCl. Diffusion coefficients (D) and diffusional exponents (n) for water transport were determined using an empirical equation. In vitro release studies indicated the dependence of drug release rates on both the extent of cross-linking and amount of AAm-g-Dex used in preparing the microspheres; the slow release was extended up to 12 h. The release indicated a non-Fickian trend for the release of acyclovir (Rokhade et al. 2007).

Rokhade et al. (2007) prepared IPN microspheres of chitosan (CS) and methylcellulose (MC) were prepared by emulsion cross-linking in the presence of glutaraldehyde (GA) as a cross-linker. Theophylline (THP), an antiasthma drug, was encapsulated into IPN microspheres under varying ratios of MC and CS, % drug loading, and amount of GA added (Rokhade et al. 2007). IPNs have shown better mechanical properties than pure CS. The cross-link density of the matrices was significantly affected by the amount of GA and MC. Theophylline encapsulation of up to 82% was achieved as measured by a UV spectrometer. Equilibrium swelling was performed in distilled water. In vitro release studies were performed in both 0.1 N HCl and pH 7.4 buffer solutions. These data indicated a dependence of drug release on the extent of cross-linking and amount of MC added during the preparation of microspheres. The release was extended up to 12 h and release rates indicated a slight deviation from the Fickian trend for the release of theophylline.

In a study Reddy et al. synthesized ghatti gum and chitosan IPN microparticles by the emulsion cross-linking method using glutaraldehyde as a cross-linker. IPN microparticles were used to deliver diclofenac sodium to the intestine. Characterization of IPN microparticles was done by SEM, DSC, and FTIR and was evaluated for in vitro drug release. FTIR studies assessed the formation of an IPN structure. It was found that the drug release from IPN microparticles was extended up to 12 h (Reddy et al. 2013).

pH-sensitive novel semi-IPNs of N,N′-dimethylacrylamide (NNDMA) and chitosan (CS) were prepared by Babu et al. (2008) in the form of microspheres by water-in-oil (w/o) emulsion technique using the chlorothiazide (CLT) drug. Scanning electron micrographs (SEM) of the microspheres indicated smooth surfaces of the spherical microspheres. Swelling studies have shown that with an increasing amount of NNDMA in the microspheres, drug containing water uptake has decreased. Cumulative release characteristics of the matrices for CLT, an antihypertensive drug, were investigated in pH 1.2 and 7.4 media. It was possible to release CLT in a controlled manner up to 12 h (Babu et al. 2008).

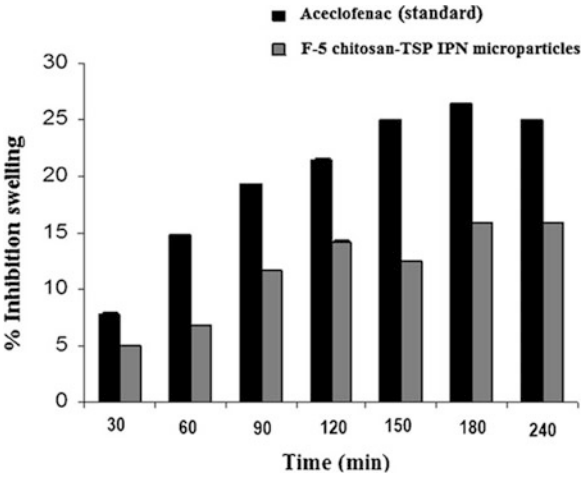

Fig. 9.2 The inhibition percentages of paw edema swelling in carrageenan-induced rat paw edema model for the standard (pure aceclofenac) and the test (F-5 chitosan–TSP IPN microparticles containing aceclofenac) at various time intervals. (Reprinted from Jana et al. (2013b). Copyright (2013), with permission from Elsevier)

In another study, Jana et al. (2013b) carried out preparation, characterization, and evaluation of glutaraldehyde cross-linked chitosan–tamarind seed polysaccharide (TSP) IPN microparticles for prolonged aceclofenac release. The drug entrapment efficiency of these microparticles was found to be 85.84 ± 1.75 to 91.97 ± 1.30%, and the in vitro drug release from these aceclofenac-loaded chitosan–TSP IPN microparticles showed sustained release of aceclofenac over 8 h following the Korsmeyer–Peppas model with an anomalous (non-Fickian) diffusion drug release mechanism. The in vivo studies exhibited sustained anti-inflammatory activity in carrageenan-induced rats (Fig. 9.2) (Jana et al. 2013b).

Rani et al. (2010) addressed the development of novel pH-sensitive IPN beads composed of chitosan–glycine–glutamic acid cross-linked with glutaraldehyde and their use for controlled drug release (Rani et al. 2010). A comparative study has been carried out on these IPN beads with the beads that of chitosan, chitosan–glycine, and chitosan–glutamic acid cross-linked with glutaraldehyde. The swelling behavior of the beads at different time intervals was monitored in solutions of pH 2.0 and pH 7.4. The release experiments were performed in solutions of pH 2.0 and pH 7.4 at 37 °C using chlorpheniramine maleate (CPM) as a model drug. The swelling behavior and release of the drug were observed to be dependent on pH, degree of cross-linking, and their composition. The results indicate that the newly constructed cross-linked IPN beads of chitosan–glycine–glutamic acid might be useful as a vehicle for controlled release of a drug. The kinetics of drug release from beads was best fitted by Higuchi's model in which release rate is largely governed by rate of diffusion through the matrix.

Chitosan and gelatin were used to prepare novel pH-sensitive stearic acid-coated IPN blend microspheres by the emulsion cross-linking method using glutaraldehyde for the controlled release (CR) of isoniazid (INH), an antituberculosis drug. Coated as well as uncoated microspheres were developed and characterized. Coated microspheres were produced in the size range of 52 μm down to 502 nm with

encapsulation efficiencies of 65–78%. Equilibrium swelling was studied in pH 1.2 and pH 7.4 buffer media, and the in vitro drug release showed the dependence of drug release on the cross-linking, blend ratio of the IPN matrix, as well as stearic acid coating. The variations in the IPN blend ratio and cross-link density controlled the drug release up to 30 h, but the coated microspheres could reduce the burst release in the gastric stomach media while enhancing in intestinal pH 7.4 media (Angadi et al. 2011).

Kulkarni and Keshavayya (2010) adopted an inexpensive and simple method for the preparation of chitosan–sodium alginate IPN beads for the release of an antibiotic, ofloxacin hydrochloride. The polymers were combined at different ratios and the exposure time to the cross-linking agent, glutaraldehyde (GA), was varied to get the beads with different processing parameters (Kulkarni and Keshavayya 2010). The beads characterized by optical microscopy indicated that they were in the size range of 30–250 μm. The degree of swelling as well as the rate of drying of beads were influenced by the incorporation of alginate into chitosan and the time of exposure to the cross-linking agent. The encapsulation efficiency varied between 76 and 86% for different formulations. According to the in vitro dissolution studies, the IPN beads showed sustained effect up to 24 hrs; increase in the alginate content and exposure time to the cross-linking agent resulted in reduction of extent of drug release.

Another research paper described the synthesis of capecitabine-loaded semi-IPN hydrogel microspheres of chitosan–poly(ethylene oxide-*g*-acrylamide) by emulsion cross-linking using glutaraldehyde. Poly(ethylene oxide) was grafted with polyacrylamide by free radical polymerization using ceric ammonium nitrate as a redox initiator. The mean particle size of the microspheres as measured by the laser light scattering technique ranging between 82 and 168 μm. Capecitabine was successfully encapsulated into semi-IPN microspheres and percentage of encapsulation efficiency varied from 79 to 87%. In vitro release studies were performed in simulated gastric fluid (pH 1.2) for the initial 2 h, followed by simulated intestinal fluid (pH 7.4) until complete dissolution. The release of capecitabine was continued up to 10 h (Agnihotri and Aminabhavi 2006).

IPN of poly(N-isopropylacrylamide) (PNIPA) and chitosan (two grades) were prepared by Alvarez-Lorenzo et al. (2005) by free radical polymerization and cross-linking of PNIPA with bis(acrylamide) in chitosan solutions (1.5 wt.% in acetic acid) and subsequent immersion in glutaraldehyde solutions to post-cross-link the chitosan (Alvarez-Lorenzo et al. 2005). The amount of chitosan that remained in the IPNs, after washing, was proportional to the glutaraldehyde concentration used in the post-cross-linking step, being only 50% of the theoretical when the post-cross-linking was omitted (semi-IPN). The PNIPA/chitosan IPNs had a notably greater affinity for diclofenac and were able to sustain the drug release for more than 8 h in 0.9% NaCl solutions or pH 8 phosphate buffer. PNIPA microdomains play an important role in controlling the release process. So, the interpenetration of networks with complementary properties, such as those made with PNIPA and chitosan, make it possible to develop drug delivery systems with improved drug loading capacity (owing to chitosan) and sustained-release behavior (owing to PNIPA).

IPN has numerous advantages as a biomaterial and is widely used as a carrier system for the delivery of drug and protein in the form of microspheres. The concepts of high swelling capacity, specificity, and sensitivity play an important role in targeting delivery of drugs. IPN can provide the resources to deliver drugs at a prolonged controlled release to specific targets. Once optimized, these targeted microspheres will provide better treatment options. So, it can be inferred that IPN-based microspheres for various drug delivery systems are expected to become a useful matrix substance for various therapeutic applications in the future.

9.4.3 Films

Chitosan possess capability to form films with good strength (Rao et al. 2010). Chitosan-based films have been widely investigated for modifying the drug release from pharmaceutical dosage forms. However, due to its solubility in the acidic pH of the stomach, endeavors are aimed at derivatizing or cross-linking it for modifying drug release characteristics (Risbud et al. 2000). Attempts have been made to co-process various polysaccharides like chondroitin (Bhise et al. 2007), sodium citrate (Chen et al. 2004), and sodium alginate (Arzate-Vázquez et al. 2012) with chitosan in order to modify the film-forming ability of the former polymers. These films have been investigated for improving drug delivery in colorectal drug release dosage forms (Naidu et al. 2009), sustained-release drug delivery systems (Kumar et al. 2004), and transdermal film formulations (Sivakumar et al. 2002). Several chitosan-based IPN films were prepared and evaluated for their physicochemical properties as well as mechanical performance for exploring their potential in food and pharmaceutical industry.

Jindal et al. (2013) performed co-processing of gum obtained from unripe fruits of *Aegle marmelos* with chitosan to improve the IPN film-forming property of the former. The results of FTIR and differential thermal analysis of films revealed maximum interaction between –COO groups of gum and $-NH_3^+$ of chitosan when they were present in equal proportion in the film. These films exhibited almost negligible zeta potential and the surface remained smooth after exposure to both acidic and alkaline pH as observed under scanning electron microscope. The contact angle and swelling index of this film in both acidic and alkaline buffers were observed to be lowest as compared to other films prepared with different ratios of gum and chitosan. The work of adhesion and spreading coefficient for this film was observed to be lowest. These results could be attributed to the optimum interaction between the –COO groups of the gum and $-NH_3^+$ of chitosan. These results indicated the ability of this film for use in modifying drug release and processed food items (Jindal et al. 2013).

Anion exchange membranes with semi-IPN network were prepared based on quaternized chitosan (QCS) and polystyrene (PS). The PS was synthesized by polymerization of styrene monomers in the emulsion of the QCS in an acetic acid aqueous solution under nitrogen atmosphere at elevated temperatures. The semi-IPN system was formed by post-cross-linking of the QCS. A hydroxyl ionic conductivity of $2.80{*}10^{-2}$ S $cm^{-}1$ at 80 °C and a tensile stress at break of 20.0 MPa at room

temperature were reached, respectively, by the semi-IPN membrane containing 21 wt.% of the PS. The durability of the semi-IPN membrane in alkaline solutions was tested by monitoring the variation of the conductivity and the mechanical strength. The water swelling of the semi-IPN membranes was discussed based on the stress relaxation model of polymer chains, and it obeyed Schott's second-order swelling kinetics (Wang et al. 2011).

In another study, a novel organic–inorganic composite membrane was prepared, using tetraethyl orthosilicate (TEOS) as an inorganic material and chitosan as an organic compound. Equilibrium and oscillatory swelling studies were conducted to investigate swelling behaviors of the membrane according to the pH of the swelling medium. Drug permeation experiments were also performed in phosphate buffer solution at pH of 2.5 and 7.5, respectively. Lidocaine HCl, sodium salicylate, and 4-acetamidophenol were selected as model drugs to examine the effect of the ionic property of the drug on the permeation behavior. The effects of membrane composition and the external pH on the swelling and drug permeation behavior of an IPN membrane could be summarized as follows: chitosan incorporated into TEOS IPN swelled at pH 2.5, while it shrunk at pH 7.5. This swelling behavior was completely reversible and the membrane responded rapidly to the change in environmental pH condition. According to swelling behavior, an increase in pH from 2.5 to 7.5 yielded an increase in the rate of drug permeation because of the shrinking of the incorporated chitosan in TEOS IPN, while decrease in pH resulted in low permeation rate. The optimal TEOS–chitosan ratio for maximum pH sensitivity existed and drug permeation was influenced not only with the external pH but also with the ionic interactions between the drug and membrane (Park et al. 2001).

It is well known that chitosan (CS) porous membranes are suitable for various applications such as controlled release of drugs (Patel and Amiji 1996; Risbud et al. 2000), wound dressings (Wang et al. 2002), scaffolds (Chung et al. 2002), and chromatographic media (Ghosh 2002). Many methods have been reported to obtain a chitosan matrix with a porous structure including the phase inversion technique (Hu et al. 2001), freeze-drying, or cryogenic-induced phase separation (Gao et al. 2003), and casting/salt leaching (Chow and Khor 2000). The pore structure is controlled by the phase separation behavior of the CS/counterpart polymer blend.

Zeng and Fang (2004) prepared a cross-linked chitosan (CS) membrane with a submicrometer porous structure by extraction of polyethylene glycol (PEG) from CS/PEG semi-IPN membrane. The content of PEG and the cross-linking agent has significant effects on the pore structure, swellability, and mechanical properties of the membranes. The membrane is pH sensitive, exhibiting reversibility and rapid response in swelling to pH changes. The swelling ratio oscillated between ca. 120 and 220 when pH was varied between 3.2 and 11. It also has good mechanical strength in both dry and swollen state. Thus, it was expected as a potential candidate for biomedical use as environmental pH condition is altered. Generally, in this study, the chitosan matrix was cross-linked so as to enhance the chemical stability and mechanical properties of the porous membrane. The pore size, controlled by the content of PEG and the cross-linking agent, was in sub-micrometer level (Zeng and Fang 2004).

Yu et al. (2006) synthesized semi-IPN membranes from 6-*O*-carboxymethylchitosan (6-OCC) and waterborne polyurethanes (WPU) with ester or ether soft segments. Results from the dynamic mechanical analysis (DMA) and scanning electron microscopy (SEM) showed that miscibility and mechanical properties of the 6-OCC/WPU semi-IPNs were varied with their compositions and the cross-linking/grafting reactions. 6-OCC had better miscibility with the waterborne polyurethane containing ester soft segments and could interfere the formation of hydrogen bonds between the ester soft segments and the NH groups in hard segments. The 6-OCC/WPU composite membranes demonstrated microphase-separated structures in which the 6-OCC particles were dispersed in the matrix of the WPU-rich phase. The 6-OCC/WPU composites were cross-linked with glutaraldehyde or cross-linked/grafted with ethylene glycol diglycidyl ether (EGDE) to form 6-OCC/WPU semi-IPN membranes. It was concluded that the 6-OCC/WPU semi-IPN membranes could be good candidates for biomedical applications (Yu et al. 2006).

Different chitosan-based interpenetrating polymer networks as drug delivery vehicle are given in Table 9.1.

Jana and Sen (2017) investigated chitosan (CS) and locust bean gum (LBG) IPN nanocomposites for aceclofenac delivery. CS-LBG IPN was synthesized by glutaraldehyde through the cross-linked method reaction mechanism and is depicted in Fig. 9.3. The nanocomposite systems controlled the burst release of the drug (Jana and Sen 2017).

9.5 Conclusions and Perspectives

Chitosan-based IPNs, as a particular category of composite materials, received great attention over the last decade owing to their improved responsiveness and mechanical properties. Chitosan-based IPNs are effective and suitable vehicles for the controlled delivery of drugs. It encapsulates a large amount of drug and release them in the body from the swelled matrix in a controlled manner. Chitosan is nontoxic, biodegradable, non-mutagenic, and biocompatible, which make it a suitable vehicle for the oral delivery of drugs. Properties of chitosan in conjunction to other additional polymers have been explored. With IPNs, it is possible to combine synergistic properties of two or more polymers. Two different types of polymer properties such as hydrophilic and hydrophobic behavior have been combined by using such system. The field of chitosan-based IPNs has an immense scope to explore their properties, which makes them suitable vehicles for oral delivery of bioactive molecules especially for localized and controlled delivery of drugs. The potential of chitosan derivatives for IPNs remains to be explored. Mucoadhesive properties of novel derivative of chitosan such as thiolated chitosan and carboxymethyl chitosan are reported to be very high. Chitosan derivatives having an environment-responsive behavior offer opportunities for oral controlled drug delivery. Cross-linker choice is also obligatory for the formulation of chitosan-based IPNs, which is expected to receive much attention in the future.

Table 9.1 Chitosan-based interpenetrating polymer networks as drug delivery vehicle

Polymers	Cross-linkers	Biomedical applications	References
Chitosan(CS) + methyl cellulose	Glutaraldehyde (GA)	Oral controlled release of theophylline	Rokhade et al. (2007)
CS + alanine	GA	Oral controlled release of chlorpheniramine	Kumari and Kundu (2007)
CS + alginate	GA	Oral controlled release of drug	Kulkarni et al. (2001)
CS + glutamic acid	GA	Oral controlled release of chlorpheniramine	Kumari and Kundu (2007)
CS + poloxamer 407	UV radiation	Sponge for wound dressing	Kim et al. (2006)
CS + hydroxyl ethyl cellulose	GA	Oral controlled release of isoniazid	Angadi et al. (2010)
CS + N,N′-dimethyl acrylamide	GA	Oral controlled release of chlorothiazide	Babu et al. (2008)
CS + polyethylene glycol	GA	Oral controlled release of isoniazid	Gupta and Ravikumar (2001)
CS + poly(dimethylsiloxane)	Hexamethylene–1,6-di-(aminocarboxysulfonate)	Bioadhesive film	Rodkate et al. (2010)
CS + poly(ethylene oxide)-g-acrylamide	GA	Oral controlled release of capecitabine	Agnihotri and Aminabhavi (2006)
CS + polyvinyl pyrrolidone	GA	Controlled release of amoxicillin	Risbud et al. (2000)
CS + gelatin	GA	Microsphere for nasal delivery of propranolol HCl	Dandangi et al. (2007)
CS + glycine	GA	Oral controlled release of chlorpheniramine	Gupta and Ravi-Kumar (2000)
CS + poly (N-isopropylacrylamide)	N,N′-methylene-bis-acrylamide	Oral pH, temperature sensitive release of cAMP	Chen et al. (2010)
CS + acrylamide-g-poly(vinyl alcohol)	Ceric ammonium nitrate	Oral controlled release of cefadroxil	Rao et al. (2006)
CS + poly(acrylic acid-co-acrylamide)	N,N′-methylene-bis-acrylamide	Superporous hydrogel for oral delivery of insulin	Yin et al. (2008)

(continued)

Table 9.1 (continued)

Polymers	Cross-linkers	Biomedical applications	References
CS + poly(acrylic acid)	GA	Controlled release of amoxicillin	Ekici and Saraydin (2007)
CS + poly(aniline)	GA	Biosensor film	Kim et al. (2005)
CS + dextran-g-acryl amide	GA	Oral controlled release of theophylline	Ahmed et al. (2009)
CS + polyacrylamide grafted guar gum	GA	Microsphere for delivery of ciprofloxacin	Kajjari et al. (2011)
CS+ poly (2-hydroxyethylmethacrylate)	UV radiation	Adsorption of lysozyme	Bayramogˇlu and Arıca (2002)
CS+ poly(*N*-isopropylacrylamide)	Formaldehyde	Temperature sensitive hydrogel	Wang et al. (2001)
CS+ polyacrylamide	N,N′-methylene-bis-acrylamide	Immobilization of redox protein hemoglobin (Hb)	Zeng et al. (2007)
CS+ aspen hemicellulose	GA	Cross-link density and swelling properties of hydrogels can be well controlled	Karaaslan et al. (2012)

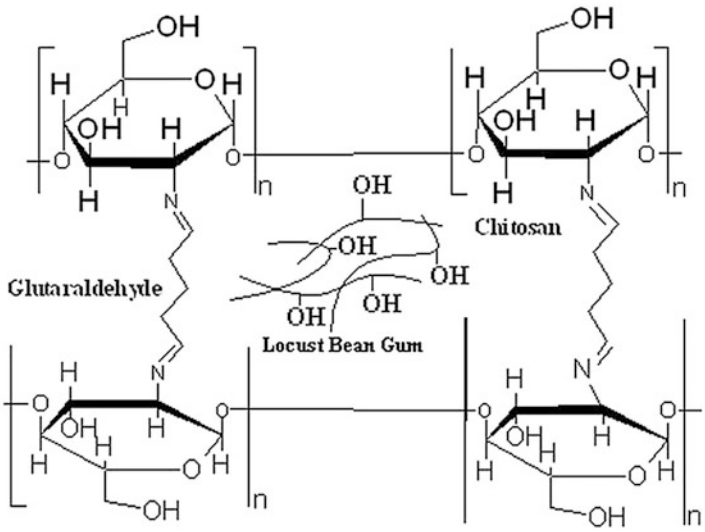

Fig. 9.3 The reaction mechanism of GA cross-link CS-LBG IPN. (Reprinted from Jana and Sen (2017). Copyright (2017), with permission from Elsevier)

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Chitosan-Based Systems in Tissue Engineering

10

M. Azeera, S. Vaidevi, J. Kumar, A. Shanmugarathinam, and K. Ruckmani

Abstract

Chitosan is a unique, naturally obtained polysaccharide derived from chitin. As a biomaterial, it attains immense interest for tissue engineering due to its biocompatibility and biodegradation which also exhibits desired properties such as bactericidal, fungicidal, and enhanced immune activity. Chitosan-based materials are mainly focused on fabricating scaffolds for tissue engineering. The ideal features of scaffolds are three-dimensional, highly porous, biocompatible, and bioresorbable, with cell attachment, proliferation, and implantation. Especially, these scaffolds have distinctive advantages, such as preservation of cellular binding and bioactive factor enrichment. This chapter highlights chitosan-based scaffolds and its composites having higher potential to be employed in bone, cartilage, liver, nerve, and musculoskeletal tissue engineering.

Keywords

Chitosan · Tissue engineering · Biomaterials and scaffolds

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_10

10.1 Introduction

In advanced research, tissue engineering is a promising multidisciplinary field that applies the principles of engineering and life sciences in turn to create living replacement parts for the body (Cigdem Arca and Senel 2008a). It uses synthetic or natural derivative, or engineered, biomaterials to restore damaged or defective tissues and organs. In tissue engineering, manipulation of scaffolds with artificial composition is capable of exciting cellular progress in their growth and development, proliferation, and differentiation. Every year, millions of people suffer from tissue injury and organ failure worldwide. The aim of tissue engineering is to develop the quality of life of millions of people by improving the tissue and the functions of organs (Cigdem Arca and Senel 2008b).

The main function of scaffolds is to provide mechanical integrity at the injured site until the damaged tissue gets repaired or regenerated, and the normal biomechanical role that needs to be restored is mentioned in Fig. 10.1. Once the function is fulfilled, the scaffolds should meet any particular requirement (Wu et al. 2014; Langer 2000). The platform should have mechanical properties that are compatible with the anatomical area and it must sufficiently accept implantation. In tissue engineering, the appropriate physical and mechanical properties initiating the production of scaffolds are one of the major criteria (Drury and Mooney 2003).

Currently, based on chitosan a range of hybrid scaffolds and further biodegradable as well as biocompatible materials have been developed. These hybrid scaffolds

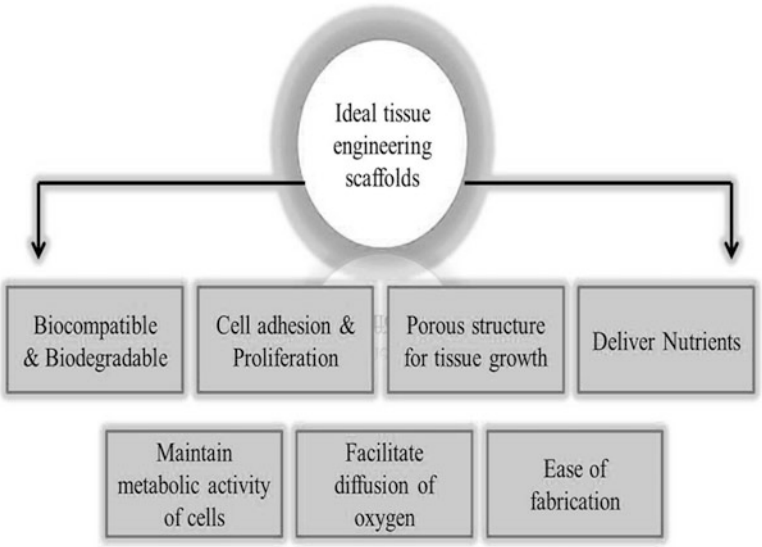

Fig. 10.1 Ideal characteristics of scaffolds for tissue engineering and tissue regeneration applications

with enhanced microstructure, swelling, mechanical support, pressure modulus, and biocompatibility are due to the property of chitosan. The materials used for the preparation of hybrid scaffolds contribute a vital role in the physicochemical properties and applications of the platform in tissue engineering (Hu et al. 2014; Leong and Cheah 2003; Liu et al. 2007). The main objective of this chapter is to strengthen the recent advancements of chitosan-based biomaterials as scaffolds for bone, liver, cartilage, nerve, and musculoskeletal tissue repair or regeneration, and its flowchart is shown in Fig. 10.4.

10.2 Chitosan: Structure and Extraction

Chitosan is characterized by three functional groups consisting of an amino and acetamido group and the hydroxyl groups in primary and secondary formation, which are reactive to chemical variation (O'Brien 2011; Raymund 2012). Chitosan is a linear, semicrystalline polysaccharide composed of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan(N-acetyl D-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-D-glucan (D-glucosamine) units (Kurita 2006). The structure of this polymer is represented in Fig. 10.2. The deacetylation method introduces a cationic charge to the polymer and enhances the interactions with negatively charged cytokines, growth factors, and phosphate groups in LPS (Khor and Lim 2003; Jayakumar et al. 2011).

In fact, the affinity for endotoxins is very strong and the removal of endotoxin contamination is quite possible by cross-linked chitosan microfiltration membranes (Rinaudo 2006). Moreover, the cationic nature of chitosan is the basis for its enhanced solubility in dilute aqueous acids, which is opposed to the insolubility of chitin, and versatile use in tissue engineering applications (Minke and Blackwell 1978). With chitosan as a heterogeneous polymer, its properties strongly depend upon the number of charged groups, molecular weight or its distribution (polydispersity index), and the consecutive order of acetylated and deacetylated residues in the chain (Minagawa et al. 2007).

In general, < 50% N-acetyl-glucosamine units were present in chitosan, and also there is no clear definition of degree of deacetylation (DD) (Lee et al. 2008). Apart from the general properties, the derivatives of chitin enhance the chitosan key properties such as bactericidal, fungicidal, and also the immune activity (Suzuki

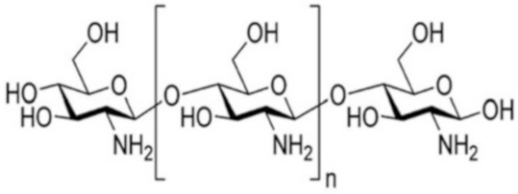

Fig. 10.2 Chemical structure of chitosan

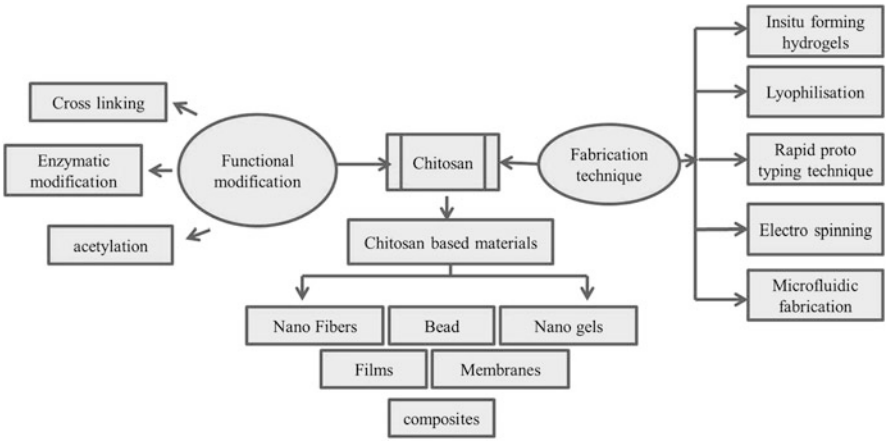

Fig. 10.3 Flowchart representing the different functional modifications and fabrication techniques for chitosan-based materials for tissue engineering applications

et al. 1984). Bactericidal activity depends on the cationic charge which has higher affinity toward the bacterial cell wall; microbial nutrient flow gets inhibited and cytoplasmic component release gets mediated (Krajewska 2004). The immune response of chitosan can be enhanced by cytokine secretion in fibroblasts and macrophages and immune cell migration, attracting polymorphonuclear cells in the process of tissue regeneration (Zhang et al. 2010; Harish Prashanth and Tharanathan 2007). In regeneration process, by attracting neutrophils and macrophages, chitosan exerts optimistic effects in wound healing and skin regeneration (Chung and Chen 2008; Kim et al. 2008). As an analgesic, chitosan may heal the wound without the formation of scar tissue (Cui and Mumper 2001; Zhao and Chang 2004). The different techniques and functional modifications for chitosan based materilas were shown in Fig. 10.3. As an excellent polymer, chitosan has wide applications in various fields such as tissue engineering, agriculture, food industry, wastewater treatment, and genetic engineering (Barnes et al. 2007a). In bone regeneration, based on tissue engineering approaches, in vitro and in vivo osteogenesis in chitosan enhances cell attachment and is helpful in the formation of natural ECM (Gomes et al. 2002). In genetic engineering, chitosan plays a vital role in the delivery of DNA, thereby providing a replacement for traditional viral genomic transfer systems (Zhang et al. 2006). Lastly, chitosan can also be used as one of the raw materials for the production of low molecular weight, water-soluble chitosan, and highly deacetylated ChOS preparations (Liu et al. 2007) (Fig. 10.4).

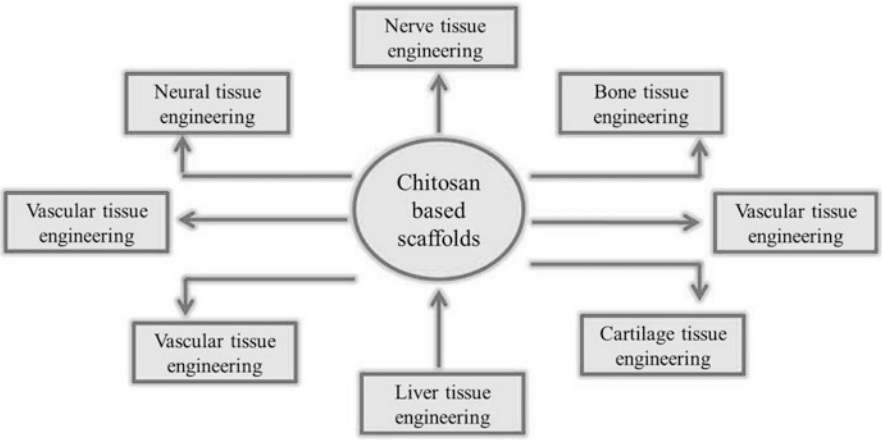

Fig. 10.4 Flowchart indicating the various tissue engineering applications of chitosan-based materials

10.3 Bone Tissue Engineering

Bone tissue engineering is an excellent field of research for developing new three-dimensional (3D) scaffolds with interconnected porous structure. These scaffolds should meet the characteristics of the tissue which is going to be replaced. Tissue engineering scaffolds should be biocompatible, osteoconductive/inductive, and mechanically suitable to restore bone which has been lost or damaged. In this aspect, artificial scaffolds based upon chitosan, organic/inorganic materials, and synthetic polymers have received greater impact in recent years due to their desirable properties for bone tissue engineering application (Zhu et al. 2015; Burg et al. 2000). Ceramics such as calcium phosphate (biphasic) (CAP), hydroxyapatite (HAP), and tricalcium phosphate (TCP) are widely used in scaffold fabrication due to their structural resemblance with the mineral components of human bone. Bioactive glass ceramics (BGCs), zirconium oxide (ZrO_2), titania (Ti), and silicon dioxide (SiO_2) are some other ceramics employed in BTE (Rezwan et al. 2006). Both natural and synthetic polymers such as poly(L-lactic acid) (PLLA) and polycaprolactone (PCL) have been studied for their applications as scaffolding materials (Rezwan et al. 2006; Hench 1991). Natural polymer-based composite materials are attaining more attention for their use in BTE. Chitosan, collagen (Col), alginate (Alg), silk fibroin (SF), and glycosaminoglycans (GAGs) were mostly used as natural polymers (Maria and Jose Maria 2004; Padilla et al. 2007; Jones et al. 2005).

A special shish kebab structure was prepared by Jing et al., using electrospinning and controlled copolymer crystallization. PCL–SK (CS–PCL 8.8) scaffolds were the

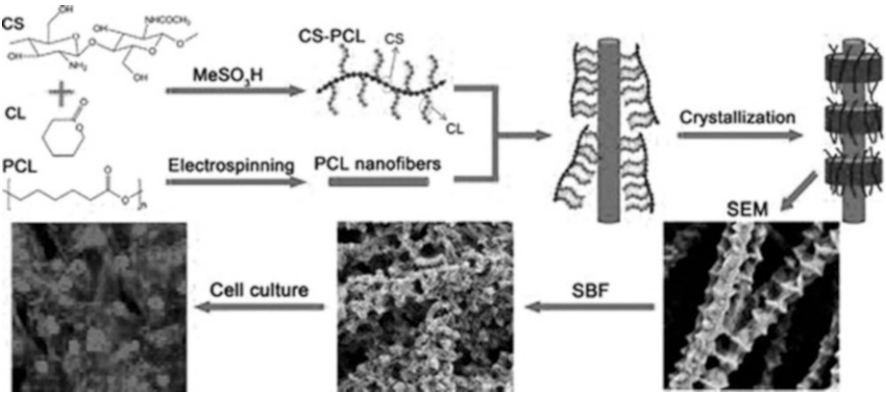

Fig. 10.5 Schematic of shish kebab-structured scaffold preparation using chitosan–polycaprolactone copolymers. (Reprinted with permission from Jing et al. 2015; Copyright © 2015 American Chemical Society)

most promising for cell growth, with enhanced cell attachment, higher cell viability, and good interaction with other scaffolds, as well as a better ability to form bones. The biocompatibility of natural biopolymers was also introduced, as shown in Fig. 10.5 (Jing et al. 2015).

10.3.1 Chitosan–Synthetic Polymer Hybrid Scaffolds

PLLA–chitosan hybrid scaffolds were developed using chitosan solution and prepared PLLA scaffolds that were used for bone tissue engineering. The size and shape of hybrid scaffold microstructures were based on the concentration of chitosan solution in which the PLLA scaffolds are soaked (Mano et al. 2008). It showed that PLLA–chitosan hybrid scaffolds are able to produce calcium phosphate precursors in its structure by dipping them into another phosphorous and calcium solution. Bioactivity tests were performed to observe the formation of apatite layers inside the hybrid scaffolds. Santo et al. (2010) prepared a hybrid scaffold based on poly(D, L-lactic acid) (PDLLA) permeated with chitosan/chondroitin sulfate nanoparticles (NPs) with the drug release profile. These hybrid scaffolds result in higher swelling character and appropriate mechanical properties for cell adhesion and support bone tissue engineering due to the chitosan or chondroitin sulfate NPs. For thermally induced stage separation and lyophilization, hybrid scaffolds based on chitosan and PDLLA *co*-glycolide were also developed for bone tissue engineering and its applications (Martel-Estrada et al. 2011). FTIR and FE-SEM studies confirmed the formation of apatite layers on the hybrid scaffolds after being saturated with stimulated body fluid (SBF). These studies showed the potential and impact of chitosan-based hybrid scaffolds in bone tissue engineering. Niu et al. (2009) reported the properties of chitosan microsphere-loaded porous PLLA scaffolds as a better carrier for BMP-2-derived synthetic peptide.

In FTIR, the results showed a strong hydrogen bond between PLLA and chitosan component. While the chitosan microsphere contents were increased from 0% to 50%, the strength of the PLLA scaffolds increased from 0.48 to 0.66 MPa, and at the same time the modulus also increased from 7.29 to 8.23 MPa. Chitosan microsphere insertion into PLLA scaffolds was initiated to neutralize the acidity of PLLA degradation products. Release studies reveal that PLLA–chitosan hybrid scaffolds show controlled release of loaded peptide when compared with the control (chitosan microspheres). The release pattern showed that it depends on the degradation of the PLLA matrix. The above result indicates PLLA–chitosan scaffolds can also be used to deliver bioactive factors in a range of non-loaded bone regeneration. Santo et al. (2010) developed hybrid scaffolds based on PDLLA consisting of chitosan–chondroitin sulfate NPs loaded with platelet lysate (PL) through the supercritical fluid-foaming technique. Release studies showed that PL has been released in a sustained behavior from the hybrid scaffolds. Due to PL and hASCs, these hybrid scaffolds have been used as multifunctional materials for bone tissue engineering applications. L-Lactide-methoxy PEG-tetrandrine nanospheres loaded with chitosan–gelatin hybrid scaffolds were prepared through the freeze-drying method for bone tissue engineering (Xiaoyan et al. 2008). As a plant derivative tetrandrine, this can also be used as a modifier for hybrid scaffolds to promote chondrocyte differentiation and secrete type II collagen. Since tetrandrine-loaded nanospheres are found inside the chitosan–gelatin scaffolds, there is sustained release of tetrandrine from the hybrid scaffolds.

10.3.2 Chitosan–Calcium Phosphate Hybrid Scaffolds

Calcium phosphate is an excellent platform for bone repair and regeneration using biomaterials even though the chemical compositions have similar characteristic of the inorganic components of bone (Hench 1998). It is essential to develop a composite material with favorable properties of chitosan and calcium phosphates. Here the studies were carried out with β-tricalcium phosphate (β-TCP), and calcium phosphate invert glasses ($CaO/P_2O_5 \approx 2.0$) were used for powder fillers to strengthen the chitosan scaffold. The composite scaffolds exhibit morphological microstructures which are examined by an environmental scanning electron microscope (ESEM). Compression testing has been performed to evaluate the elastic modulus and yield strength of the scaffolds with various ratios of calcium phosphates to chitosan.

Calcium phosphates also induce the initial adsorption of serum proteins when compared with pure polymers with hydrophobic surfaces. Therefore, it is essential to develop and to study the calcium phosphate–chitosan composite scaffolds which are suitable for bone repair and regeneration. Preparation of calcium phosphate–chitosan composite scaffolds and their uses in bone tissue engineering and drug delivery has been reported. The calcium phosphate powders were incorporated into macroporous chitosan matrices for the preparation of composite scaffolds. It showed that these composite scaffolds have excellent bioactivity and biodegradability and significantly

improved the tensile strength and Young's modulus (Zhang and Santos 2000). The scaffolds were suitable for repairing cartilage defects, wound healing, and drug delivery, but not suitable for load-bearing orthopedic implants, because the calcium phosphate powders were incorporated physically and interaction between the organic and inorganic phases was weak. Furthermore, to increase the mechanical strength of the chitosan composite scaffolds, a novel scaffold has been developed, namely, three-dimensional macroporous calcium phosphate embedded with bioceramics and porous sponge biopolymer (Sundararajan and Howard 1999). The scaffolds produced showed higher mechanical strength, biocompatibility, bioactivity, and higher surface area-to-volume ratio.

10.3.3 Chitosan–Bioactive Glass Hybrid Scaffolds

Chitosan induces wound healing and also has bacteriostatic effects (Cascone et al. 2001). Chitosan combination with synthetic polymers that exhibit a rigid effect to improve the mechanical properties of the produced materials that are less soluble in water is also examined (Liu et al. 2004). Chitosan is efficient, and its production is less expensive and ecologically remarkable (Cascone et al. 2001; Liu et al. 2004; Bergera et al. 2004; Berger et al. 2004). Bioactive ceramics have been largely used due to their properties of hosting the bone cells and promoting the formation of a continuous bone–ceramic interface, thereby permitting an implant fixation mechanism. Bioceramics usually have mechanical properties which are quite different from those of natural tissues, particularly a high expandable modulus and low toughness. Bioactive glasses are essential bioceramic materials and have been utilized for the repair and reconstruction of diseased bone tissues (Rezwan et al. 2006). This bioactive glass promotes the formation of bone tissue on their surface that will bond to the surrounding tissues when it is implanted in the living body. A common feature of bioactive materials is the formation of an apatite-like layer on the surface when they come in contact with physiological fluids/solutions that mimic the human plasma (Rezwan et al. 2006; Hench 1991). On the other hand, bioactive glasses typically have low mechanical properties, particularly in a porous form when compared to cortical as well as cancerous bone (Rezwan et al. 2006; Hench 1991; Maria and Jose Maria 2004; Padilla et al. 2007). Thus, the information confines the use of these materials in various applications. Another alternative is being considered and studies the composites and hybrid production systems. Therefore, the expansion of organic–inorganic (O–I) hybrids was considered as a promising approach for the preparation of bioactive scaffolds (Montserrat et al. 2006; Chiellini et al. 2003; Pereira et al. 2000).

Composites that include bioactive glass phase of synthetic and biological polymers have higher potential capability of connecting bioactive behavior with appropriate mechanical properties. In particular, based upon the biodegradable polymers, the composite materials were associated with inorganic bioactive glasses, especially enhanced to engineer the scaffolds as it can offer a good balance between toughness and strength that also progress the mechanical properties when compared

with individual components (Jones et al. 2005; Pereira et al. 2005; Montserrat et al. 2006; Chiellini et al. 2003). These compounds were synthesized through a hybridization method, whereas two different components, that is, organic and inorganic, are combined together. Sol–gel allows the inclusion of polymers of various state changes into inorganic silica, thereby producing organic–inorganic hybrid biomaterials (Jones et al. 2005; Pereira et al. 2005; Montserrat et al. 2006). Depending on the strength of the interaction between the two phases, the hybrids can be divided into two classes. The first is weak bound components including hydrogen or van der Waals interactions, and the second one is differentiated by strong covalent bonds (Sanchez and Ribot 1994). Another characteristic of fundamental importance in designing materials for bone engineering is related with porous and pore structure of the delivery system. Hence, special attention has been paid for the synthesis of composites and porous bioceramic structure to allow growth of bone tissue (Maria and Jose Maria 2004; Jones et al. 2005; Padilla et al. 2007; Pereira et al. 2005). The suitable porosity, pore size distribution, and also the bioactivity support the adhesion, nucleation, and thereby growth of bone tissue specifically achieving complete integration with living bones. The chemical composition of scaffold tissue engineering is highly crucial for resorbability, osteoinductivity, and osteoconductivity with interconnected porous structure along with internal pore structure for vascular growth (Maria and Jose Maria 2004; Montserrat et al. 2006; Willi and Chandra 2006). Apart from engineering the bone tissue design, the biomaterials also support extensive loading and tensile/torsion stresses, provide the mechanical property compatible with natural bone tissue (Hench 1991; Maria and Jose Maria 2004; Padilla et al. 2007). Lastly, it also induces cell anchorage and reassembles the extracellular matrix that integrates with the surrounding tissue. Therefore, in spite of the complexity of the system, this challenge should be overcome in order to attain a proper replacement of bone tissues using synthetic materials (Maria 2006).

10.3.4 Chitosan–Hydroxyapatite Hybrid Scaffolds

Bone has been considered as a natural nanocomposite that contains hydroxyapatite nanocrystallite in a collagen-rich environment enclosed with non-collagen proteins. Alongside, it provides a structural support and the bone serves as a reservoir of calcium phosphate ions which are involved in abundant metabolic functions. The significant role of bone in humans and appropriate synthetic hybrid materials were used for various biomedical applications like injured bone regeneration, in an effort to mimic its structure and composition. Among various synthetic materials for bone grafting, nanocomposites are restored instead of polymers, metals, ceramics, and composites because of their advantageous properties such as, large surface area, strong bonding, improved mechanical performance, and flexibility. In mechanical properties, mandatory necessities for the bone substitutes must include biocompatibility, porous nature with interconnected pores to permit nutrient transport and also metabolic waste disposal, biodegradability, and a temporary scaffold for the generation of new tissues (Thomas et al. 2006; Karageorgiou and Kaplan 2005). An ideal

bone substitute must possess a macrostructure similar to natural bone in terms of biochemistry and micro−/nanoscale surface topography that can formulate favorable binding sites to regulate and control cellular tissue (Hollister 2005; Ruiz et al. 2008). Along with various biodegradable polymers, chitosan is receiving attention for its interesting features such as bacteriostatic/bactericidal properties, biocompatibility with human tissue, biodegradability, growth regulation, and osteoconductivity. A serious negative aspect of chitosan scaffolds is its extremely low mechanical strength, making it unsuitable for load-bearing and orthopedic applications. A necessary step is to increase the mechanical strength to enhance biological properties for the development of novel synthetic chitosan-based composites/hybrids (Pek et al. 2008) with the aim of developing a three-dimensional bioactive support suitable for matching the mechanical properties of bone, that is, high strength and low elastic modulus. Hydroxyapatite (HAP) and its derivatives were extensively studied and used to strengthen ECM-like polymer scaffolds because of its great similarity to the mineral components of bone (Kane and Roeder 2012; Jelen et al. 2013; Murugan and Ramakrishna 2005).

Moreover, the porous structure, volume fraction, shape, and dimension of HAP particles strongly induce the mechanical properties of composite scaffolds. Additionally, it has been revealed that nanocrystalline HAP (nHAP) showed better results than microcrystalline HAP in adhesion, proliferation, and differentiation, as well as biomineralization (Murugan and Ramakrishna 2005; Dorozhkin and Epple 2002). Pek et al. (2008) successfully prepared the collagen nanoapatite composite by freeze-drying the collagen slurry with carbonated apatite (CAP) and HAP nanocrystals with inorganic component. Scaffolds with an optimized CAP and HAP nanocrystal mixture resulted in superior compressive stiffness (37.3 ± 2.2 MPa) that yields strength (2.7 ± 0.1 MPa), because of the perfect matching of the HAP and CAP nanocrystals with the apatite structure and crystallite size of bone trabecular. In fact, a higher volume fraction of grain boundaries which is associated with nanocrystalline apatite also strengthens the matrix more than conventional coarse-grained HAP crystals (Pek et al. 2008). Herein, a composite scaffold acts as a candidate for bone tissue engineering applications, by approaching freeze-drying homogeneous dispersion of chitosan and HAP precursors. Thus, a chitosan is acid soluble, while HAP forms in the solution with pH > 7, with respect to achieving homogeneous nano-hydroxyapatite or chitosan composite scaffolds.

10.4 Cartilage Tissue Engineering

Cartilage is an avascular tissue containing chondrocytes and has a self-repairing capacity even after injury (Hunter 1743; Buckwalter and Mankin 1997, 1998). The cartilage chondrocytes are responsible for the synthesis and also maintenance of the extracellular matrix (ECM), which is composed of a hydrated collagen network (~65 wt.% of tissue dry weight, dw) and then highly charged proteoglycan gel (~25 wt.% dw), proteins, and glycoproteins (~15 wt.% dw) (Grigolo et al. 2001). The success of cartilage tissue engineering is based on the selection of scaffold

fabrication. Synthetic polymers like poly(L-lactide) (PLLA) and poly(D,L-lactide-co-glycolide) (PLGA) (Hsu et al. 2002; Kawanishi et al. 2004) are used to prepare scaffolds for cartilage regeneration. Natural polymers like chitosan (Lin et al. 2007), hyaluronic acid (Zhao et al. 2012), collagen (Pieper et al. 2002), alginate (Barnes et al. 2007b), and demineralized bone matrix are also used for scaffolds because of their good biocompatibility. One disadvantage of natural polymers is their lower mechanical properties. It is reported that many synthetic polymers support cell growth in the dedifferentiated state leading to tissue deposition, where the natural polymers support the cellular growth as well as the regeneration of injured tissues (Angele et al. 2004). Collagen is considered as a good template for tissue remodeling (Pieper et al. 2002; Barnes et al. 2007b; Kim et al. 2011). Chitosan is obtained from the deacetylation of chitin. The positive charges may also protect glycosaminoglycans (GAGs) from hydrolysis (Barnes et al. 2007b; Kim et al. 2011; Hodde 2002; Sechriest et al. 2000). Chitosan was reported to induce cartilage growth and repair (Hodde 2002; Sechriest et al. 2000). Chondrocytes were found to grow slowly on the chitosan surface because of positive charge (Foda et al. 2007). In order to gain suitable biomaterials for tissue regeneration, combinations of different materials were employed in tissue engineering. Even though type B gelatin is negatively charged, the combination of gelatin and chitosan formed a polyelectrolyte complex (PEC) due to the ionic interaction between the two molecules (Sechriest et al. 2000; Foda et al. 2007; Kuo and Wang 2010). This results in gel formation and an improvement of its mechanical properties. Therefore, chitosan–gelatin complexes were used in drug delivery. Chitosan–gelatin scaffolds were seeded with chondrocytes to promote growth of the elastic cartilage in vivo (Kuo and Wang 2010).

Choi et al. developed injectable hydrogels using methacrylated glycol chitosan (MeGC) and riboflavin (RF) as an aqueous initiator that allows gelation in situ upon exposure to visible blue light (VBL). Cartilaginous extracellular matrix (ECM) components such as type II collagen (Col II) and chondroitin sulfate (CS) play a crucial role in chondrogenesis. Direct usage of both components is limited due to instability and rapid enzymatic degradation. Further, the incorporation of native Col II or CS into chitosan hydrogels thereby increases chondrogenesis and enhances cellular condensation, as shown in Fig. 10.6. This process was mediated by the binding of Col II to integrin-α10 and increased the cell matrix adhesion. These findings exhibit the potential benefit of cartilage ECM-modified chitosan hydrogels to promote cartilage regeneration (Choi et al. 2014).

10.4.1 Chitosan-Based Fibrous Scaffolds

Chitosan is recognized by its degree of deacetylation (DD), the percentage measurement of free amine groups along with the chitosan backbone. Generally, the material is considered as chitosan when it becomes soluble in dilute acidic solutions, while DD is 60%. Because of its solubility, chitosan favors chitin for a wide range of applications. Chitin and chitosan were biocompatible, biodegradable, nontoxic,

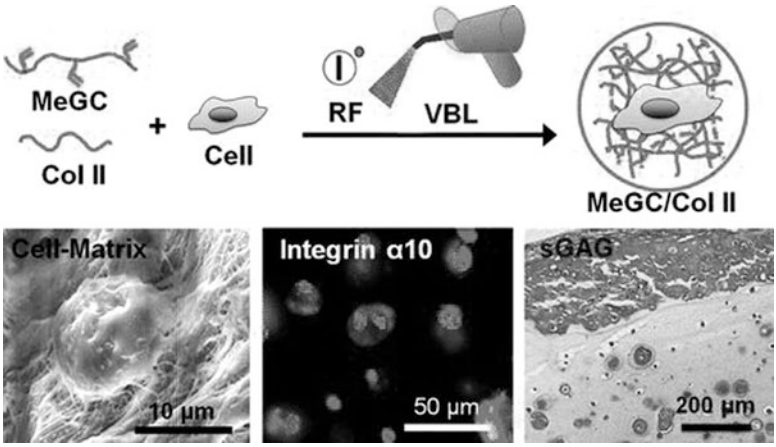

Fig. 10.6 Injectable hydrogels were synthesized using photopolymerizable chitosan and riboflavin (RF) as an aqueous initiator that allows gelation in situ upon exposure to visible blue light (VBL). (Reprinted with permission from Choi et al. 2014; Copyright © 2014 American Chemical Society)

antimicrobial, and hydrating agents. Chitin and chitosan are easily prepared into gels, membranes, nanofibers, beads, microparticles, nanoparticles, scaffolds, and sponges. The number of promising applications of nanoscale thin films and fibers of chitin/chitosan was reported (Roberts 1992). Recently, much attention has been focused on electrospinning process as a unique technique; it can produce polymer nanofibers with diameter in the range of micrometers to nanometers, depending on the polymer used and the processing conditions. While electrospinning, a high voltage is applied to produce jets of the electrically charged polymer solution. These jets were dried to form nanofibers, which are assembled on a target as a nonwoven fabric. These nanofibers are used in various kinds of applications, because it has several properties such as high specific surface area and porosity. Nanofibrous nonwoven fibers, containing chitin or chitosan, yield potential applications in areas such as filtrations, drug release, dentistry, tissue engineering, enzyme carriers, wound healing, cosmetics, medical implants, and biosensors (Nagahama et al. 2008).

Among these, a polyglycolic acid (PGA) mesh has also been extensively studied and serve as the best reference to compare novel scaffolds. PGA is easily extracted into fibrous and open lattice sponges, allowing control of the structural characteristics of its matrix. Although this synthetic material is not biomimetic, clinical applications still remain limited due to biomechanical properties of the material. Chitosan has been attracted recently as a candidate for cartilage repair because of its excellent biocompatibility and structural similarity of naturally present glycosaminoglycans (GAG) in the extracellular matrix of cartilage. Chitosan is formed by alkaline deacetylation of chitin, and the second one is rich natural polysaccharide, primarily obtained as a subproduct of shellfish, such as crabs and shrimps. Significant attention has been consequently focused on chitosan-based

materials and their applications in the field of orthopedic tissue engineering. Chitosan has been molded into porous structures which sustain chondrogenesis. The physical properties of the 3D scaffold also affect the cellular behavior, especially in determining the performance of a tissue-engineered construct (Jayakumar et al. 2005). Although the structural characteristics of 3D matrices may influence cell proliferation and matrix production, more efforts have been focused on altering the chemical composition rather than the biomaterial structure examined for cartilage engineering. With chitosan as a biomaterial for tissue engineering, most studies on pure chitosan scaffolds have also been focused on sponges or hydrogel. Therefore, fibrous scaffolds are more attainable than sponges and films which stimulate the fibrous nature of the native cartilaginous extracellular matrix. The increased cell proliferation and GAG production were reported when chondrocytes were cultured on tiny fibers composed of poly(L-lactide) (Madhumathi et al. 2009).

10.4.2 Chitosan-Based Scaffolds

Chitosan is a linear polysaccharide similar in structure to glycosaminoglycans (GAGs) present in the native cartilage ECM. This property is really important for cartilage tissue engineering and permits the development of different types of scaffolds. Another advantage of chitosan is its ability to be molded into various shapes supporting the formation of different pore-sized structures. It has an intrinsic antibacterial activity with high biocompatibility. Chitosan has been used in various blends to produce different types of scaffold, like hydrogels, amino acid immobilization, and drug delivery (Wang and Hon 2003). It has been reported that novel blends of synthetic polymers with chitosan were developed which have been employed for biomedical applications. Studies have been demonstrated in preparing chitosan-based scaffolds expected for cartilage regeneration, which shows the evidence of favorable responses in vitro (Pillai et al. 2009).

While developing cartilage-specific extracellular matrix, components such as collagen II and GAG play a vital role in regulating the expression of chondrocytic phenotype, promoting chondrogenesis both in vitro and in vivo. It is demonstrated that the chosen biomaterials should offer a biochemical and biomechanical environment for cellular regeneration of the hyaline cartilage at the injured site in the long term. For example, the hydrogel containing chitosan and hyaluronan with the entrapped chondrocytes and meniscal cells, with a mechanical property of 15% and tensile strain 0.5 Hz for 10 min/day continued for 43 days, resulted in substantial GAG increase and collagen production (all >31%) over the static controls, and it does not significantly affect the proliferation and viability of cells (Marimuthu and Kim 2009).

The natural materials that were suitable for the preparation of scaffold include proteins such as collagen, keratin, fibroin, and elastin and polysaccharides such as chitosan, hyaluronan, and alginate. Once these materials were processed into scaffolds, it imparts features in the pore archi tecture, namely, pore size, shape, and distribution; elasticity, including time-dependent deformation, and modulus;

and surface energetic factors such as balance between hydrophobic–hydrophilic and molecular mobility. Moreover, induced chemical activity; environmental response concerning pH, stress, and temperature; the surface micro- and nano-topography; biodegradation method; and metabolism of biodegradation products are all very important features. In vivo, the cell–tissue constructs were exposed to harsh conditions of a synovial joint, which is subjected to hypoxia, and the synovial fluid. Further, the latter is an ultrafiltrate from the blood plasma without fibrinogen, supplemented products from the synovial lining cells, and chondrocytes. The main difference is the presence of ca. 2 mg/ml hyaluronan and a protein content as low as ca. 30 mg/ml. When compared with blood plasma, the composite mixture of growth factors and cytokines was found within the synovial fluid that stimulates or suppresses the chondrogenic and antichondrogenic actions. It is one of the best tools in cartilage repair; thereby cells were obtained from the patient, seeded on a scaffold, and implanted at the place of a cartilage defect (Egli et al. 2011).

10.4.3 Chitosan-Based Composite Scaffolds

Cartilage tissue plays an important role in human health and usual lifestyle. However, these tissues can be injured by many issues, such as natural degradation, trauma, and degenerative disease. The poor blood supply to the cartilage and the low mitotic ability of chondrocytes result in low self-repair ability of the articular cartilage (AC). It is necessary to assist a method to repair the damaged cartilage tissues with few side effects. Due to the special structure and properties of cartilage tissues, effective methods for repair of defect have to be established. Present autologous or allograft implantation methods have few drawbacks, including lack of material source, immunological rejection, long recovery time, and cross-infection with donors. To overcome these issues, cartilage tissue engineering uses chondrocytes, in the injury site and to stimulate their growth and differentiation, finally degrading in respect to matrix remodeling enzymes, which get released as tissue repair progresses. Cartilage tissue engineering can create a more durable and functional replacement of degenerated tissue in vivo. Furthermore, the regenerated tissue survives in mechanical joint conditions. As a promising biomaterial, chitosan has wonderful bioactivities, including nontoxicity, antimicrobial activities, biocompatibility, biodegradability, and superior physical properties (Cheung et al. 2015). The application of CS has been considered in various applications, such as wound healing, tissue engineering, and drug delivery. CS can also be processed in a broad range, including gels, fibers, films, and sponges. Especially, the hydroxyl groups of chitosan can be easily complexed with other materials. The tissue engineering approach is used for repair and regeneration which is based on the polymer scaffolds that support, reinforce, and, in some cases, organize the regenerating tissue.15 Recently, different materials for cartilage tissue engineering have been developed, such as chitosan, collagen, bioceramics, calcium phosphate, and hydroxyapatite (Huang et al. 2009). Natural or its derived polymers belong to the natural living body. The most important advantages of natural polymers are biocompatibility, and

their biodegradable products were nontoxic even when it is implanted. Some natural polymers also possess the ability to trigger gene expression. The sources of natural polymers are very limited and usually require some complex postprocessing. The synthetic polymers are preferable for their high porosity towards chondrocyte, mechanical properties, cartilage tissue growth, and adjustable properties. However, biological compatibility is one of the weaknesses of synthetic polymers when applied to cartilage tissue engineering. Furthermore, bioceramics exhibit excellent physical properties, but its fragility and degradation properties are limited in AC defect repair. Much effort has been made to develop scaffolds with porous interconnected network structures which were well developed with many advantages.

Different types of chitosan composite scaffold are also studied for its AC defect repair. Man et al. (2016) studied allogenic chondrocytes with chitosan hydrogel (CS)–demineralized bone matrix (DBM) hybrid scaffold (CS/DBM) which repairs rabbit cartilage injury. The scaffold was also prepared by inserting DBM into CS hydrogel solution by incubation at 37° C for 10–15 min. The CS, DBM, and CS/DBM scaffolds were studied in vivo to repair rabbit cartilage injury and the mechanical properties of repaired tissues. Microfracture (MF) is a common treatment for cartilage defect. The hardness and elastic modulus of the repair tissue were enhanced for CS/DBM compared with CS and DBM alone, which results in tissue repair by improving its mechanical stability. Furthermore, Shivaprasad et al. (Manchineella et al. 2016) successfully developed biocompatible pristine and melanin composite silk fibroin scaffolds with antioxidant and electroactive properties. Both antioxidant and electrical conductivity functions of composite scaffolds supported the proliferation and induced differentiation of cells. It is studying the effects of melanin on chitosan or chitosan or silk fibroin composite scaffolds discussed above in the field of cartilage tissue engineering. Scaffolds composed of three or more phases have also been studied. Huaping Tan et al. (2009) have prepared gelatin/chitosan/hyaluronan scaffolds for cartilage tissue engineering in various studies.

10.5 Liver Tissue Engineering

Tissue engineering is employed to repair, replace, maintain, or enhance the function of a particular tissue or organ. The basic requirement for designing polymeric scaffolds includes high scaffold porosity with appropriate pore size distribution, high surface area with a rate of degradation matching to neotissue formation which is required for structural integrity to prevent the pores from collapsing during neotissue formation, biocompatibility, nontoxicity, promotion of adhesion, migration, proliferation, and differentiation as well as other cellular functions (Jayakumar et al. 2010).

10.5.1 Chitosan–Collagen Matrices

Chitosan has distinct features such as low toxicity, non-immunogenic, and biodegradable, which enable a great interest toward its biomedical applications. Chitosan is a positively charged biopolymer that can interact with structural molecules present in the extracellular matrix. Blending collagen with natural polymers can enhance the performance of collagen scaffolds.

Similarly, synthetic polymers were blended with collagen and used as scaffold with optimal mechanical and biological properties for specific tissue engineering applications. Herein, the synthetic polymer affords mechanical support to the structure of scaffolds, while collagen present in the surface and inside of the scaffolds provides cell recognition signals to promote cellular behavior and development. Therefore, a collagen–chitosan scaffold with homogeneous structure can be fabricated by polyelectrolyte complexation through blending of anionic collagen and cationic chitosan. Further, the relationships between the component ratio or cross-linking methods and stiffness, swelling, degradation, and cytotoxicity of the collagen–chitosan scaffold were also studied (Martinez et al. 2015).

10.5.2 Chitosan-Based Microfibers

Microfluidic fabrication was utilized to synthesize pure chitosan microfibers and characterize their mechanical, chemical, and diffusion properties and evaluate its capability for liver tissue formation. Further, HepG2 cells were cultured on chitosan-based microfibers showing a higher liver-specific function that was confirmed by albumin secretion and urea synthesis (Lee et al. 2010).

10.6 Nerve Tissue Engineering

Nerve defects play a major role in movement disorders of related muscles, bringing physical and psychological problems to the patients. Mature neurons have little capacity for replication, and if the nervous system is impaired, it can hardly heal itself. It has been proven that chitosan is a potential candidate for nerve tissue engineering due to good nerve affinity towards different cell types, that is, neural stem cells, PC12 cells, neuro-2a neuroblastoma cells, or N1E-115 cells, derived from mouse neuroblastoma C1300.

10.6.1 Chitosan-Based Membranes

Porous conductive chitosan/polyaniline/graphene (PAG) showing proper conductivity, mechanical properties, and biocompatibility may be used as scaffold for nerve tissue engineering. In vitro biodegradation studies confirmed that conductive scaffolds exhibited relatively slower degradation compared to pure chitosan/gelatin

scaffolds. In addition, in vitro cytotoxicity assay revealed that a huge number of attached Schwann cells were found on scaffolds with 2.5 wt. % PAG compared to other ratios of PAG-incorporated scaffolds (Baniasadi et al. 2015).

10.6.2 Chitosan-Based Hydrogels

A new method for fabrication of chitosan-based hydrogel implants intended for peripheral nervous tissue regeneration was developed using electrodeposition technique using a solution of chitosan and organic acid. It was proven that the structure of the implant, its dimensions, chemical composition, morphology, and mechanical properties can be tuned by changing the concentration of the chitosan solution. Here hydroxyapatite was employed to enrich the mechanical strength of the implant and served as a source for calcium ions. Moreover, the developed method allows controlling the calcium concentration, the essential component influencing axonal regeneration, within the structure of implants. The results of the physicochemical evaluation, proinflammatory assays, and in vitro biocompatibility testing showed good suitability of implants for their further application in nerve regeneration (Nawrotek et al. 2016).

Photo-cross-linkable chitosan hydrogels were developed and encapsulated with neural stem cells (NSCs), which enable the differentiation of NSCs into tubulin-positive neurons and astrocytes that proved to be a suitable scaffold for neural tissue engineering (Valmikinathan et al. 2012). Li et al. developed nerve growth factor (NGF)-loaded heparin/chitosan scaffolds via electrostatic interaction and confirmed its utility in nerve regeneration. Heparin immobilization, NGF loading, and NGF release quantitatively and qualitatively characterized in physicochemical properties including morphology, wettability, and composition were measured, respectively. The major findings showed that heparin immobilization and NGF loading did not cause a change in bulk properties of chitosan scaffolds except for morphology and wettability. The preimmobilization of heparin in chitosan scaffolds could enhance the stability of loaded NGF. The effect of NGF-loaded heparin/chitosan scaffolds on nerve regeneration was evaluated by culturing the Schwann cells for different periods, further proving that the developed scaffolds promote the morphology development of Schwann cells. The NGF-loaded heparin/chitosan scaffolds can obviously improve the attachment and proliferation of Schwann cells in vitro (Li et al. 2017).

10.7 Musculoskeletal Tissue Engineering

The musculoskeletal system includes bone, cartilage, and spine which are exciting tissue engineering targets as the need for their replacement is high. Bone is one of the most common transplanted tissues, next to blood. Cartilage has also been the focus of significant tissue engineering research for the last decade due to its lack of self-

repair ability when it is lost due to trauma, disease, or congenital abnormalities. Cartilage acts as a cushion and lubricating surface for proper joint function.

For musculoskeletal tissue engineering, scaffolds made out of silkworm and silk have been widely utilized for anterior cruciate ligaments, bone, and cartilage tissue engineering. Unlike many other scaffolds, a silk scaffold promotes cell attachment and cell spreading without any functional modifications. Silk possesses slow degradation kinetics and biocompatible properties compared to PGA and collagen. In addition, extracellular matrix components (ECM) such as hyaluronic acid (HA), chondroitin sulfate (CS), matrigel, and collagen-based hydrogels are used as scaffolds for cartilage and skin regeneration. Other naturally derived materials such as alginate, chitosan, fibrin, and agarose were used to synthesize hydrogels for tissue engineering applications.

Alginate has been widely used in a range of biomedical applications including drug delivery and cell transplantation. Alginate is oxidized to a low extent (5%), with a degradation rate depending on the pH and temperature of the solution. Alginate is ionically cross-linked with calcium ions to form gels, which are degraded within 9 days in phosphate buffer saline solution. Finally, the use of these degradable alginate-derived hydrogels greatly improved cartilage-like tissue formation in vivo, as compared to alginate hydrogels (Kamal et al. 2001). Collagen hydrogels are excellent candidates for musculoskeletal tissue engineering scaffolds because cells can easily adhere onto the gels, and the scaffold can provide appropriate biological signals to the cells. Both collagen I and collagen II are often employed as a scaffold for cartilage engineering.

10.8 Conclusion

Chitosan and its composites with organic, inorganic, and polymeric materials, a group of porous scaffolds, have been fabricated for both tissue engineering and regenerative medicine. These chitosan-based scaffolds are biocompatible and biodegradable, as an excellent platform which has desirable physicochemical and biological properties. Chitosan-based scaffolds received greater attention due to their potential activity as biomaterials for bone, cartilage, liver, nerve, and musculoskeletal tissue regeneration. Chitosan-based scaffolds require adequate stability and mechanical strength for efficient bone and cartilage tissue engineering applications. The above requirements can be fulfilled, by using different types of hybrid scaffolds based on chitosan and other biocompatible materials. These hybrid scaffolds have desirable properties such as highly porous structure with sufficient mechanical properties for cell adhesion to promote bone and cartilage tissue engineering. The bioactive chitosan-based hybrid scaffolds were used to deliver bioactive materials for cell migration, differentiation, and proliferation or drug molecules to enhance the therapeutic effects in tissue engineering approaches.

Acknowledgments The authors wish to acknowledge the DST-sponsored National Facility for Drug Development (VI-D&P/349/10-11/TDT/1), Nanomission program of the Department of Science and Technology (DST), Ministry of Science and Technology of India (DST/SR/NM/NS-19/2009), and Technical Education Quality Improvement Programme (TEQIP-PHASE II) for their support in this work.

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Chitosan-Based Nanoformulation as Carriers of Small Molecules for Tissue Regeneration

11

Shoba Narayan

Abstract

This chapter traces the growth of nanoformulations and the role of biopolymers in the field of medicine and nanomedicine in particular. Keeping in mind the vast literature available in this field, the chapter provides the reader an insight into the developments relating to use of one of the biopolymers, viz., chitosan. While highlighting the developments, extensive care is taken to provide an overall understanding of the subject while providing the literature support for detailed analysis. The chapter highlights the significance and facile maneuverability of chitosan to yield hydrogels and polyplexes that are ideally suited for tissue engineering applications. The chapter closes with the introduction to 3D printing technologies which are likely to take over the tissue regeneration field in a massive way.

Keywords

Chitosan · Nano-formulation · Tissue regeneration · Hydrogels · Polyplexes · Biopolymers

11.1 The Growth of Nanoformulations

Drug dosage given to a patient is determined from that concentration that can create a pharmacological action in the organ/tissue of concern, effectively and safely. The methodology employed for drug delivery through the ages has been determined from how best the target organ/tissue can be reached by the drug. It is a common belief that the therapeutic compound should be hydrophilic, should be available in a consistent dose at the site, and should have a predictable exposure at the site of

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_11

action. However, when the active component is partially hydrophilic, the drug dosage is increased, leading to side effects.

Further, even with hydrophilic therapeutic components, their release onto cells/organs other than the target site also causes undesired effects. A new range of products for targeted and sustained release is on the anvil. The advent of combination therapy, where an assembly of multiple drugs into a single platform enables synchronous therapy, has opened up the scope for nanoparticle-based formulations (Mu et al. 2018).

Nanoformulations can be used as imaging (magnetic, optical, photoacoustic) agents, drug carriers, therapeutics, and monitoring devices/sensors in healthcare. Till very recently, the focus of nanoformulations was more toward the delivery of the drug in a manner in which the toxicity of the incorporated drug is reduced (De Jong and Borm 2008). The use of nanoformulations as drug carriers has been based on advantages such as (a) increased exposure of diseased cells to therapeutic agents, (b) improved efficiency in treatment through prolonged circulation of drug, (c) protection of entrapped drugs from degradation, and (d) higher uptake of drugs by cells through EPR effect and/or endocytosis mediated by receptors (Fernandez-Fernandez et al. 2011). Various forms of nanoformulations have been developed with improved solubility and activity of drugs, which requires further modifications before large-scale production (Tan et al. 2012).

As of 2012, according to a paper of Weissig et al., there are 33 marketed nanotherapeutics and 122 therapeutics in clinical trials (Weissig et al. 2014). The approved liposomal formulations carry drugs/active compounds such as amphotericin, daunorubicin, cytarabine, morphine, doxorubicin, influenza virus antigens, vincristine, mifamurtide, and verteporfin.

Among the other nanocarriers, the polymeric nanocarriers have gained attention as safe and effective delivery vehicles. Oral formulations based on polymeric nanocarriers have also attracted attention, with biodegradable polymers providing for low immunogenicity and biocompatibility (Kanwar et al. 2012). Classic examples for biodegradable polymers are the natural products, predominantly polysaccharides such as alginate, chitosan, starch, etc.

The natural polymers, which are also known as biopolymers, are organic molecules present in living systems. They structurally consist of amino acids, nucleotides, etc. that can form peptides, polysaccharides, polyesters, or polyphenols (Rehm 2010). Nanotechnology approaches have led to these biopolymers to be potential candidates for wound care applications. Sahana and Rekha had in 2018 brought out a review where wound management has been addressed from the biopolymer perspective (Sahana and Rekha 2018).

11.2 Nanoformulations Based on Biomolecules

Biomolecules find use as drugs, as drug carriers, or as target specificity providers for nanoparticle platforms.

11.2.1 Biomolecule-Based Therapies

Therapies based on biomolecules such as peptides, proteins, RNA, etc. have emerged in the past decade but with limited applications arising from poor stability and inability to cross cell membranes (Lino and Ferreira 2018). Formulations aimed at release of biomolecules by responding to endogenous stimuli have been reported. External stimulus, such as light, ultrasound, and magnetic waves, can also enable controlled release of the biomolecule from the formulations (Mura et al. 2013).

Among the light-triggerable systems, recent series of nanocarriers have been based on NIR light rather than UV-Vis radiation. Poor uptake by skin and tissue resulting in better depth of penetration has been possible with NIR irradiation (Henderson and Morries 2015). Presence of NIR labile compounds, such as coumarin derivatives, in the nanocarrier enables the increase in the energy of the NIR irradiation (Babin et al. 2009). Photolabile compounds can be cleaved by light, leading to generation of ROS and are gaining much attention. The use of engineered bacteriophages for delivery of such photosensitizers has been reported (Chen et al. 2016). Some of the photosensitizers such as phthalocyanine are hydrophobic and are delivered through liposomes (Sreeram et al. 2010).

11.2.2 Biomolecules as Target Specificity Providers

For DNA production, target specificity providers such as folate or vitamin B9 are important (Dehaini et al. 2016). Folate receptors are overexpressed during cancer and folate linked to nanoparticle helps target the drug. Folate functionalization of nanoparticles of iron oxide also has been reported for magnetic resonance imaging. Riboflavin, simple sugars such as galactose and glycans, peptides and more specifically cell-penetrating peptides, and proteins such as those of transferrin family, lipoproteins, adhesion proteins, etc. have been reported for functionalization of nanocarriers to target diseased cells (Bae and Park 2011).

11.3 Chitosan-Based Drug Delivery Systems

A partial (66–95%) deacetylation of chitin leads to chitosan (mol. wt 3800–20,000 Da) and characteristically contains glucosamine and N-acetylglucosamine residues. Chitosan is known for its potential for (a) sustained discharge of active agents; (b) facile aqueous synthesis of nanoparticles; (c) presence of free amine groups, from polyamines, for cross-linking; (d) being cationic, easy cross-linking with metal ions through ionic gelation; and (e) increased residual time during adsorption through muco-adhesive character (Jagani et al. 2013). This is in addition to features common of polysaccharides such as low toxicity, immunogenicity, biocompatibility, and enzymatic degradability. One of the significant advantages of chitosan-based nanoformulations is in its ability to enhance dispersibility of hydrophobic drugs it carries (Du et al. 2013). Solid-cored liposomes based on drug-carrying chitosan nanoparticles and phospholipids have been reported to

overcome the poor encapsulation efficiency and fast leakage associated with conventional liposomal carriers (Huang et al. 2005). The applications of chitosan nanoparticles for buccal delivery, colon-specific delivery, and gene delivery have been reviewed by Sonia and Sharma (Sonia and Sharma 2011). Engineered chitosan-based nanoplatforms are increasingly finding application for simultaneous drug delivery and imaging (Swierczewska et al. 2016). Chitosan dressing containing neuropeptide like neurotensin and immunomodulators has been reported to decrease inflammations and increase fibroblast formation and deposition of collagen (Moura et al. 2014).

11.4 Small Molecules for Regenerative Medicine

Gene therapy alters or modifies defective/missing gene sequence by introducing external genetic material to organs (Jagani et al. 2013; Mulligan 1993). Small interfering RNA (siRNA) triggers the expression of gene of interest. Nanoformulations are designed to improve the transfection of siRNA and molecules like DNA or RNA into the cells without degradation. Engineered nanoformulations also provide for controlled release, cellular internalization, and safety during blood circulation (Sun et al. 2014). Light-responsive formulations have been reported for siRNA delivery. This includes nanocarriers such as gold nanorods, gold nanospheres, deblock polymers, polymeric nanoparticles, etc. (Lino and Ferreira 2018). By disruption of epithelial junctions, chitosan brings about cellular internalization (Kotze et al. 1998).

Molecules like pDNA and siRNA can be transported through non-viral vectors like biopolymer such as chitosan, PLGA, and gelatin (Fathabadi et al. 2012). The positive-charged amine groups of chitosan under slightly acidic conditions bring about electrostatic interactions with phosphate-bearing nucleic acids, leading to formation of polyelectrolytic complexes (Rudzinski and Aminabhavi 2010).

Targeted delivery to cisplatin-sensitive lung cancer has been possible using chitosan carrying epidermal growth factor receptors (Nascimento et al. 2016). Addition of PEG and the formation of stable siRNA-loaded chitosan nanoparticles enhance the transfection efficiency (Sun et al. 2016; Rudzinski et al. 2016). Modification of the primary amines with 4-imidazole acetic acid gives rise to amines of higher order in the polymeric structure (Ghosn et al. 2010), which in turn brings about enhanced solubility. Such structures also enhance the siRNA-mediated knockdown in cells (Ghosn et al. 2010).

The expression of reporter genes is promoted by carriers such as chitosan. Though chitosan-DNA complexes have long life, low protein affinity, and immunotoxicity, the transfection efficiency is lower than other cationic molecules and dendrimers (Medberry et al. 2004; Gao et al. 2011; Mohammadi et al. 2011). There have been reports on enhancement of cationic charge of chitosan by linking with cationic groups and moieties such as polyarginine, PLGA, etc. (Noh et al. 2010; Nafee et al. 2007). Neutral and anionic molecules such as PEG and cyclodextrin are also added to chitosan to increase the particle stability, reduce aggregation, elongate

plasma circulation times, and bring in sustained delivery of DNA (Teijeiro-Osorio et al. 2009; Duceppe and Tabrizian 2009).

Heparin has an affinity to bind to chitosan, leading to release of the DNA from the chitosan-DNA complex. The ratio of chitosan-amino group to DNA phosphate group influences the stability of the chitosan-DNA complex against molecules such as heparin. By increasing the ratio of amino group to phosphate group, the premature extracellular release of DNA when the chitosan-DNA complex interacts with anionic components can be overcome (Veilleux et al. 2018).

The generation of polyplex complexes of chitosan by addition of anionic molecules has opened up a new range of drug delivery vehicles. Chitosan-alginate polyplex systems have been reported to be homogenous and stable in blood plasma pH and demonstrated low drug leakage (Brezaniova et al. 2017). It has been reported that chitosan-siRNA polyplexes are able to treat multiple diseases through gene silencing. Polyplexes coated with hyaluronic acid are non-cytotoxic and pH neutral (Veilleux et al. 2018). Injectable gene delivery carrier based on the polyplex glycol chitosan-methyl acrylate-polyethylenimine has been reported to demonstrate osteogenic differentiation and phenotype expression of mesenchymal cells, thus showcasing the polyplexes as potential targeted gene delivery vehicles (Wen and Oh 2014; Bae et al. 2016). In a similar manner, oleoyl-carboxymethyl-chitosan-hyaluronic acid polyplexes have been reported as oral gene delivery carriers (Liu et al. 2016). Complexity of the chitosan-based gene delivery vehicles also brought in several advantages. For instance, the deposition of chitosan, polyethyleneimine, and nucleic acids on spherical gold nanoparticles enhanced the transfection efficiency up to 1000 times (Saengkrit et al. 2011).

11.5 An Insight into the Challenges of Tissue Engineering

11.5.1 The Need for Tissue Engineering

Functional tissues and organs perform systematic physiological functions through a collective effort of trillions of cells. Injury or disease leaves many types of tissues incapable of regeneration, classical example being those subjected to burns, spinal cord trauma, etc. While tissues of bone are generally regenerating type, they may not completely regenerate and may have large defects. Illnesses such as heart failure, renal failure, liver cirrhosis, diabetes mellitus, etc. call for transplantation procedures using grafting methods (Bianconi et al. 2013). One of the major shortfalls of grafting methods is the shortage of donor organs, which is coupled with high cost, cumbersome operative procedures, organ rejection, etc. Tissue engineering thus focused its attention on harnessing the native capacity of cells to replace, repair, and regenerate (Griffith and Naughton 2002).

The fundamental understanding of the underlying concepts of cellular and molecular biology coupled with the increased relevance toward research in biomaterials and their applications has led to value addition to the subject of tissue engineering (Risbud and Sittinger 2002). Tissue engineering focuses on delivery

and organization of appropriate cells to restore defective tissues (Minuth et al. 1998). In short, the presence of appropriate cells like stem cells, a supporting scaffold, and bioactive molecules that can enhance cell differentiation and regeneration is the requirement of tissue engineering.

Stem cells are unformed and undifferentiated cells that turn into specialized cell types through self-renewal and differentiation (Strauer and Kornowski 2003). Adult stem cells, also known as hematopoietic and mesenchymal cells, are found in mature tissues, for example, the bone marrow. These cells find use in organ regeneration. For the regeneration, the extracellular matrix (ECM) microenvironment needs to be engineered in such a way that it can facilitate the regeneration (Lane et al. 2014).

11.5.2 Engineered Scaffolds

An idealistic material or engineered material, broadly described as scaffold, is a transient structure that facilitates cell regeneration by way of evaluation, treatment, augmentation, or restoration (Chen and Liu 2016; Gu et al. 2014) and degrades with formation of natural ECM and new tissue (Lu et al. 2000). The advantages and disadvantages of various biological materials as scaffolds have been discussed widely (Mogosanu and Grumezescu 2014). To obtain the advantages of various types of scaffolds onto a single matrix, functionally graded scaffolds (FGS) have been designed (Scaffaro et al. 2017). The FGS are categorized as bilayered or multilayered, based on the number of layers that contribute to the whole structure. The FGS can be mono- or multiphasic and can even be designed with the same material but possessing significantly varying properties such as porosity. Scaffolds used for osteochondral repair are generally bilayer structures, with each layer bringing in variable properties for easy healing. Scaffolds meant for tendon-bone interface, human dermis, etc. are multilayered and possessing variable porosity, chemical cues, drug, etc. Chitosan-hyaluronic acid hydrogels in the bilayer format and prepared through freeze-drying/electrospinning techniques have been reported to have variable material composition, shapes, and porosity, with potential application in the ligament tissue regeneration (Liu et al. 2019).

11.5.3 Three-Dimensional Scaffolds

Complex three-dimensional architecture based on supportive matrices such as collagen, chitosan, alginate, and biodegradable polymeric scaffolds has been created to mimic the in vivo environment (Atala et al. 1994; Risbud et al. 2001). The three components of tissue engineering collectively have to ensure that the cells collected from patients are differentiated and provide mechanical stability in a short time.

Ideally, a scaffold (a) is a three-dimensional porous structure with interconnected pores to promote cell growth and transport of nutrients and metabolic waste, (b) is compatible and resorbable in such a way as to have controllable degradation and resorption of the tissue, (c) possesses surface chemistry for associated activities of

tissue growth, and (d) possesses mechanical properties that match with tissues available in the site of implantation (Hutmacher 2000).

The challenge of obtaining desired mechanical stability along biocompatibility has been addressed through use of natural polymeric gels, such as those based on chitosan, alginate, collagen, etc. Preparation of nanocomposites of these natural biopolymers with metal/metal oxides and polymers improves the mechanical properties, without compromising the biocompatibility.

11.5.4 Material Modification of Scaffolds

From the above literature, it is clear that no single scaffold has all the properties desired for a scaffold. In order to advantageously utilize the positive features of a biological material and overcome the drawbacks associated, the material can be physically or biochemically modified to overcome the shortcomings (Cao et al. 2014).

Physical techniques like compression, filtration, and irradiation with UV light are employed to improve the porosity and biomechanical properties. For instance, the scaffolds employed for cartilage repair are expected not to contract during cell culture, and such physical techniques enable the scaffolds to prevent cell-mediated contractions. There are reports that dehydrothermal treatment and UV light irradiation would make chondrocytes produce higher glycosaminoglycan and collagen content while improving cell attachment (Rowland et al. 2013). Similarly, compression and filtration of scaffolds improve the biomechanical and biochemical properties. Recent efforts on microfluidic technology have demonstrated that scaffolds with interconnected pores and higher porosity can be generated by this technique (Mueller-Rath et al. 2010).

Biochemical modification includes conjugating peptides on the surface of the scaffolds. For instance, the presence TATVHL peptide grafting onto polyethylene oxide/chitosan scaffold improved the proliferation of chondrocytes in the constructs (Kuo and Wang 2012). There are also reports that the cell adhesion improved with the presence of CDPGYIGSR peptide (Kuo and Wang 2011).

A smart three-dimensional scaffold that has the ability to orchestrate the regeneration of cells in a manner in which the normal cells would have is the ideal goal of tissue engineering. Proliferation, migration, and differentiation of stem cells through the scaffold require growth factors. The dosage of the growth factors is of concern as erroneous dosages could lead to adverse effects. The delivery of the growth factors either is carried through the scaffold or separately and needs to be tunable (Subbiah and Guldberg 2019). There are instances in literature where the scaffold itself serves as controlled or sustained delivery vehicle for the growth factors. In spite of having several types of scaffolds or biomaterials, the sensitivity of growth factors toward environmental conditions such as temperature, pH, etc. could lead to loss of therapeutic potency, requiring optimization of scaffolds for delivery of growth factors (Subbiah and Guldberg 2019).

Improving the properties of 3D scaffolds through multiple biopolymers is of growing interest. Some of the disadvantages of chitosan-based scaffolds have been reported to be offset by composite scaffolds based on chitosan, gelatin, and graphene oxide (Saravanan et al. 2017). The growth of graphene oxide for biological applications has been phenomenal. The hydroxyl and carboxyl groups present in the graphene oxide form chemical bonds with chitosan in the scaffold matrix, thus providing stability and interfacial strength (Yu et al. 2017). Using techniques like directional freezing, aligned porous chitosan-graphene oxide scaffolds that mimic the bone tissues can be prepared (Liu et al. 2018a). Such aligned scaffolds have the advantage of inducing cell growth in the longitudinal direction.

11.5.5 Hydrogels of Chitosan

Coating, low-temperature oxidation, and blending various chitosan derivatives using plasma and surfactant are employed to engineer the chitosan surfaces. In general, processing of chitosan into membranes, nanogels, films, nanofibers, hydrogels, beads, and bandages has been reported (Vukajlovic et al. 2019). The variability of structures under each of these categories is a unique feature of chitosan. For instance, hydrogels of chitosan can be formed by cross-linking between two chitosan molecules, formation of polymeric networks between heterogeneous monomers or polymers, or ionic-type cross-linking (Chaudhari et al. 2016; Wu et al. 2017). Reaction of amino groups of chitosan and thiol groups of cell membrane glycoproteins also yields hydrogels (Bernkop-Schnurch et al. 2001). The hydrogels form imine, amide, nitro, H-bonds, etc. with the host tissue (Ghobril and Grinstaff 2015). Reaction takes place between negatively charged tissue surfaces, sulfonic acid groups, and glycosaminoglycans present in the extracellular matrix and the amino groups of chitosan hydrogels (Jiao et al. 2019).

Wu et al., in an article published in 2017, have tabulated various composite hydrogels that find applications in drug delivery (Wu et al. 2017). Interestingly, the composite hydrogels, such as chitosan-alginate, chitosan-PVA, chitosan-PEG, etc., had over 90% encapsulation efficiency (Xu et al. 2007; Sarmento et al. 2007). The natural polymers that have been coupled with chitosan for drug delivery included alginate, collagen, gelatin, etc. (Qian et al. 2019). UV-irradiated chitosan hydrogel grafts are able to inhibit local infections as they are resistant to *E. coli*.

Preparation of hydrogels has parallels to procedures employed for modification of chitosan. Schiff base reactions between amine groups present in carboxymethyl chitosan and the aldehydic groups present in oxidized hyaluronic acid result in injectable hydrogels (Li et al. 2014). Similarly, reaction between oxidized dextran and chitosan results in injectable tissue adhesives having more than five times adhesive strength than fibrin glue (Balakrishnan et al. 2017). Facile chemistries such as use of EDC-NHS cross-linking between chitosan and compounds with carboxyl groups are also employed for preparation of hydrogels (Jiang et al. 2016).

11.5.6 Incorporation of Growth Factors in Hydrogels

Upregulation of growth factors, such as the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), etc., helps rebuilt defective tissues and accelerate fibroblast and endothelial growth (Ferrara et al. 2003; Liu et al. 2017). Release of growth factors slowly and in low concentration avoids side effects of sudden overexpression of VEGF, such as oncogenesis, dermatomyositis, etc. (De Francesco et al. 2017). Both drug and hydrogel properties influence the release profile. While drugs with more hydrophilic character are released faster, highly porous hydrogels with shorter degradation times also allow greater diffusion of drug. It has been demonstrated that the porosity and pore structure of chitosan hydrogels have a direct bearing on the rate of release of growth factors, with larger pores releasing the drug faster (Jiao et al. 2019; Cai et al. 2017).

11.5.7 Chitosan-Based Wound Healing Materials

The skin regulates body temperature, prevents loss of water, and inhibits bacterial and viral infections from entering the body. Being the outer cover of the body, the skin is also subjected to day-to-day abuse as well as damage from burns, cuts, bruises, etc., causing significant loss of function. Among the two methods of tissue transplantation – allogeneic and autologous – the autologous is more preferred as allogeneic has complications such as tissue rejection by immune system. However, the autologous is limited by the availability of replacement tissue (Supp and Boyce 2005). The chosen way forward has been bioengineered skin which couples the development in wound dressing and tissue engineering (Kirsner et al. 1998). Larger surface area availability, reduced demand for donor tissue, ability to incorporate antimicrobial and hemostatic functions, entrapment of growth factors for tissue regeneration, etc. have enabled the development of artificial wound dressing materials (Boateng et al. 2008). In addition to this, stimuli that can apply low-level stretching to accelerate migration of cells into a gap, leading to faster wound closure, have also been reported (Toume et al. 2017). The effectivity of loading of multiple drugs and stimuli is a complex process as they have to interact with the scaffold and also with the tissues and cells. Thus wound healing is considered to be three parts comprising of inflammation, proliferation, and remodelling (Li et al. 2007). Of the three, the inflammation is a response to the skin trauma, which is preceded by coagulation of blood. The movement of neutrophils and adipose cells to the site of the wound removes infection, which is followed by endothelial cell proliferation and wound healing (Chandika et al. 2015). Thus an ideal healing material should provide rapid healing at a reasonable cost. Among the naturally occurring polymers for wound dressing application, the ability to activate fibroblasts and regulate the deposition and arrangement of collagen fibers has been found to be better with chitosan (Kokabi et al. 2007). Simpler chitosan hydrogel-based dressings such as chitosan solution exposed to ammonia, heat-treated chitosan hydrogels, etc. have been shown to enhance re-epithelization, without inflammatory reaction in the wound or systemic toxicities (Mayol et al. 2014; Ribeiro et al. 2009). Electrospun nanofibers of carboxyethyl chitosan-polyvinyl alcohol have been found to be

good wound dressing material with properties ideal for promoting cell attachment and proliferation (Zhou et al. 2008), while electrospun chitosan fibers carrying silver nanoparticles were effective as antibacterial treatment in wound care (Lee et al. 2014).

Chitosan hydrogels have been found to be effective even where conventional wound healing is impaired. For instance, foot ulcers from diabetic neuropathy and peripheral vascular disease affect about 15% diabetic population, of which 80% patients go through foot amputations (Brem and Tomic-Canic 2007). Such foot ulcers are easily healed through the use of chitosan hydrogels as they are able to release bioactive payloads in a sustained manner at the wound site (Binning et al. 2019). Chitosan cross-linked to collagen, chitosan with varying levels of deacetylation, chitosan-dextran hydrogels, and chitosan-alginate-polyglutamic acid hydrogels are today available commercially for treating diabetic foot ulcers.

3D cell cultures are used to study the cell behavior and interaction of cells with external environment. Based on this, researchers have demonstrated that 3D cell cultures on porous structures such as those based on synthetic L-glutamic acid, gelatin, and chitosan have good elastic behavior and facilitate cell growth and differentiation better than 2D structures (Yan et al. 2013; Song et al. 2015; Huebsch and Mooney 2009). Further the differentiated cells secreted specific cytokines that recruited surrounding cells to repair defective tissues.

Slow and sustained biodegradation which matches the rate of new tissue formation is a major requirement for scaffolds for tissue engineering application, as they enable the steady replacement of the scaffold with the regenerated tissues. Chitosan scaffolds carrying N-acetyl-β-D-glucosamine can release them in a time-bound manner and kick start fibroblast proliferation and increase collagen and hyaluronic acid content at the lesion site during tissue healing (Bano et al. 2017). The possible mechanism by which the biodegradability of chitosan scaffolds helps tissue repair and anti-inflammatory response has been explained as twofold – first where the hydrogels kill bacteria, thus inhibiting the enhanced inflammatory response induced by the pathogen, and second the regulation of macrophages to M2 phenotype (Deng et al. 2017; Fan et al. 2015).

11.6 Small Molecules Entrapped Chitosan-Based Matrices for Tissue Regeneration

Manufacture and delivery of target-specific small molecules using magnetic nanofactories have been reported (Fernandes et al. 2007). Wen and Oh have brought out an excellent review on the strategies to modify chitosan to develop chitosan-based biomaterials, self-assembled micelles, nanogels, and meshes (Wen and Oh 2014). High payload amorphous drug-chitosan nanoparticle complexes (also known as nanoplexes) are supersaturating drug delivery systems with enhanced bioavailability. For instance, the low solubility and high metabolic rate of curcumin result in low efficacy and poor bioavailability, which are overcome through nanoformulations based on chitosan (Yallapu et al. 2013), involving cross-linking agents such as disuccinimidyl tartrate (Nguyen et al. 2016). Encapsulation of

curcumin in poly(lactic-co-glycolic-acid) microspheres of chitosan and aloe vera has also been reported for advancing wound healing and skin regeneration (Liu et al. 2019). For encapsulating hydrophilic payloads such as proteins, inverse miniemulsions based on cross-linked glycol chitosan or glycol chitosan protected by water-soluble molecules such as monosodium glutamate have been reported (He et al. 2016).

11.7 Nano-Based Chitosan Platforms for Tissue Regeneration

The significance of polymeric nanoparticles as drug carriers, where drugs are either adsorbed or covalently bound to the carrier surface, is growing phenomenally. Carries can also have varying types and number of binding sites to carry target-specific receptors, sensors, and imaging tools. With specific reference to chitosan nanoparticles, the large number of lone pair electron provides for high binding power arising from empty orbitals, thus making chitosan nanoparticles ideal for drug and gene delivery, biosensors, etc. (Guo et al. 2010; Verma et al. 2012; Anitha et al. 2012).

Techniques employed to generate chitosan nanoparticles have been reviewed elsewhere (Zhao et al. 2018). The significant advantages of employing chitosan nanoparticles for delivery of polyphenols, antibiotics, peptides, and genes are schematically summarized in Fig. 11.1.

The easy maneuvering of chitosan scaffolds for tissue engineering applications is because of its structural similarity with glycosaminoglycans present in ECM. Chitosan nanoparticles and composites can be tailored to have tunable degradation, biocompatibility, sustained release of drugs, variable porosity, etc., thus making it easy for their application as scaffolds. A recent article by Baranwal et al. highlights several chitosan scaffolds that can be used for cell regeneration (Baranwal et al. 2018).

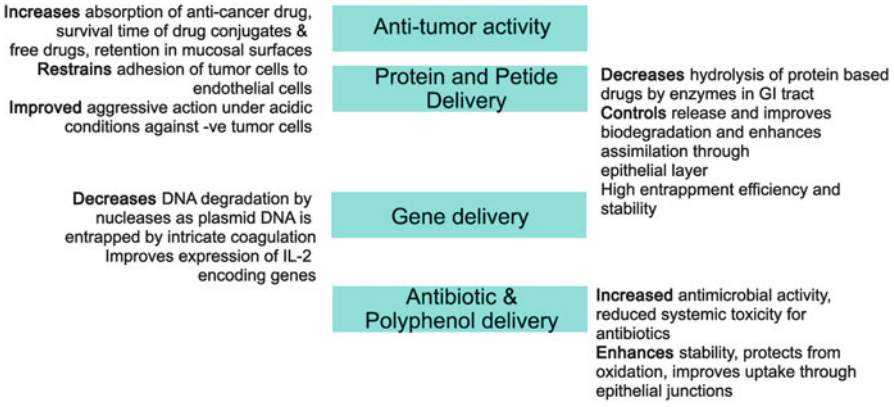

Fig. 11.1 Schematic description of the advantages of chitosan nanoparticles as delivery vehicles

Techniques like electrospinning have facilitated the preparation of composite scaffolds of chitosan. For instance, chitosan-silk fibroin composite scaffolds with very narrow fiber diameter and uniform blending have been reported through electrospinning (Qasim et al. 2018). Intermolecular hydrogen-bonded carboxyethyl chitosan-silk fibroin-PVA also has better biocompatibility and faster skin regeneration ability (Zhou et al. 2013).

11.7.1 Integration of Metal/Metal Oxide to Chitosan Matrices

Organic/inorganic hybrid network structures comprising organic material such as chitosan and metal/metal oxide nanoparticles have been explored in tissue engineering, for antibacterial activity and faster healing. The popularly employed metal and metal oxide nanoparticles for this purpose are Ag, Cu, Ti, and Zn/ZnO (Sirelkhatim et al. 2015; Joorabloo et al. 2019). Formation of bonds between metal ions and the growth factor incorporated in chitosan scaffolds can enhance the nanoparticle-induced wound healing. It has been postulated that ZnO brings about cell death by leaking of DNA cytoplasmic liquid through alteration of the electrical balance of the bacterial cell wall.

Addition of nano-hydroxyapatite to chitosan, even at 1 wt%, increases the compression modulus of the scaffold significantly (Saxena et al. 2018). High Young's modulus, controlled swelling behavior, and reduced water uptake are obtained from 3D scaffolds prepared using chitosan- and ethylene glycol-functionalized hydroxyapatite (Depan et al. 2011). Synthesis of Ag nanoparticles in chitosan solution followed by freeze-drying has been reported to yield a porous sponge, which provided for good swelling and water retention properties, low or no toxicity, thus making them ideal as wound dressing materials (Ran et al. 2019).

11.7.2 Chitosan-Carbon-Based Nanomaterial Scaffolds

Carbon-based nanomaterials consist of single- and multiwalled carbon nanotubes, fullerenes, nano-diamonds, and graphene, possessing high mechanical strength, conductivity, and optical properties. Reinforcing known biomaterials with carbon-based nanomaterials has resulted in better drug delivery systems, scaffolds with higher mechanical strength, and so on. The inherent softness of hydrogels and fibrous scaffolds can easily be overcome by incorporating carbon-based nanomaterials in the scaffolds. Another advantage of incorporating carbon-based nanomaterials is to bring in electrical conductivity in the scaffolds, which are otherwise insulating. This has advantageous applications in cardiac tissue engineering as propagation of electrical signals stimulates electrophysiological functions.

Mechanical strength and electroactive properties of carbon materials are advantageously used for stimulated and directional growth of cells on scaffolds (Lau et al. 2008). Multiwalled carbon nanotube doping onto chitosan scaffolds provided for high conductivity, porosity, and biocompatibility. However, preparation of carbon

nanotubes involves metal catalysts that occur as impurities in the scaffolds, leading to cytotoxicity. Graphene thus becomes an ideal choice for scaffold applications (Fan et al. 2010).

Exfoliation of graphite yields graphene, which has almost the same properties as that of carbon nanotubes. It is a structurally robust, highly flexible, and thin material (Kim et al. 2009). Under acidic conditions, oxidation of graphite produces graphene oxide. Graphene oxide is water soluble and has hydrophilic groups that can be functionalized based on end use (Dreyer et al. 2014). Though reports on graphene-/graphene oxide-based scaffolds are scarce, the large surface area of graphene oxide has been used for π-π stacking of drugs such as doxorubicin (Liu et al. 2018a; Zhang et al. 2010).

Composites of graphene are reported to have good electrical and thermal conductivity and mechanical stiffness. Graphene-chitosan composite films prepared without use of catalysts are biocompatible to cells (Fan et al. 2010).

By using density functional theory, the interactions among functionalized graphene and groups such as -COOH-, OH-, and NH_2- present in the chitosan have been studied by Zhang et al. (Zhang et al. 2016). The interfacial interaction between repeat units of chitosan and intrinsic graphene was weak. The study did not reveal any electron transfer between chitosan and graphene, and the increase in mechanical strength observed in experimental works was attributed to the properties of graphene. The authors also predicted that such materials would eventually fail. The interaction energies increased with -COOH and -NH_2- functionalization of graphene. With multifunctional graphene such as -COOH- and NH_2-modified, COOH- and OH-modified, or NH_2- and OH-modified composites, the COOH- and OH-modified graphene had the highest interfacial interaction energy, thus indicating that modified graphene interaction with chitosan would lead to scaffolds with better mechanical strength.

11.8 Current Challenges in the Use of Chitosan-Based Matrix

As of 2017, 116,000 patients are awaiting organ transplantation in the USA alone. Though tissue engineering has been conceived as the alternative, lack of complexity, inability to transport nutrients or exchange oxygen through interconnected pores, cell death due to circular cell morphology, etc. have been cited drawbacks (Li et al. 2018).

Morphology of cells is influenced by the stiffness of scaffold. Scaffolds having stiffness less than 5 kPa result in cells with circular morphology. A scaffold stiffness higher than 20 kPa is preferred for elongation and spreading of encapsulated cells (Ghobril and Grinstaff 2015). Reduced stiffness of chitosan hydrogels is often reported, which is possibly attributed to the acid environment employed for obtaining higher solubility in chitosan as the solubility is limited in neutral solutions (Hu et al. 2016). There are reports that the incorporation of metal/metal oxide nanoparticles increases the stiffness of the nanocomposite (Badawi et al. 2017). In spite of the advancements discussed in this chapter, low mechanical strength, batch-

to-batch quality variations, poor to low delivery of growth factors, and insufficient vascularization are still the challenges associated with chitosan scaffolds (Levengood and Zhang 2014). However, the advantages that chitosan offers as a base material and the engineering possibilities on its structure indicate the possibility of overcoming the slated disadvantages.

The future role of chitosan in tissue regeneration would significantly depend on clinical trials using the developed scaffolds. Clinical trials are also hampered by the regulation on use of chitosan to areas such as wound dressing, food supplement, and cosmetic industry. Further, research needs to address how critical components such as chemical variability of chitosan could affect the ultimate use of the scaffold in tissue regeneration. In spite of all the advancements, the emergence of 3D printing technologies is considered as the way forward for tissue regeneration.

11.9 Future Role of Chitosan in Tissue Regeneration

11.9.1 3D Printed Scaffolds

The concept of layer-by-layer assemblies of gold nanoparticles and chitosan discussed earlier in this chapter is the same in the case of 3D printing. The hydrogels discussed in this chapter have significant advantage arising from their hydrated structure and ability to form 3D scaffolds, subject to researchers overcoming the issue of structure maintenance by biopolymer filaments, owing to their low mechanical properties.

The use of 3D printing techniques for producing biomaterials with appropriate cells has opened up a new area of science called bioprinting, with applications in tissue engineering and transplantation. Precision and controllable deposition of cells is one of the significant advantages of this technique. Reports on successful 3D printing of bone tissues, skin, and pancreatic tissues have been forthcoming. Challenges encountered with respect to incorporation of growth factors, porosity, throughput, etc. are likely to be overcome with 3D printing. Success of bioprinting depends on the printer and the bio ink. Newer bioprinters are today coming up in the market, along with bio inks that are based on hydrogels, cell preloaded polymers, polymers with ECM, etc. (Pati et al. 2015). Using polycaprolactone, PLGA, and tricalcium phosphate and mesenchymal stromal cells, Pati et al. generated 3D-printed scaffolds that had a futuristic possibility as off-shelf bone graft substitutes.

However, the ability of the polymeric material to maintain the cells in the appropriate physiological state is a major concern. Added to this is the challenge of patterning multiple cell types which are the norm in native tissues, as cell-cell interactions are required to boost cell functionality. Matching the properties of polymeric inks and the hydrogel-based inks where polymeric inks have better mechanical properties but poor degradability while hydrogels have favorable environment for cell growth but poor mechanical properties is another challenge in the way forward for bioprinting (Ozbolat et al. 2016). The antibacterial properties of

chitosan-poly-epsilon-caprolactone bioprinted scaffolds have been reported to be good and comparable to electrospun scaffolds (Tardajos et al. 2018).

Mechanical properties of natural polymers such as chitosan have been reported to be improved by chemical cross-linking of chitosan to alginate, gelatin, and collagen (Yan et al. 2005). The aqueous solubility of biological materials such as chitosan is also a major challenge, which are generally overcome through chemical methods. However, the chemical methods could turn out to be potentially toxic due to reactions occurring during the bio ink formulation. This perhaps attributes to the reports that the extent to which chitosan could be employed in bio ink was only 4% of the total polymer weight (Liu et al. 2018b) as on date.

Yet another aspect that has not been studied with reference to chitosan is the rheological properties. The deformation associated with external force, which is very significant in printing needs to be evaluated for the hydrogels. There are reports that methacrylated hyaluronic acids in triblock polymeric hydrogels positively influenced Young's modulus of the constructs in a dose-dependent manner (Mouser et al. 2017). As in the case of hydrogels, the introduction of metal oxide nanoparticles improves the compressive modulus of the printed scaffolds (Rasoulianboroujeni et al. 2019).

Taking clue from earlier research involving hydrogels of gelatin with chitosan and PVA, where the visco-elastic properties were found to increase with chitosan concentration (Rodriguez-Rodriguez et al. 2019), the preparation of such complex or polyplex hydrogels could be a way forward for chitosan-based bio inks. In a similar approach, suspension of phosphate and chitosan in acidic solutions could be turned into hydrogels whose rheological properties could be tuned by varying the chitosan concentration (Mouser et al. 2017).

Changes in the technology of 3D printer technologies would also require changes in the bio ink. For instance, 3D printing techniques need to be able to produce accurate scaffolds, which in many cases are soft materials. However, the stiffness of currently printed scaffolds needs to be higher so as to avoid breakage of the filaments on its own weight. A cryogenic printer, where the ink in solution form transforms to a solid at sub-zero temperature, was conceptualized to build stable 3D structures (Adamkiewicz and Rubinsky 2015). Tan et al. have reported the development of a hydrogel ink which on rapid cooling using solid carbon dioxide provides for a matrix with less than 1 kPA compressive stiffness (Tan et al. 2017). Though the cryogenic printing is in its native state, the development of appropriate printers and inks could lead to the generation of soft tissues.

Some researchers consider 3D printing as a disruptive technology which has a potential to bring about wide changes in the manner in which tissue regeneration is performed. This would however require development of appropriate hydrogels whose transformation into filaments has the requisite durability and capability to carry drugs and other growth factors.

Research in the near future could enable surgeons to replicate and replace tissues with printed organs, thus improving quality of life (Colaco et al. 2018).

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Chitosan-Based Systems for Theranostic Applications

12

V. Balan, S. Malihin, and Liliana Verestiuc

Abstract

The ideal theranostic approach is capable of several functions ranging from diagnosis to treatment with accurate targeting of cancer-specific cells. Therefore, the newest generation of theranostic systems offers opportunities to combine passive and active targeting, environmentally responsive drug release, molecular imaging, and other therapeutic functions into a single biomedical platform. To achieve this purpose, biomedical researchers have developed various systems composed of organic or inorganic materials. Due to its remarkable physicochemical and biological properties, chitosan and its derivatives have been employed in the development of composite theranostic systems able of prediction, real-time monitoring, and assessment of the therapeutic responses. This review outlines recent developments of chitosan-based systems for theranostic applications and analyzes them in terms of performances and limits. Conclusions and future perspectives will both synthesize the state of the art of the chitosan applications in theragnosis and the authors' point of view about insights toward biopolymer unexploited implications in this field.

Keywords

Theranostic · Chitosan · Bioactivity · Nanoparticles · Scaffolds

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_12

12.1 Theranostics: A Novel Approach to Combine Diagnosis, Treatment, and Subsequent Imaging

12.1.1 Concerns for Theranostic Systems

Cancer is the leading cause of worldwide death for centuries, accounting millions of deaths annually (Jemal et al. 2011). Based on data statistics reported by the International Agency for Research on Cancer (GLOBOCAN) in 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million existing cancer patients worldwide. An increase is estimated to about 16 million new cancer cases per year by 2020. Current promising therapies for cancer, including surgery, chemotherapy, and radiotherapy, are still limited due to the following: (i) lack of selectivity, (ii) multidrug resistance, and (iii) severe toxic side effects (Chowdhury et al. 2016).

In this context, extensive research has been attracted in the development of complex site-specific therapies based on nanotechnology (Liu et al. 2016; Lei et al. 2017; Zhang et al. 2016). Several advantages of nanosystems that can be exploited in cancer management are mentioned below (Zhen et al. 2014):

1. Due to their small size, nanosystems are able to escape renal clearance, easily permeate through the leaky blood vessels of tumor tissues, and accumulate inside the tumoral cells.
2. Their high surface area increases their loading capacity for both therapeutic and imaging agents.
3. They are able to be functionalized with targeting agents in order to selectively accumulate in the cancer cells.
4. They are composed of biocompatible materials, and their biodegradation products are nontoxic.
5. They can be designed for personalized medicine, as the drug therapeutic efficacy can be easily monitored by combining both therapeutic and imaging elements in a single structure.

Recently, a promising site-directed application in the field of nanotechnology relies on a new concept, known as *theranostic*, which involves simultaneous procedure of therapeutic and diagnostic approaches for personalized medicine (Schleich et al. 2013). There are several definitions of *theranostics*, also spelled as *theragnostics*, in some reports. They have been defined as a smart integrated system that assures therapy, diagnosis, and monitoring of the treatment response through imaging techniques (Warner 2004). Therefore, they can provide diagnosis followed by therapy and treatment followed by diagnosis or codeliver imaging and therapy components (Chen and Wong 2014). The ideal theranostic approach is capable of several functions ranging from diagnosis to treatment with accurate targeting of cancer-specific cells. The newest generation of theranostic systems offers opportunities to combine passive and active targeting, environmentally responsive drug release, molecular imaging, and other therapeutic functions into a single biomedical platform.

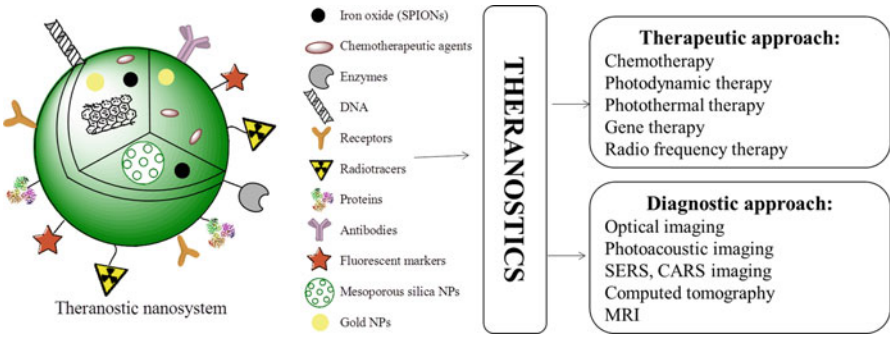

Fig. 12.1 Schematic diagram of the main components for theranostic nanosystems

In particular, a theranostic nanosystem is able to deliver an anticancer agent with triggered or controlled release, coupled to tumor-specific targeting of the microenvironment, while simultaneously allows sensitive tumor imaging with high diagnostic accuracy (Kumar and Srivastava 2015; Jeelani et al. 2014; Zhao et al. 2016). The main components of theranostic nanosystems include the therapeutic agent (payload), payload carrier, targeting ligands, and signal emitter (imaging agent). A schematic representation of the main components for theranostic nanosystems is depicted in Fig. 12.1. The therapeutic agent may be represented by anticancer drugs, nucleic acids, therapeutic proteins, or peptides; meanwhile the signal emitter consists of inorganic nanoparticles, such as gold nanoparticles (AuNPs), carbon nanotubes (CNTs), silica nanotubes, quantum dots (QDs), and superparamagnetic iron oxide nanoparticles (SPIONs). These inorganic nanoparticles exhibit unique optical, electrochemical, and magnetic characteristics. In most cases, the payload carriers are expressed as organic nanosystems, including here polymeric micelles and nanoparticles, liposomes, or dendrimers comprised of biocompatible materials with multifunctional groups such as –OH, –COOH, $–NH_2$, and –SH. Both therapeutic and imaging agent can be loaded in the theranostic nanosystem structure or attached to its surface by covalent or electrostatic bonds. The targeting moieties could be represented by proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other receptor ligands (peptides, vitamins).

Some of the tumor imaging modalities are represented by magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), fluorescent imaging through fluorescent agents, ultrasound imaging by nano-/microbubbles, etc.; meanwhile therapeutic approach can be accomplished by chemotherapy, photodynamic and photothermal therapy, and gene or radio-frequency therapy.

12.1.2 Materials Used in Theranostic Applications

12.1.2.1 Iron Oxide Nanoparticles

As a family of high-performance biomedical materials, theranostic nanosystems based on functionalized superparamagnetic iron oxide nanoparticles (SPIONs) could represent the best compromise between excellent magnetic properties and reduced toxicity, evidenced by extensive in vitro and in vivo tests (Mahmoudi et al. 2012) and by quantitative evaluation of biodistribution and local therapeutic effects (Dürr et al. 2013; Tietze et al. 2013). Current studies have been dedicated to the development of functionalized SPIONs for theranostic approaches, and encouraging results have been reported (Dadras et al. 2017; Shen et al. 2013). An important motivation for the choice of theranostic nanosystems in cancer therapy is represented by the patient safety and compliance (Ling et al. 2011).

Indeed, magnetic functionality can be exploited to render the functionalized SPIONs as theranostic nanosystems, particularly as a diagnostic tool and a targeting drug carrier in cancer therapy. If the diagnostic approach is accomplished by magnetic resonance imaging (MRI), the therapeutic path can be achieved by using one of the following techniques described below or by mixing the advantages of all three. First, by using magnetic-field-mediated guidance, it is possible to increase the site specificity and selectivity of the therapy, reduce side effects, and ensure chemotherapy cost-effectiveness (Tietze et al. 2015). Second, by conjugating a targeting molecule, the therapeutic efficacy can be enhanced by site-specific accumulation (Holohan et al. 2013). Third, functionalized SPIONs can be designed to incorporate both hydrophilic and hydrophobic chemotherapeutic agents (doxorubicin, docetaxel, paclitaxel, curcumin, etc.) and deliver them to the tumor cells (Jhaveri et al. 2014).

A strategy to develop a theranostic nanosystem capable of tumor cell-targeting, imaging, and drug delivery was proposed by Sahu et al. (2012). This nanosystem is composed of a magnetic material (magnetite) coated by a polymeric layer represented by N-carboxymethyl chitosan (N-CMC), functionalized with a fluorescent dye and a targeting ligand (folic acid) and loaded with doxorubicin. This targeted delivery system may also be used for tumor cell detection and labeling applications in vivo (Sahu et al. 2012). Likewise, Fan et al. (2011) functionalized magnetic composites based on iron oxide and O-carboxymethyl chitosan (O-CMC) with folic acid in order to improve their biocompatibility and ability to target specific tumor cells. MR imaging and tumor histological analysis demonstrated that this system could target folate receptor-positive tumor cells in vivo (Fan et al. 2011). Si et al. (2010) improved the antitumor effect of genistein using a biocompatible superparamagnetic drug delivery system, based on covalently attached genistein onto cross-linked carboxymethylated chitosan–iron oxide nanoparticles. Their results indicated that this system could be promising for future multifunctional chemotherapeutic applications (Si et al. 2010).

12.1.2.2 Gold Nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) represent an attractive opportunity for developing drug delivery systems with tunable features, due to their size, unique physical properties

combined with chemical inertness, stability, biocompatibility, optical properties in the near-infrared (NIR) region (Xia et al. 2011a, b), and ability to be functionalized with desired targeting ligands, specific antibodies, or drugs (Huang et al. 2013). The most common applications of AuNPs in cancer therapy were photothermal therapy (PTT) and radiation therapy. AuNPs coated with chitosan and polyacrylic acid and loaded with cisplatin were effective as theranostic tool able to combine near-IR photothermal therapy and chemotherapy (Chen et al. 2013a). Moreover, these nanosystems proved an enhanced antitumor effect of cisplatin if photodynamic therapy was included as an additional option (Chen et al. 2013b).

12.1.2.3 Quantum Dots (QDs)

Quantum dots (QDs) are semiconductor nanomaterials with fluorescent, optical, and electronic properties. They exhibit several advantages over conventional organic dyes for both in vitro and in vivo imaging (Sabaeian and Nasab 2012), such as broad excitation spectra, excellent photochemical stability, and narrow, symmetric, and tunable emission spectra as a result of quantum confinement effect. The most commonly used QDs include cadmium-based QDs, carbon QDs (CQDs), and graphene QDs (GQDs). Conventional QDs have a multilayer structure comprised of a metallic core cadmium and its derivatives, cadmium selenide (CdSe) and cadmium sulfide (CdS), and a coating material that prevents the core leaking or photobleaching (Kulkarni et al. 2019). Novel types of QDs particles based on cadmium telluride (CdTe) have been studied and proved to be more stable and efficacious than conventional QDs (Kim et al. 2015a, b). Narayanan et al. (2017) proposed a synergistic therapy of manganese-doped zinc sulfide (ZnS) QDs conjugated with chitosan nanoparticles and cisplatin and proved their effectiveness by in vitro cytotoxicity tests on HeLa cells. Hua et al. (2017) developed QDs functionalized with mercaptosuccinic acid (MSA), ethylenediamine, and chitosan in order to be used as a versatile tool in mitochondrial-targeted photodynamic therapy (PDT).

12.1.2.4 Carbon Nanotubes (CNTs)

CNTs are tube-shaped materials that can be divided into single-walled CNTs (SWNTs, 0.4–2.0 nm in diameters, 20–1000 nm in lengths) and multi-walled CNTs (MWNTs, 1.4–100 nm in diameters, $\geq$1 μm in lengths). SWNTs exhibit attractive optical properties that can be exploited for biological imaging (Welsher et al. 2008) and can be functionalized by covalent binding, adsorption, and electrostatic interactions in order to develop drug delivery carriers with high biocompatibility and enhanced water solubility. A theranostic nanosystem based on SWCNTs functionalized with fluorescent chitosan and folic acid has been tested by Wu et al. The nanosystem showed water dispersibility, excellent cytotoxicity (60–74%) on HeLa cell lines when exposed to 635 nm laser irradiation, and selective folate receptor-mediated endocytosis (Wu and Zhao 2016). Qi et al. (2015) developed a theranostic nanoformulation using oxidized CNTs wrapped with glycosylated

chitosan and loaded with DOX for hepatic tumors and demonstrated by in vivo studies that the nanosystem showed higher drug accumulation in the tumor as compared to DOX.

12.1.2.5 Mesoporous Silica Nanoparticles (MSNs)

MSNs exhibit unique properties such as stability and resistance to degradations induced by pH, heat, and mechanical stress, uniform and tunable pore size, high surface area and large pore volume allowing for high drug loading, and internal and external functional surfaces available for selective modification (Kumar et al. 2018). Highly charged polymers like chitosan, polyacrylate, and polyaspartate have been frequently employed as capping agents in mesoporous silica nanoparticles in order to achieve pH responsiveness (Shah and Rajput 2018). For instance, chitosan-capped MSNs were used to deliver curcumin in a pH-dependent manner to glioblastoma cells (Ahmadi Nasab et al. 2018); meanwhile pH-responsive alginate/chitosan multilayer-modified magnetic mesoporous silica nanocomposites were designed for gene therapy and cancer treatment (Yang et al. 2017). A photosensitizer chlorin e6 (Ce6) and antitumor drug doxorubicin (Dox) were adsorbed onto the MSNs and successfully achieved reversing multidrug resistance in cancer (Yang et al. 2017).

Using another strategy, Samykutty et al. (2018) developed an acidic pH targeted wormhole mesoporous silica nanoparticle (V7-RUBY) to serve as a novel tumor-specific theranostic nanosystem detectable using multispectral optoacoustic tomographic (MSOT) imaging. These nanoparticles presented both large loading capacity for chemotherapeutic agents (paclitaxel or carboplatin) and favorable release kinetics combined with tumor-specific targeting and gatekeeping. The IR780 imaging dye is carried as cargo, and chitosan controls the release of the dye to orthotopic ovarian tumors. The size and shape of the biocompatible V7-RUBY have essential features including the ability to translocate into the cytoplasmic compartment.

12.1.2.6 Lipid-Based Nanoparticle Platform: Liposomes, Solid Lipid Nanoparticles (SLNs), and Nanostructured Lipid Carriers (NLC)

Liposomes are small spherical-shaped vesicles with bilayer membrane structures composed of phospholipids able to encapsulate either hydrophilic or hydrophobic agents. The most commonly used phospholipids in the preparation of liposomes are polyethylene glycol and phosphatidylcholines (Elgqvist 2017). Noteworthy to mention that a PEGylated liposome formulation of doxorubicin (DOX), namely, Doxil®/Caelyx, was the first formulation approved for clinical use (Barenholz 2012). Liposomes can be designed to respond to changes in light (Leung and Romanowski 2012), temperature (Park et al. 2013), or acid (Mamasheva et al. 2011). Recently multifunctional and theranostic liposomes have been highlighted in the literature (Charron et al. 2015; Haeri et al. 2016; Perche and Torchilin 2013). However, there are some drawbacks such as rapid clearance from the bloodstream, instability of the carrier, high production cost, and fast oxidation of some phospholipids.

As an alternative, SLN that contains a matrix composed of solid lipid nanoparticle and modified SLN – nanostructured lipid carriers (NLC) – that contains a matrix composed of liquid lipid and solid lipid have been proposed. SLNs exhibit several

advantages including stable formulations, excellent reproducibility, and drug protection from degradation; meanwhile NLC assures enhanced drug loading and less drug expulsion during storage (Iqbal et al. 2012). Both SLN and NLC have been successfully functionalized with targeting agents and achieve efficient drug release in a controlled manner.

Salva et al. (2015) developed chitosan-coated liposomes for the co-delivery of siHIF1-a and siVEGF. The expression level of VEGF mRNA was markedly suppressed in breast tumoral cells (MCF-7 and MDA-MB-435 cells) transfected with the chitosan-coated liposomes designed, and the co-delivery greatly enhanced in vitro gene-silencing efficiency. In addition, chitosan-coated liposomes showed 96% cell viability.

12.1.2.7 Dendrimers

Dendrimers are nano-sized materials (1–15 nm), with regularly and highly branched three-dimensional architecture, monodisperse structure, high water solubility, and high payload. One of the most studied dendrimers was polyamidoamine (PAMAM) dendrimer. Recently, PAMAM dendrimers and chitosan-based nanohybrids were developed, and a significant reduction in the hemolytic toxicity of dendrimers after inclusion into chitosan was noted (Zhou et al. 2015). Zhang et al. (2015) prepared a controllable aptamer-based self-assembled DNA with significant stability, biocompatibility, high drug payload, and intracellular drug delivery efficacy for theranostic applications in cancer. It is important to mention that some dendrimer-based nanohybrids have shown promising outcomes in preclinical studies (Kesharwani et al. 2015; Nounou et al. 2015).

12.2 Chitosan: A Programmable Polymer for Theranostic Applications

12.2.1 Advantages of Chitosan

Chitosan has several advantages in biomedical applications, such as biocompatibility and controlled biodegradability. Due to its antimicrobial, bioadhesive, and bioresorbable nature, chitosan has been tested in medical fields, such as wound dressing, tissue engineering and regenerative medicine, controlled drug delivery, and antimicrobial agent. Its functionality and ability to form complexes with iron oxides and other inorganics and the processability in nanoparticles and nanocapsules made these materials very useful in theranostics, hyperthermia, and biosensing or cancer therapy (Anitha et al. 2014; Vunain et al. 2017). Chitosan has remarkable healing activity, and as all polysaccharides, it is a great moisturizing agent due to its water-retention capacity. It is a cholesterol-lowering product and can inhibit the development of a number of parasites and germs (*E. coli*, *Pseudomonas*, *Candida albicans*) (Gallaher 2003).

Compatibility of chitosan with physiological medium depends on the preparation method (because the residual proteins generate allergic reactions) and on the deacetylation degree – biocompatibility increases at higher deacetylation degree.

Chitosan actually proved to be more cytocompatible in vitro than chitin because the number of positive charges increases and the interaction between cells and chitosan increases as well, which improve biocompatibility (Croisier and Jérôme 2013). Chitosan with a high degree of deacetylation exhibits a high level of biocompatibility with the human biological environment. Thus, polymers with acetylation rates of 4 to 15% were tested, and the inflammatory response of the body after the implantation of the material was monitored. For chitosan with a degree of acetylation of 4–8%, only reduced inflammation was observed which did not cause biocompatibility problems. In contrast, for chitosan with a 15% acetyl degree, a collagen capsule around the implant was observed and infiltration of the implant matrix with neutrophils associated with the inflammatory response. All of that confirmed the significance of degree of acetylation prior to chitosan selection for use in tissue engineering (Barbosa et al. 2010).

Chitosan exhibits a high adhesion to the epithelial and mucosal tissues covering the tissues. Clinical trials on chitosan-containing materials have not reported inflammatory or allergic reactions in the case of implantation, injection, topical application, or ingestion by the human body. In vivo and in vitro cytotoxicity tests revealed the compatibility of chitosan films in contact with keratinocytes and fibroblasts, as well as the insignificant influence of acetylation. Chitosan-based materials in contact with skin lesions adhere to fibroblasts and promote the proliferation of keratinocytes and therefore tissue regeneration (Dai et al. 2011).

The mucoadhesion of chitosan is due to its positive charge which interacts with negatively charged residues in the mucin, the glycoprotein that composes the various mucous. Mucoadhesion is directly related to the positive charge density of chitosan and therefore with deacetylation degree. If the deacetylation degree of chitosan increases, the number of positive charges also increases, and mucoadhesion is improved (Sogias et al. 2018).

The hemostatic activity of chitosan is explained also by the presence of positive charges on chitosan backbone and their interactions with negatively charged membranes of the red blood cells. Due to its positive charges, chitosan can also interact with the negative part of cells' membrane and contributes to the reorganization and opening of the tight junction proteins, explaining the permeation enhancing property of this polysaccharide (Ibrahim and El-Zairy 2015).

The antimicrobial activity of chitosan combines two types of interactions of the positive charges:

(a) The interactions with negatively charged groups at the surface of cells, and as a consequence, the membrane cells' permeability are modified.
(b) Interactions that involve the binding of chitosan with the cell DNA (through protonated amino groups), which leads to the inhibition of the microbial RNA synthesis.

The polycationic nature of chitosan also explains the chitosan analgesic effects: The amino groups can protonate in the presence of protons that are released in the inflammatory area, resulting in an analgesic effect (Cheung et al. 2015).

The chitosan biodegradability is based on its polysaccharidic nature, which consequently contains breakable glycosidic bonds. Chitosan is actually degraded in vivo by several proteases and mainly lysozyme (Muxika et al. 2017). By degradation nontoxic oligosaccharides of variable length are formed which are incorporated in the metabolic pathways and finally excreted. The degradation rate of chitosan is mainly related to its degree of deacetylation but also to the distribution of *N*-acetyl-D-glucosamine residues and the molecular mass of chitosan (Kumar et al. 2004).

Considering all properties of chitosan, it was and will be tested in many biomedical and pharmaceutical applications, such as drug delivery and 3D scaffolds for tissue engineering or 2D scaffolds, especially for wound-healing purposes, and micro- and nanoparticles for diagnostics, cancer therapy, and bone implants (Bružauskaitė et al. 2015). Within the framework of the chitosan biocompatibility, it was notably approved by the Food and Drug Administration (FDA) for use in wound dressings (Gianino et al. 2018).

12.2.2 Chitosan Structure and Properties

12.2.2.1 Chitosan Structure

Chitin is one of the most abundant biopolymer and has attractive functional properties such as bioactivity, biocompatibility, biodegradability, and very good mechanical strength. Despite of these important features, it has limited biological applications due to its poor solubility (Saikia et al. 2015; Pillai and Sharma 2009). The solubility is reachable by chitin conversion into CS through chemical or enzymatic processes. Chemical deacetylation involves the chitin reaction with hydroxides at high temperatures, and a wide range CSs can be obtained when the deacetylation degree of CS reaches about 50%. In acidic environment, the amino groups are protonated, and the polymer becomes cationic, allowing it to interact with diverse types of biological substrates (Martinou et al. 1995). Basically, CS is a random copolymer formed by D-glucosamine and *N*-acetyl-D-glucosamine units, linked by 1,4 glycosidic linkages (Fig. 12.2).

Chitosan is a semicrystalline polymer and shows polymorphism depending on its physical condition: A crystallized state the orthorhombic system, like chitin, and two types of chitosan (with a high degree of deacetylation and in the form of free amine

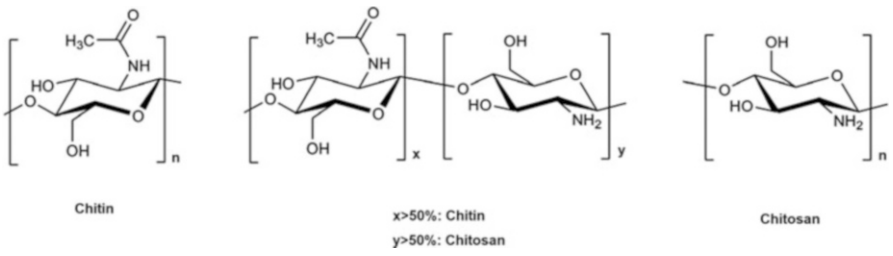

Fig. 12.2 Structure of chitin and chitosan

and chitosan in salt form, with weak degree of deacetylation which is more disordered) can be distinguished. The crystallinity may vary considerably with the origin of the biopolymer and extraction procedure. It is maximum for both 0% deacetylated chitin and fully deacetylated chitosan (100% deacetylated) (Crini et al. 2009).

12.2.2.2 Deacetylation/Acetylation Degree of Chitosan and Molecular Weight

The deacetylation of chitin involves the elimination of acetyl groups from the molecular chain, leaving behind a complex with a high degree of chemical reactive groups (NH_2). Chitosan is a linear polysaccharide, composed of glucosamine and *N*-acetyl glucosamine units linked by 1,4 glycosidic bonds; the content of glucosamine is called the degree of deacetylation (DD).

$$\text{DD is defined as the ratio} : \frac{GlcN}{GlcNAc + GlcN} \times 100,$$

where *GlcNAc* and *GlcN* are the number of *N*-acetyl-D-glucosamine and D-glucosamine units, respectively (Czechowska-Biskup et al. 2012; Correia et al. 2017). DD and the distribution of the acetyl groups along the main chain have a significant effect on the solubility of chitosan and the conformation and flexibility of macromolecular chains, which are important parameters for the use of this biopolymer in biomedical applications. Chitosan has a degree of deacetylation up to 95%, and for a complete deacetylation, repeated alkaline treatments are necessary (Hirai et al. 1991). Some authors reported that the acetylation degree (AD) of chitosan, corresponding to the proportion of *N*-acetyl-D-glucosamine units relative to the total number of glycosidic units, is a structural parameter that influences overall charge, reactivity, and biological properties (Hamdi et al. 2019).

Chitosan can have very high molecular weights (up to 3.000.000 Da), when the polymer was extracted with controlled parameters, but in general they are weaker, between 10.000 Da and 1.500.000 Da. Molecular weight influences the polymer solubility, solution viscosity, and polymer processability. The physical properties of chitosan are dependent on a number of parameters, such as the molecular weight, DD, sequence of the amino and the acetamido groups, and the biopolymer purity (Kaur and Dhillon 2014).

12.2.2.3 Chitosan Solubility

Chitin is insoluble in most organic solvents, but chitosan is soluble in dilute acidic solutions (generally, at pH <6.0). At acidic pH, the amines are protonated and become positively charged; chitosan becomes a water-soluble cationic polyelectrolyte. The primary amino groups of chitosan have a pKa value of 6.3 and make the biopolymer a strong base. At pH above 6.3, the amine group of chitosan is again deprotonated, and the polymer loses the charges and becomes insoluble (Zivanovic et al. 2014).

Chitosan is able to form quaternary nitrogen salts at low pH values. Therefore, organic acids such as acetic, formic, and lactic acids can dissolve CS. The most

frequently used solvent is acetic acid (1–2% in water) at about pH 3.5–4.0. Chitosan is also soluble in 1% hydrochloric acid and dilute nitric acid but insoluble in sulfuric and phosphoric acids. Concentrated acetic acid solutions, especially at high temperature, cause depolymerization of chitosan (Qin et al. 2006).

Due to the presence of hydroxyl and amino groups on its backbone, chitosan can be easily modified through various methods in the aim to improve its physicochemical properties or to extend its applications. Various chemical approaches have been tested for modifying chitosan, including quaternization, N-alkylation, hydroxylalkylation, carboxyalkylation, thiolation, and glycation (Mourya et al. 2010). Physical modifications imply the use of electromagnetic radiation and sonication and enhance properties, such as solubility in aqueous solutions, and modulate surface charges and absorption efficiency.

12.2.2.4 Chitosan Properties

As a biological origin-based polymer, chitosan is a promising biomaterial due to its availability, solubility, modulation of properties, non-toxicity, biocompatibility, and biodegradability. There is a great increasing demand for chitosan for several biological and medical applications. In the last decade, various chitosans and their derivatives have been extensively explored in new applications as antioxidant and free radical scavenging entity (Anraku et al. 2018). Chitosan properties are strongly connected with molecular mass and deacetylation degree (Fig. 12.3).

Degradation of Chitosan

Various enzymes are involved in chitosan degradation, including chitosanases, chitodextrinases, chitotriosidases, chitobiosidases, and chitobiases. Chitosanase converts chitosan in products with lower molecular weight (oligosaccharides) by hydrolysis of glycosidic link β (1-4) of the saccharide units; the oligosaccharides are not harmful and are, often, further degraded by β-glucosaminidases before removing from the body (Guarino et al. 2015). Chitosan has been shown to degrade in vivo, and the degradability by lysozyme is one of the most important properties of chitosan especially for medical and pharmaceutical applications (Szymańska and Winnicka 2015). Enzymatic hydrolysis of chitosan leads to *N*-acetyl-D-glucosamine and D-glucosamine. Glucosamine plays an important physiological role in biochemical processes that occur in vivo and participates in detoxification functions in the

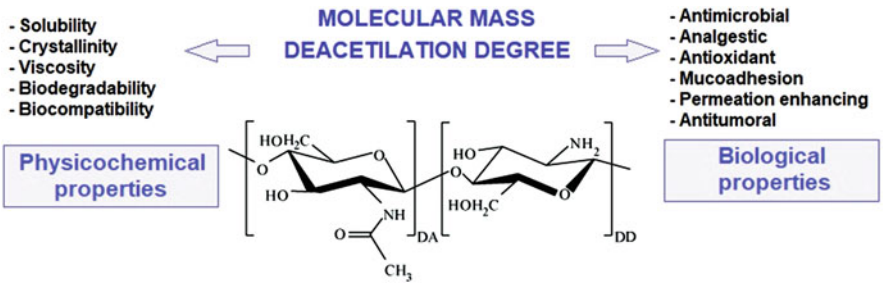

Fig. 12.3 Chitosan properties

liver and kidneys; it present anti-inflammatory properties, hepatoprotective, antireactive and hypoxic effect (Azuma et al. 2015).

Many studies have shown that chitosan is a biodegradable polymer and that it degraded in vivo mainly by lysozyme, which is ubiquitous in the human body (4–13 mg/l in serum and 450–1230 mg/l in tears); however, the biodegradation is dependent on some factors, including pH, type of chitin or chitosan, and chitosan preparation method (Dutta et al. 2011). In general, both rate and extent of chitosan biodegradability in living organisms are dependent on the degree of deacetylation (DD) (Younes and Rinaudo 2015).

It is likely that distribution, degradation, and elimination processes are strongly dependent on molecular weight (MW). Possible sites of degradation, inferred due to the localization of chitosan, are estimated to be located in the liver and kidney. Chitosan has also been administered on tegument. Skin substitutes based on chitosan/collagen were relatively stable over time compared to collagen alone when implanted in rabbits (Ma et al. 2003). After oral administration, chitosan should present some degradation in the gastrointestinal tract. The digestion of chitosan, occurring predominantly in the gut, was found to be species dependent with hens and broilers being more efficient digesters (67–98% degradation after oral ingestion) than rabbits (39–83% degradation). In the same study, the digestion of N-stearoyl chitosan was negligible, indicating that the enzymatic degradation is dependent on chitosan's NH_2 availability. Chitosan degradation rate can be affected by the polymer's MW and degree of acetylation. In these conditions, N-substitution may affect enzymatic degradation, and it should be considered when new derivatives are suggested in formulations for systemic administration (Biswas et al. 2014).

Antimicrobial Properties

Biomass is a naturally abundant source of sustainable biopolymers, and in the last years, increasing environmental awareness has led to growing interest in the development of green compounds with improved performance. Chitosan is a cationic polysaccharide exhibiting both film-forming properties and bioactivity. Due to the quaternization of the amino group, chitosan is known for its natural antifungal and antibacterial activity and also shows abilities to retain included antimicrobial substances. The basic mechanism for its antimicrobial activity is based on the interactions between the positively charged amine groups and negatively charged cell membrane, causing disruption and release of intracellular compounds (No et al. 2002; Vaz et al. 2014). Additionally, two other mechanisms have been also identified as synergists:

1. Chelation of metals by chitosan functional groups, thus inhibiting bacterial enzyme activity.
2. In the case of yeast cells, chitosan segments are able to penetrate the cell membrane and inhibit the RNA synthesis (Kong et al. 2010).

The antimicrobial activity of chitosan against *L. monocytogenes* and yeast *C. albicans* was demonstrated. Chitosan with higher DD (92%) exhibited more remarkable antibacterial property against *S. aureus* (Zhuang et al. 2019). The ability of these compounds to kill or inhibit a wide range of microorganisms makes possible to be used for a number of applications, from hospital surfaces and medical devices to building materials and filtration devices. The biomedical application possibilities for chitin and chitosan into several broad areas of tissue engineering, bone substitutes, and wound dressings have been detailed by Anitha et al. (2014). Laurencin et al. studied biologically active chitosan matrices for tissue engineering application and reported that antimicrobial chitosan matrices could be an alternative to antimicrobial agents because they remove the biofilm problems, like microbe resistance to treatment, the invasion into immune system, and building of bacteria reservoirs which can seed infections elsewhere (Laurencin et al. 2008).

Mucoadhesive Properties of Chitosan

The excellent mucoadhesive properties of chitosan have often been linked to its ability to interact with negatively charged mucins by electrostatic attraction (Hejjaji et al. 2018). However, some authors have highlighted the complexity of the mucoadhesive interactions of chitosan and have suggested that hydrogen bonds and hydrophobic effects may also have certain functions (Nikogeorgos et al. 2015). Mucins are a family of complex high molecular weight glycoproteins secreted by the epithelial tissues (intestinal, respiratory, urogenital tracts). They consist of linear or branched oligosaccharides attached to the protein backbone. These glycoproteins are mostly carbohydrate (five sugars *N*-acetyl glucosamine, *N*-acetylgalactosamine, galactose, and fucose and sialic acid (*N*-acetylneuraminic), and traces of mannose and sulfate esters (Varki et al. 2009). The high concentration of sialic acid and sulfate ester leads to a negative charge on mucin which is the main reason for its mucoadhesive properties. Depending on the physiological conditions and physiochemical properties such as pH, the carboxylate group of sialic acid residues on mucin can interact with the positive charges (protonated amino group $–NH_3^+$) on the chitosan through electrostatic and hydrogen bonds (Leal et al. 2017). The pH of the medium as well as the presence of other chemicals can change the relative contributions of each type of physical interaction. Hydrophobic and hydrophilic interactions are also very important (Szymańska and Winnicka 2015). The presence of active functional groups (such as amines and hydroxyls) in chitosan opens broad opportunities for its chemical derivatization. There are a number of well-known chitosan derivatives; some of these are even commercially available. The most important chitosan derivatives relevant for pharmaceutical applications include trimethyl chitosan, glycol chitosan, carboxymethyl chitosan, and semi-acetylated chitosan. Numerous papers have been published on the use of chitosan-based materials for the preparation of transmucosal medications (Ways et al. 2018; Tzaneva et al. 2017).

Antioxidant Activity of Chitosan

In the last decade, extensive researches have been focused on the antioxidant and free radical scavenging abilities of chitosan and its derivatives and in vitro but also in vivo using animal models, and clinical trial tests strongly suggest that chitosan and

its derivatives will find new applications as natural biological defenses against the consequences of oxidative stress (Anraku et al. 2017; Anraku et al. 2018). Chang et al. studied the influence of chitosan molecular mass on antioxidant and antimutagenic properties. The authors used seven chitosan samples with MWs ranging from 2.2 to 300.0 kDa and demonstrated that the antioxidant and antimutagenic properties of chitosans were inversely related to chitosan MW. Chitosan generally exhibited higher antioxidant property when scavenging H_2O_2 and DPPH (2, 2-diphenyl-1-picrylhydrazyl radical, superoxide anion) free radical than when chelating Fe^{2+} (Chang et al. 2018).

Some derivatives of chitosan were tested as materials with improved antioxidant properties. Tan et al. prepared cationic chitosan derivative with 1,2,3-triazole and *N*,*N*,*N*-trimethyl moieties using cuprous-catalyzed azide–alkyne click chemistry approach and tested the antifungal and antimicrobial properties. The chitosan derivative bearing 1,2,3-triazole exhibited enhanced antifungal activity against four kinds of plant-threatening fungal strains and strong scavenging effect against superoxide radical as well as improved reducing power. The authors consider that the behaviors of the cationic chitosan derivative with 1,2,3-triazole are due to the introduction of quaternary ammonium and 1,2,3-triazole moieties and consider the new combination a very promising material for biomedical applications (Tan et al. 2018).

Chitosan could significantly reduce serum free fatty acid and malondialdehyde (MDA) concentrations and increase antioxidant enzymes' activities such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), indicating that this biopolymer regulated the antioxidant enzymes' activities and decreased lipid peroxidation. Chitosan and its derivatives have been indicated as dietary food additives and functional factors for their antimicrobial, hypocholesterolemic, and immune-stimulating effects (Xia et al. 2011a, b). Antioxidant activity of chitin, chitosan, and their derivatives can be attributed to in vitro and in vivo free radical scavenging activities, and therefore, these polymers may be used as functional ingredients in food formulations to promote consumer health and to improve the shelf life of food products (Ngo and Kim 2014).

Concerning the disadvantages of using chitosan-based material for biomedical applications, the variability and heterogeneity of the polymer is of great importance. Indeed, the difficulty of controlling the distribution of the acetyl groups along the backbone makes it difficult to get reproducible initial polymers (Bellich et al. 2016). There is a need for a better standardization of production processes in order to prepare reproducible initial polymers with the same characteristics; changes in the specifications of the polymer may significantly change its performances.

12.2.3 Chitosan Modification for Theranostics

Taken into consideration its biocompatibility and vast derivative potential, chitosan has proven to be an excellent choice for the design of theranostic systems. In order to accomplish its purpose as an efficient vehicle for both therapeutic and imaging

agents, chitosan must undergo a series of modifications. Depending on the final purpose, chitosan can be derived using specific molecules to enhance its properties. In Fig. 12.4 a few chemical modifications of chitosan are presented, and the remarkable properties of obtained derivatives are highlighted.

For instance, hydrophilic molecules such as glycol or PEG (polyethylene glycol) are being used as a means to enhance the water solubility of chitosan in basic or neutral conditions. As a result, PEGylated chitosan nanoparticles possess improved hemocompatibility and higher cellular uptake and are excellent vehicles for hydrophobic agents such as ibuprofen or paclitaxel. The enhanced properties of the glycol-modified chitosan nanoparticles have major implications in the passive targeting of tumors via the EPR (enhanced permeability and retention) effect, ensuring a longer circulation time and a higher accumulation of the nanocarriers at the tumor site (Zhang et al. 2002).

Choi et al. (2018) have developed a series of echogenic nanoparticles based on glycol chitosan for the dual imaging of cancer. The team has chemically conjugated iodine-contained diatrizoic acid to the glycol chitosan backbone for CT imaging purposes, followed by the encapsulation of perfluoropentane, a US imaging agent, through the oil-in-water (O/W) emulsion method. In addition to the comprehensive diagnosis provided by the CT/US dual-modal imaging, the study has shown an accurate and rather fast accumulation of the nanocarriers at tumor level, which can be assessed as a result of the glycol-modified chitosan and its properties. Furthermore, as seen in a study conducted by Min et al. (2015), echogenic glycol chitosan nanoparticles can be engineered not only for diagnostic purposes but also for the treatment of cancer. The study focuses on the development of glycol chitosan nanoparticles that can simultaneously be employed as a vector for cancer-targeted ultrasound imaging and ultrasound-triggered drug release. The nanoparticles were synthesized by O/W emulsion method and encapsulate both perfluoropentane and a hydrophobic anticancer drug (docetaxel).

It is essential for theranostic nanosystems that are not characterized by active targeting to exhibit proficient tumor-homing abilities and reduced nonspecific uptake by other tissues. Therefore, to assure a precise treatment and monitoring of the disease, the nanoparticles should have to benefit from an efficient EPR effect. The EPR effect is strictly dependent on their characteristics such as particles' size, morphology, and chemical interactions with different biological media. As proven in many studies, glycol and PEG modifications of chitosan increased the nanoparticles' abilities to delivering therapeutic or imaging agents to specific cancer sites via EPR (Prabaharan 2015).

In order to induce the formation of nanoparticles through self-assembly, hydrophobic molecules such as palmitoyl or caproyl can be added to the chitosan backbone through the N-acylation of the NH_2 group. These modifications resulted in improved polymeric network stability and increased the ability to encapsulate hydrophobic agents inside the nanoparticle core. In addition, the N-acylation of chitosan entails a rather simple process, making it both a time- and resource-efficient method (Fathi et al. 2018).

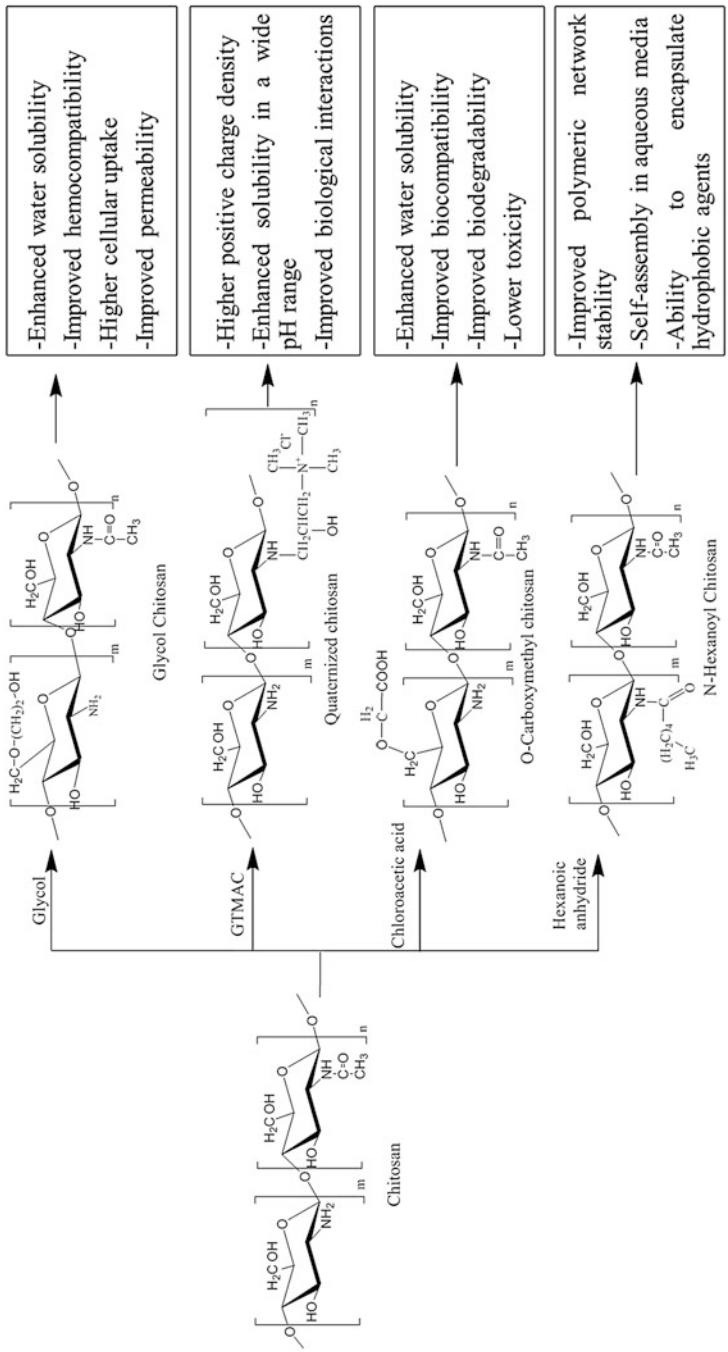

Fig. 12.4 Chemical modifications of chitosan for theranostic applications

Hsiao et al. (2015) proposed a nanosystem that combines oleic acid-stabilized iron oxide nanoparticles (OA-IONP) with a functionalized polysaccharide – an amphiphilic copolymer of hexanoyl-chitosan-PEG (CP6C) – and encapsulates paclitaxel (PTX) in its hydrophobic region. Chlorotoxin was then conjugated onto the nanocarrier surface to act as a targeting peptide which binds to a certain molecule (viz., MMP-2) that is overexpressed in the majority of brain tumors. The inner core of the nanocarrier comprising of OA-IONP is bound to the outer shell through hydrophobic interactions. These particles have excellent superparamagnetic properties and can be tracked via MR imaging, thus enabling real-time observation of the ongoing treatment.

The selection of the material used in the development of a drug carrier is of critical importance. The selected polymer must possess specific characteristics such as the ability to protect its cargo from enzymatic or hydrolytic degradation and to enhance its interactions and permeability across biological barriers. Native chitosan is a polycationic saccharide, which makes it soluble in various acidic media, but its positive charges are limited to the number of conjugations and ionic complexations it undergoes, and so are its interactions and stability. Quaternized chitosan, instead, exhibits a higher density of positive charges and enhanced solubility in a wider pH range, making possible better interactions at a biological level and also to effectively permeate gastrointestinal junctions (Desai 2016).

Nanocarriers based on quaternized chitosan (*N*-trimethyl chitosan, *N*-diethylmethyl chitosan, *O*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride/O-HTCC, *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride/HTCC, etc.) have been previously designed. For instance, Soares et al. (2016) developed O-HTCC iron oxide nanoparticles. The team of researchers synthesized O-HTCC-coated Fe_3O_4 nanoparticles loaded with anticancer drug DOX through ionotropic gelation method. Taking into consideration the behavior of chitosan compared to its quaternized counterpart at different pH values, the current study has concluded that drug release profile is influenced, with O-HTCC-Fe_3O_4 nanoparticles exhibiting an increase in the amount of DOX released compared to CS-Fe_3O_4 at a pH value of 4.5. Also, it has been observed that the presence of Fe_3O_4 fastens the release.

Another study conducted by Huang et al. (2019) explored the synthesis of CuS nanoparticles as a photothermal agent stabilized using quaternized chitosan. CuS is responsive to near-infrared (NIR) light and is able to convert photon energy to thermal energy, which results in the destruction of the cellular medium. Also, Cu ions, when activated by an appropriate light wavelength, can generate reactive oxygen species (ROS) through redox reactions and undergo oxidative processes at protein and DNA level. These processes can generate inflammation and initiate apoptosis, leading to extensive tumor damage. Moreover, fluorescence emission occurs as a result of relaxation from the excited state of the compound, which can be used as a source for imaging applications. On the other hand, CuS nanoparticles present high levels of biological toxicity and are insoluble in water; therefore, their surface has to be modified in order to achieve more desirable characteristics. Thanks to its unique properties, quaternized chitosan has been chosen as a stabilizer and

proved to be an excellent choice for enhancing CuS nanoparticles' therapeutic activity. The study concluded that engineered nanoparticles induced low toxicity in all vital organs compared to the control group.

To further enhance the properties of chitosan in terms of water solubility, biocompatibility, biodegradability, and low toxicity, the biopolymer can undergo certain modifications which enable better interactions at macromolecular level and increased cellular uptake. Carboxymethyl substituents can be added at the primary hydroxyl group of chitosan (*O*-carboxymethyl chitosan) or both amino and hydroxyl sites (*N*,*O*-carboxymethyl chitosan). Depending on the purpose meant for the nanocarriers, *O*-carboxymethyl chitosan can be an option if further conjugations are needed, as it presents amine and carboxyl sites available for modifications (Ji et al. 2011).

In a study conducted by Li et al. (2014a, b), the team engineered a theranostic system based on a core–shell structure, composed of $Fe_3O_4@SiO_2$ coated with O-carboxymethyl chitosan-folic acid (OCMCS-FA). The main challenge when dealing with $Fe_3O_4@SiO_2$ nanoparticles is their low biocompatibility and low specific cellular uptake. Hence, OCMCS has been chosen as coating for its excellent water solubility, low toxicity, and reduced immunogenic response; meanwhile FA has proved to enhance targeting potential. The novel nanosystem not only combines two targeting mechanisms (magnetic- and receptor-mediated targeting) but is also characterized by improved biologic interactions and increased safety, thanks to its OCMCS outer layer.

Thiolated derivates of chitosan are the main choice for engineering nanosystems with enhanced mucoadhesion. A higher mucoadhesivity is a desirable feature for a theranostic system, as it enables a prolonged contact which results in increased concentration of nanosystems at their specific delivery site. The enhanced mucoadhesion of thiolated derivates of chitosan is a result of the covalent interactions that take place between the thiolated polymer and the cysteine-rich subdomains of mucus glycoproteins via disulfide bonds (R–S–S–R′) (Kast and Bernkop-Schnurch 2001).

Yang et al. (2016) have designed hybrid self-assembled nanoparticles based on the modification of chitosan with folic acid. The team studied the possibility of engineering a complex nanostructure that includes both an encapsulated chemotherapeutic drug (docetaxel) and VEGF shRNA (vascular endothelial growth factor short hairpin RNA) gene-silencing therapy as a means to combat multidrug resistance in cervical cancer. Furthermore, in order to assess the evolution of the synergistically applied therapy, researchers included in the structure two fluorescence imaging agents, fluorescein isothiocyanate (FITC) and carbon quantum dots (C-dots).

12.2.4 Types of Chitosan-Based Systems for Theranostic Applications

12.2.4.1 Self-Assembled Nanoparticles

Self-aggregated nanoparticles, also known as polymeric micelles, have unique core–shell structure, including a hydrophobic core that provides a loading space for the lipophilic and poorly water-soluble drugs and hydrophilic shell that assures stability in the aqueous media. Polymeric micelles based on biocompatible and biodegradable polymers represent a promising research area, as remarkable properties of these biopolymers can significantly improve their biological behavior as theranostic nanosystems able to defeat cancer drug resistance (Yang et al. 2014; Zhu et al. 2015).

Several representative works have explored the self-assembly ability of amphiphilic modified chitosan (AMC) derivates as a feasible strategy to design various drug delivery platforms, with promising results as chemotherapeutic systems (Chiu et al. 2010; Yhee et al. 2014), the AMC self-assembled micelles being more stable than the most of low molecular weight surfactant-based micelles (Larsson et al. 2013).

The nanoscale size of polymeric micelles allows their efficient accumulation in tumor tissue through the EPR effect by passive targeting approach, but at the same time, their surface may be functionalized with a series of small compounds with targeting functions such as folic acid (Wang et al. 2011), arginine–glycine–aspartic acid (Zhao and Zhai 2013), or biotin (Yang et al. 2014). Among all, biotin (vitamin B7 or H) is known to be involved in various cellular functions such as cell growth or signal transduction (Vadlapudi et al. 2012). Furthermore, it has been revealed that biotin receptors are overexpressed on the tumoral cell surface, especially breast cancer cells.

In this context, Balan et al. (2016) have synthesized biotinylated N-palmitoyl chitosan through the reaction of *N*-palmitoyl chitosan with biotin, via carbodiimide chemistry, and developed self-assembled nanoparticles loaded with docetaxel. Nanoparticles exhibited a good drug loading ability and a pH-dependent release profile of drug, susceptibility to biodegradation, and hemocompatibility, properties which can be further exploited in drug delivery applications.

Recently, Yu et al. (2018) proposed an effective strategy to deliver gambogic acid (GA) to the liver using *N*-octyl-*N*-arginine-chitosan micelles. Gambogic acid (GA) is a potent anticancer agent that suppresses the signaling pathway of nuclear factor-kappa B and connects to the transferrin receptor. After exposing the human hepatocellular carcinoma, HepG2 cells, to GA-loaded micelles for 2 h, a high cellular uptake was noted. Furthermore, in vivo studies indicated that the micelles could increase the half-life (1.5–2.0-folds) and showed a high biodistribution to the liver.

Because cancer exhibits abnormally high local acidities compared to normal tissues (pH 7.4), smart drug delivery systems that are triggered by environmental conditions have been developed to enhance cancer therapeutic efficacy while limiting side effects. For instance, Lim et al. (2013) designed smart chitosan-based magnetic nanocomposites capable of pH-sensitive doxorubicin release and

MR-guided imaging. In vivo therapeutic efficacy trial of these theranostic nanocomposites showed effectiveness in delaying tumor regression.

12.2.4.2 Cross-Linked Chitosan-Based Nanoparticles

Self-assembled polymeric micelles are formed and held together by weak physical interactions, which entails poor stability for in vivo administration of hydrophobic drugs. Hence, there are a number of risks associated with the use of these micelles as carriers, such as the possibility of dissociation if the dilution is made below the critical micelle concentration (CMC) and, as a result, the release of the therapeutic agent off-site. Micelle dissociation can occur also due to nanoparticle–blood interactions, when certain blood components may cause variations in the kinetic stability of the suspension. It is essential for a drug delivery system to exhibit a high stability profile for as long as needed before reaching its target (Lu and Park 2013). Therefore, stability-enhancing strategies have been extensively used as a means to enhance nanoparticle stability in vivo and maintain their integrity. Some of these strategies include crosslinking and mineralization through deposition of inorganic materials.

Chitosan is a polycationic polymer with several highly reactive functional groups that enable the formation of three-dimensional networks through both ionic and covalent crosslinking, which result in structurally stable nanoparticles. Ionic crosslinking of chitosan is a rather facile process, which can be conducted in mild conditions, at room temperature, in the absence of organic solvents (Swierczewska et al. 2016). The ionic complexation may occur between the positively charged chitosan and other negatively charged structures, which can be either polymers or small molecules. Sodium tripolyphosphate (TPP) is commonly used as a crosslinking agent for chitosan-based drug delivery systems and has been broadly used for this purpose. Its polyanionic structure enables the formation of physical bonds between ionized hydroxyl and amine moieties. Similarly, ionic complexation can take place between chitosan and other negatively charged polysaccharides: hyaluronic acid, (Deng et al. 2014) alginate (Thu et al. 1996), or chitosan and certain inorganic salts, such as calcium chloride (Kalliola et al. 2017).

Zahraei et al. (2016) developed a theranostic nanosystem composed of nanoparticles that combine a manganese zinc ferrite core as a negative contrast agent and an outer shell made of ionically cross-linked chitosan. The crosslinker was TPP, a safe, nontoxic polyanion. For comparison, PEG and dextran have also been used as coating materials, as their behavior is different at certain pH values. The study concluded that the hydrophilicity of the coating material is responsible for the proton relaxation rates. Thus, the closer water molecules are to the magnetic moment, the better the nanoparticles act as an imaging agent. Chitosan and dextran have been found to exhibit higher relaxivity values compared to PEG, but in turn, the PEG coating reduces particle aggregation. Furthermore, dextran and chitosan nanoparticles have shown interesting potential for hyperthermia.

Polyelectrolyte complexation can also take place between chitosan and an anionic polysaccharide such as alginate. The formation of this complex is based on the interaction between the protonated NH_2 groups of chitosan and deprotonated COOH

groups of alginate. Also, it has been found that the network of the newly formed complex can be strengthened by combining polyelectrolyte complexation with ionotropic gelation. TPP can be used as a crosslinker, but studies show that Ca^{2+} ions help alginate to bind to the chitosan 100 times better than in their absence (Sankalia et al. 2007; Tiwari et al. 2015). These findings were also confirmed by a study conducted by Türkoğlu and Taşcıoğlu (2014). The team has reached the conclusion that Ca^{2+} considerably influences the formation of a stable network between chitosan and alginate. Moreover, they have discovered that combining Ca^{2+} ions with anionic salts in the gelation process can result in stronger and more stable complexes rather than using Ca^{2+} or anions alone.

Theranostic systems based on this specific type of crosslinking are very promising and have proven to be efficient in both imaging and therapeutic approaches. For example, Yang et al. (2017) have engineered a novel multifunctional colloidal nanosystem which encompasses the photosensitizer chlorin e6 (Ce6) and the chemotherapeutic agent doxorubicin (DOX). The nanocarriers have a complex structure, with a magnetic Fe_3O_4-Au core, a functionalized silica mesoporous shell, alternatively covered by biocompatible chitosan–alginate multilayers and functionalized with P-glycoprotein small hairpin RNA (P-gp shRNA) in order to fight multidrug resistance. The layers of chitosan–alginate not only ensure the nanosystem's biocompatibility but are also pH-sensitive gatekeepers, having the important role of releasing the therapeutic agent in specific acidic environments.

Covalent or chemical crosslinking means that two or more molecules engage in the formation of covalent bonds with the purpose to obtain a stronger three-dimensional structure. Chemical bonds are far stronger than physical bonds and ensure more stability to prepared nanomaterials (Li et al. 2018). However, one of the main concerns is that covalent crosslinking might be too stable, and therefore the obtained nanoparticles might release insufficient amounts of therapeutic agent or might even negatively influence normal physiology due to prolonged circulation (Qiu et al. 2007). Chitosan can be chemically cross-linked using agents such as glutaraldehyde (Laroui et al. 2010) or genipin (Ding et al. 2017).

A theranostic strategy based on the synthesis of microspheres capable of delivering radiotherapy for unresectable liver tumor has been developed by Cho and Choi (2018). The so-called in vivo generator is comprised of chemically cross-linked chitosan microspheres loaded with $^{166}Dy/^{166}Ho$, a nontoxic parent–daughter isotope pair. This pair is able to participate in enzymatic reactions, and more than that, ^{166}Ho's paramagnetic properties can be exploited for medical imaging purposes using single-photon emission computed tomography and magnetic resonance imaging. The microspheres were prepared by W/O emulsion, and glutaraldehyde-saturated toluene was added as crosslinker. The study concluded that the biocompatible particles have real prospects of treating intractable liver tumors through high-energy radioactive decay as well as monitoring malignant cells via MRI or SPECT.

Song et al. (2015) developed nanocarriers for photothermal therapy (PTT) based on PEGylated chitosan. The ultrasmall nanoparticles (5 nm) were obtained by reverse microemulsion, and genipin was used as a covalent crosslinker. Indocyanine green (ICG) has been used as an imaging and PTT agent as it possesses remarkable

photochemical and photobiological properties (Li et al. 2013). In spite of these characteristics, ICG has several drawbacks regarding thermal degradation, aqueous degradation, and photodegradation which can be successfully overcome by its encapsulation in a chitosan-based nanocarrier. The particles exhibited good dispersibility, good stability at various pH values, and low cytotoxicity and, most of all, succeeded in suppressing tumor growth in mice.

12.2.4.3 Chitosan-Based Nanocapsules

The most common protocols of drug loading on the theranostic nanosystems imply either covalent binding or entrapment of the drug into a polymer layer (Mahmoudi et al. 2011). In some cases, covalent linkage can be too strong to be cleaved by cellular enzymes, in particular if access to the drug is hindered by the polymeric coating (Shkilnyy et al. 2010); meanwhile the entrapment of the drug within a polymer often leads to a burst effect (fast initial release) and/or to a low release in the absence of stimuli (Yang et al. 2010).

The use of nanocapsules can overcome these drawbacks. Nanocapsules can be defined as nano-vesicular systems that exhibit a typical core–shell structure in which the drug (in liquid/solid form or as a molecular dispersion) is confined to a reservoir or within a cavity surrounded by a polymer membrane or coating (Anton et al. 2008; Letchford and Burt 2007). These structures are usually prepared by nanoprecipitation or double emulsification methods. Nanocapsules can also carry the active substance on their surfaces or be embedded in the polymeric membrane (Khoee and Yaghoobian, 2008). The advantages of nanocapsule systems as nanotherapeutic tools include high encapsulation efficiency, maintenance of drug levels within a desired range by reducing their systemic distribution, and the possibility to administrate lower but more accurately targeted doses of the cytotoxic compounds (Pinto et al. 2006). The preparation and biomedical applications of nanocapsules have been reviewed in several excellent articles (Mora-Huertas et al. 2010; Levine et al. 2008). For diagnostic purposes, nanocapsules have also been applied in a variety of imaging modalities including fluorescence microscopy, PET, and MRI for noninvasive monitoring (Sun et al. 2007; Kelly et al. 2005). The theranostic applications of nanocapsules using NIR agents in combination with different chemotherapeutics (e.g., paclitaxel and irinotecan) have been tested in different cancer models.

In the last years, magnetic nanocapsules based on biocompatible polymers are emerging as excellent nanocarriers, especially for the delivery of chemotherapeutic drugs (Ashjari et al. 2012; Chiang et al. 2014; Ling et al. 2011). In this regard, Balan et al. (2015) prepared, through a double emulsion method, doxorubicin-loaded magnetic nanocapsules based on *N*-palmitoyl chitosan and magnetite. Magnetic nanocapsules exhibited suitable magnetic saturation, superparamagnetic behavior, and good ability to incorporate a chemotherapeutic agent. These characteristics, correlated with the biocompatibility and biodegradability of magnetic nanocapsules, are good premises for the suitability of proposed magnetic nanocapsules as drug delivery system for therapeutic agents.

12.3 Chitosan in Clinical Applications: Key Challenges

12.3.1 Drug Delivery, Bioimaging, and Hyperthermia

The progress in nanotechnology has contributed to the development of multifunctional nanosystems that enable specific delivery of imaging agents and therapeutic drugs to target diseased tissues for cancer imaging and therapy. A series of noninvasive imaging modalities is employed for the detection of cancer at early stages and monitoring the drug fate in living systems, in real time (Fang and Zhang 2010). These noninvasive imaging modalities include positron emission tomography (PET), magnetic resonance imaging (MRI), X-ray computed tomography (CT), single-photon emission computed tomography (SPECT), ultrasound (US), optical fluorescence, and near-infrared fluorescence (NIRF) imaging (Lee et al. 2012; Rhee et al. 2014). Among them, primarily molecular imaging techniques including PET/SPECT and optical imaging are characterized by high sensitivity; meanwhile primarily morphological/anatomical imaging techniques like CT, US, and MRI are highlighted by high spatial resolution (Huang et al. 2012). Recently, integrated molecular/anatomical systems (e.g., SPECT/CT) have been adopted to combine their strengths in order to obtain more accurate data (de Smet et al. 2013; John et al. 2013).

In another study, Agyare et al. (2014) designed targeted theranostic nanocarriers for MRI-based early detection and treatment of cerebrovascular inflammation. They developed polymeric nanocarriers loaded with Magnevist®-conjugated chitosan and cyclophosphamide and further functionalized with putrescine-modified F(ab′)2 fragment of anti-amyloid antibody, IgG4.1 (pF(ab′)24.1). The developed theranostic nanosystem-enabled imaging of cerebrovascular amyloid by both MRI and SPECT and caused decrease in pro-inflammatory cytokine production by amyloid beta-challenged BBB endothelium than free drug.

Glycol chitosan-based nanoparticles (GC NPs) have been intensively studied as theranostic nanosystems. For instance, Kim et al. (2010) developed a promising nanoconstruct based on GC-5β-cholanic acid conjugate labeled with a near-infrared fluorophore (cyanine 5.5, Cy5.5), with high tumor-targeting characteristics, good colloidal stability, and strong NIRF signals in tumor tissues after passing through the in vivo filtration system of the liver and spleen (Na et al. 2011; Kim et al. 2010). Likewise, Kim et al. (2015a, b) prepared a fibrin-targeted CT based on GC NPs for visualization of cerebrovascular thrombus and guiding thrombolytic therapy. Micro-CT imaging allowed quick detection of cerebrovascular thrombi and tracking of tissue plasminogen activator-induced thrombolysis.

Yoon et al. (2014) utilized two distinctive formulation strategies of modified glycol chitosan-based NPs (GCNPs) with the aim to encapsulate doxorubicin (DOX) or a complex Bcl-2 siRNA for gene therapy (siRNA-GCNP) while carrying imaging agents on their surfaces. The team reported that theranostic nanosystems were able to overcome drug resistance in cancer by downregulating antiapoptotic defense mechanisms of cancer cells while triggering apoptosis. A similar siRNA delivery strategy was also proposed to suppress P-glycoprotein, the protein responsible for

the efflux of chemotherapeutics in multidrug resistance, and in vivo results indicated that a combination therapy with subtherapeutic doses of DOX can effectively inhibit tumor growth in MCF-7/ADR tumor-bearing mice (Yhee et al. 2015).

Hyperthermia, used alone or in combination with other cancer therapies, treatment is generally well tolerated and, if the temperature does not exceed 45 °C, rarely affects normal tissues, this being one of the main advantages over other treatment techniques (Habash 2018). The approach pursued by Srinivasan et al. (2013) to obtain a multifunctional vector capable of in vivo imaging and also hyperthermic effects in different cancer cell lines consisted in conjugating IR820, a NIR fluorescent dye, with chitosan.

Lin et al. (2015) designed a protease-mediated drug release nanosystems based on PEG-/chitosan-coated magnetic nanoparticles with the cytotoxic agent – methotrexate (MTX) – attached to the surface via a protease-cleavable amide linker. The structural similarity between MTX and folate-enabled cellular uptake into folate-receptor-overexpressing HeLa cells and intracellular protease-mediated drug release. At the same time, the nanosystems allowed in vivo-targeted MR and fluorescence imaging in HeLa tumor growth in Sprague-Dawley rats.

Another approach was proposed by Wang et al. (2016a, b) who used SPIO nanoparticles coated with chitosan-PEG-grafted polyethyleneimine as nanovectors for the delivery of targeted siRNA to orthotopic hepatocellular carcinoma (HCC) xenografts in a mouse model. In this case, the negatively charged Luc gene-targeting siRNA was loaded to polymer-coated magnetic nanoparticles through electrostatic interaction with PEI segments. PEG linkers were further conjugated on the surface to protect siRNA and facilitate conjugation with antibody against human glypican 3 (GPC3) receptor. Upon intravenous injection, the functionalized nanovectors enabled specific targeting ability to HCC and significantly suppressed Luc expression of the tumor.

A facile approach to fabricate a tumor-specific theranostics for targeted DOX delivery and magnetic resonance (MR) imaging was established by Xie et al. (2019). The team developed a hybrid cluster bomb by co-precipitation of polyethylene glycol (PEG)-modified chitosan (CS), oleylamine-modified Fe_3O_4 (OA-Fe_3O_4) nanoparticles, and DOX. The in vitro studies indicated that clusters could be uptaken into HepG2 cells to deliver DOX into the cell nuclei with enhanced anticancer efficacy in comparison with free DOX. In the tumor intracellular microenvironment, the stimuli-responsive hybrid cluster bombs disassembled and re-self-assembled into the OA-Fe_3O_4 nanoparticle clusters with higher Ms for MR imaging-guided diagnosis or hyperthermia.

12.3.2 Chitosan in Bioresponsive Tissue-Engineered Scaffolds

The main objective of tissue engineering is to obtain functional tissues that allow implantation, regeneration, or replacement of functionally inactive biological tissue. In order to achieve this objective, some conditions (mechanical, structural, and functional) must be respected. At the tissue level, cells are organized in a three-

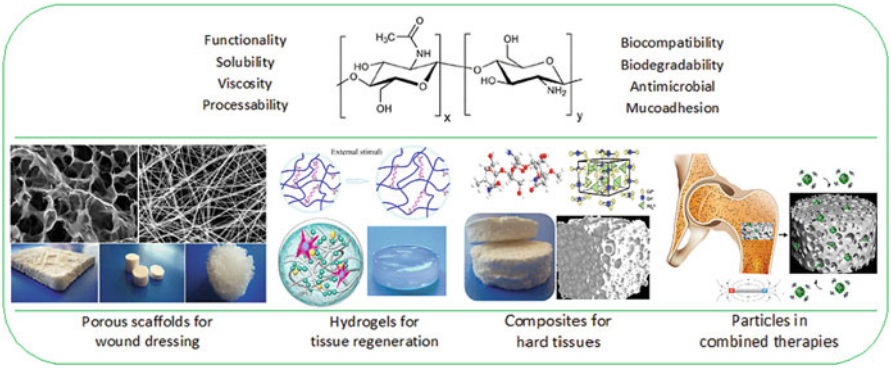

Fig. 12.5 Chitosan in tissue engineering

dimensional form and are surrounded by macromolecules of the extracellular matrix (collagen, proteoglycans, glycosaminoglycans, glycoproteins). On the other hand, the extracellular matrix is the supportive environment for the growth and differentiation of cells. Therefore, in tissue engineering, specific cells, support matrix, and signal biomolecules work together for a successful treatment or implantation. A scaffold should stimulate the adhesion and growth of cells that migrate from neighboring tissues or allow the cell differentiation into porous structure, ensuring cellular nutrition and excretion of metabolites. Some important properties should be met by any biomaterials for tissue engineering: (i) biocompatibility; (ii) porous structure with interconnected pores; (iii) promoting cell attachment, differentiation, and proliferation; and (iv) suitable mechanical properties for handling and stimulating the cells' adhesion and differentiation of mechanical properties (O'Brien 2011). The scaffold should not induce acute or chronic response, should be biodegradable, and, finally, should be manufactured into a variety of shapes (Zhu et al. 2005).

Due to its remarkable properties, chitosan is a good candidate for the preparation of scaffolds, which could substitute the missing or damaged tissue and organ and allow cell attachment and proliferation (Cai et al. 2017). Several methods have been developed to finely shape chitosan hydrogels and foams (or sponges) as 3D scaffolds applicable for soft or hard tissue engineering (Ikeda et al. 2014; Chaudhari et al. 2016). Various types of scaffolds with potential applications in soft and hard tissue engineering are presented in Fig. 12.5.

Hydrogels are interesting biomaterials considering their compatibility with a majority of living tissues. Generally, they are soft and bendable, which minimizes the damage to the surrounding tissue during and after implantation in the human body (Feksa et al. 2018). The mechanical properties of hydrogels can mimic those of soft body tissues, which allow the gels to insure both functional and morphological characteristics of the repairing tissue (Geckil et al. 2010), and therefore hydrogels are often used as biomedical scaffolds for tissue replacements and drug and growth factor delivery systems (Tang et al. 2012). Various chitosan hydrogels have been

developed, presenting either reversible or irreversible gelation. Chitosan can be physically associated, coordinated with metal ions, or irreversibly/chemically cross-linked into hydrogels (Ahmadi et al. 2015).

The amino and hydroxyl functional groups of chitosan provide chitosan derivatives with improved properties and applications for tissue engineering. Acrylic acid-grafted chitosan exhibits improved swelling properties, higher mesh mechanical resistance, and reduced cytotoxicity (Rusu et al. 2016). Chitosan crosslinking is a necessary process to improve the stability and durability of chitosan in applications as tissue engineering-controlled drug delivery. The chemical crosslinking of this polymer is irreversible and is achieved either by covalent or ionic crosslinking or both. Each type of crosslinking has advantages and disadvantages, chemical crosslinking being more resistant, but even more toxic, while the mechanical strength of physical hydrogels is lower but does not present biocompatibility problems. Physical hydrogels are preferred for achieving soft tissues and controlled drug delivery systems due to higher susceptibility to changes in pH, temperature, or ionic strength (Sood et al. 2016).

Chitosan is biocompatible and biodegradable, the products resulting from the degradation process being nontoxic. This biopolymer is used as a dressing material and hemostatic agent in treating skin lesions. A commercial product using this feature is the HemCon bandage that has the ability to stop severe bleeding by adhering to negatively charged cells as well as by attracting negatively charged red blood cells that seal the wound. The dressing is applied by pressing on the wound, bleeding, letting it act for up to 48 h. Removal occurs when saline is applied over dressing (Khoshmohabat et al. 2016). Chitosan can be used as a tissue engineering matrix with applications in the regeneration of bone, cartilaginous, cutaneous, and nervous tissue. Network porosity is of great importance for the swelling capacity, cell adhesion, and cell proliferation. The interconnected pore network allows cell migration and proliferation to the damaged tissue, replacing the tissue in the injured area. Various types of chitosan-based hydrogels with biomedical and pharmaceutical applications are reported in literature. They show some common features such as biocompatibility, biodegradability, and lack of cytotoxicity, which recommend chitosan in further tests to improve the products obtained from this biomaterial (Hamedi et al. 2018; Pellá et al. 2018).

Hydrogels containing different ratios of chitosan and collagen were made by initiating gelation using β-GP and temperature by Cho et al. (2005). This process was performed at physiological pH and temperature, so living cells could be incorporated directly into the hydrogel matrix. The presence of collagen in chitosan-collagen-like materials has been associated with increased cell proliferation as well as increased gel density and resulting rigid matrix. Chitosan content has been associated with improved osteogenic differentiation, as assessed by gene expression and the presence of osteogenic markers. Beta-glycerophosphate has been shown to be an osteogenic supplement when added to stem cell cultures derived from human bone marrow stromal cells (hBMSC). It was also used as a catalyst to cause a sol–gel transition in chitosan solutions at physiological pH and temperature. This form of chitosan hydrogel has been investigated for use in tissue engineering of cartilage

because the polysaccharide nature of chitosan resembles cartilage glycosaminoglycans and has shown good compatibility with hBMSC and chondrocytes. The use of β-GP as an agent to cause both the gel-to-gel transition in chitosan and the collagen fibrinogenesis presents the possibility of pure chitosan composites, pure collagen, and chitosan–collagen composites without the need for freeze-drying or chemical crosslinking. Therefore, it is of great interest to manufacture a chitosan–collagen composite that supports cell survival but can also be injected and gelled in situ to effectively fill tissue defects (Wang and Stegemann 2010; Wang et al. 2013).

Neovascularization is an essential part of regenerating tissues that provide food and nutrients to cells. There is a real need for proangiogenic biomaterials to help heal wounds. Degradation studies were tested in three different environments, such as phosphate-buffered saline (PBS), lysozyme, and hydrogen peroxide, and relatively greater degradation was observed in hydrogen peroxide. The hydrogel loaded with thyroxine (TLH-1)-stimulated angiogenesis. The collagen/chitosan hydrogel has a good biocompatibility with effective epithelial and nerve restoration. Both collagen (Coll) and chitosan (CS) have been used in a variety of bioactive materials for different biomedical applications (Aleem et al. 2017).

Angiogenesis is a complex process involving the formation of new blood vessels from the preexisting ones. Davis et al. (2006) reported the proangiogenic effect of thyroxine (T4) at physiological concentrations. They reported evidence that the hormonal effect was initiated at the endothelial cell plasma membrane. In addition, thyroid hormones regulated cellular metabolic activity as well as cell proliferation. The preparation, biocompatibility, and angiogenic potential of the chitosan/collagen loaded with thyroxine were reported. Synthesis of essentially three CS/Coll hydrogels with variable concentrations of thyroxine and control (without T4) was compared. Porous hydrogels were synthesized by freeze gelation, and thyroxine was loaded by the physical loading method. The synthesized materials showed a good level of degradation: 16% weight loss in PBS solvents and over 60% weight loss in lysozyme, after 6 days. In addition, these materials were placed on the chorioallantoic membrane in fertilized chicken eggs from day 8 to day 14 to investigate their angiogenic growth and tissue in this membrane potential. It has been observed that low-concentration thyroxine material (TLH-1) significantly stimulates angiogenesis compared to the high-concentration thyroxine material (TLH-10). Overall, TLH-1 has been shown to be suitable materials for future tissue engineering applications. The hydrogels have shown good levels of in vitro degradation (Aleem et al. 2017).

Chitosan can be used in regenerating surgery as a substitute for cartilage or bone (osteocompatible and osteoconductive properties). The bone consists of an organic collagen matrix and a mineral component. The bone is composed of 2030 wt% collagen and 60–70% wt% inorganic hydroxyapatite-like crystals and water. The formation and the maintenance of the bone is controlled by a variety of factors (genetic, dietary, hormonal, and physical activity) (Palmer et al. 2008).

The bone thus undergoes constant remodeling. Normally there is a balance between bone formation and bone breakdown. Globally, rates of bone disease are dramatically increasing due to as follows:

1. The elderly populations living longer, outliving their joint replacements.
2. Failure due to loosening of implant (5–10 years lifetime).
3. A non-negligible percentage of hip replacement operations are repeat procedures.

Bone disease, such as osteoarthritis (degradation of joints, including articular cartilage) or osteoporosis (reduced bone mineral density leading to increased risk of fracture), is dramatically increasing. As a result, chitosan-based matrices, associated with other polymers, might be used to elaborate implants with required properties. Recently, chitosan and its derivatives were described as good candidates to regenerate cartilage because they degrade while new tissues are formed, with no inflammatory reaction or toxic degradation products (Bressan et al. 2011). As the chitosan is transformed into glucosamine in the body, many studies concern its use in the form of injections into the knee to stimulate cartilage repair. Indeed, the surgical techniques of damaged cartilage repair require the formation of a blood clot on the lesions. Incorporation of chitosan in liquid blood allows an adherent and more stable blood clot, which leads to better repair of the cartilage, thanks to this chitosan-blood implant. Glucosamine is well-known for its anti-pain and anti-inflammatory effects and for preventing deterioration of the articulations. Chitosan derivatives ensure a progressive release of the monomer (Marchand et al. 2009).

Chitosan has a critical role in cell attachment and growth. Indeed, epithelial cells can grow on a film of chitosan. In addition, it is also a good candidate for synthesizing scaffolds because of its biocompatibility, biodegradability, non-toxicity, high affinity toward proteins, and antimicrobial affinity. The main disadvantage of chitosan-based matrices is its poor mechanical properties. To improve them, crosslinking can be carried out. To overcome this problem, researchers are studying new hybrid scaffolds made up of chitosan and synthetic polymers, prepared by chemically cross-linked chitosan and synthetic polymer for potential use in skin engineering applications (Costa-Júnior et al. 2009).

12.3.3 Bio-sensing Applications

Biosensors are finding diverse applications in clinical diagnosis, environmental monitoring, and specific biomolecule release (Prasad et al. 2016). Extensive researches have been done toward biosensor designing due to its specificity, fast detection time, and high selectivity to detect analytes (proteins, DNA/RNA, cells), within the miniaturized settings (Yager et al. 2008). A biosensor consists of a transducer combined with a biologically active molecule that converts the biochemical response into a quantifiable signal. It is comprised of three basic components: a detector, a transducer, and a signal processor. The transducer can be optical, acoustic, electrochemical, and calorimetric, depending on diagnosis and the analyte characteristics. Biosensors have been broadly studied based on various detection principles: conductometric, amperometric, potentiometric, and voltammetric (Malhotra et al. 2017). Different types of sensing systems have been tested, like enzymes (Sakamoto et al. 2015), DNA/RNA (Linardy et al. 2016), antibodies,

receptors, organelles, and animal cells/tissues (Darsanaki et al. 2013). Some considerations should be followed in biosensor designing: (i) it should work in a wide range of pH and temperature conditions, (ii) it should have a wide dynamic range and high sensitivity, and (iii) it should involve facile fabrication steps (Jolly et al. 2016). Various biomaterials have been tested to develop various types of personalized biosensing prototypes. These include natural biomaterials such as chitosan and collagen, metal oxides and carbon nanotubes (CNT), quantum dots, and grapheme (Maxwell et al. 2015; Li et al. 2014a, b).

Chitosan has proven to be a suitable modifier of transducer surface by enabling mild yet stable immobilization of biological recognition elements (BREs). Other biological polymer that can act as surface modifier (such as polylysine, polyglutamate, and poly-arginine), alginic acid, and hyaluronic acid have been tested. Chitosan seems to have the greatest potential to be used in sensor development by offering attractive benefits like nontoxicity, low cost, and solubility in aqueous solution at mild acidic pH (6.3) which ensures immobilization of BREs without actually causing their denaturation (Baranwal et al. 2018).

Substrate selection is a key step in device fabrication as it provides the foundation on which to construct the biosensor. A substrate that conforms more the contours of the surface indicates a large contact area for sample collection such as body fluids. Such devices can often also be used in combination with added functionalities (e.g., drug delivery) (Wang et al. 2016a, b).

Material selection considers the location of intended use (internal or external to the biological system). Characteristics of bendability, stretchability, conformability, and fracture resistance are obtained by two approaches: by selection/synthesis of flexible and stretchable materials with good inherent or supplemented electronic characteristics or by using rigid conductive or semi-conductive materials, imparting flexible attributes via specific geometry or structural designs (Zhu and Moran-Mirabal 2016).

Bio-derived materials have received recent consideration for green and sustainable bioelectronics because of their biocompatible and biodegradable characteristics (Muskovich and Bettinger 2012). Various, naturally derived biomaterials have been proposed as substrates for flexible devices such as silk proteins, polysaccharides, and gelatin. Bio-derived materials such as chitin and chitosan have been reported for electrochemical biosensing as components, substrates, or surface modifiers for thin film electrodes (Xu et al. 2019).

The availability of aqueous chitosan solutions near-physiological conditions contrasts with its parental polymer, chitin, and makes exploitation of chitosan more biocompatible and thus widens the range of its applications. Well-adherent thin films of both materials can be simply prepared using their dilute solutions in casting, spinning, or dip-coating procedures, resulting in structures of density and porosity that are adjustable by the number of casting/dipping/spinning repetitions and by the specific composition of the casting/dipping/spinning solutions (Muzzarelli et al. 2003).

Chitin and chitosan possess good biocompatibility, have potential to form uniform films and hydrogels, and contain multiple oxygen- and nitrogen-based

functional groups that can be chemically modified. A stable and reproducible long-term response is achieved only when the matrix-induced degradation of the immobilized BRE occurs at a negligible rate. The compatibility of the immobilizing layer with the BRE is still crucial for durable sensor performance, but in addition, the sensor's composition and tip design should not trigger a local inflammatory host response, as there is a risk of sensor fouling by cellular release of absorbable proteins and lipids or immunoprotective fibrous tip encapsulation. For in vivo applications, the aim must therefore be to construct the biosensor from materials that are as nontoxic as possible for both the BRE and the tissue under study. As expected for biological polymers, the tissue compatibility and low immunogenicity of chitin and chitosan have been confirmed in many clinical trials (Cheung et al. 2015).

Chitin and chitosan contain abundant functionalities ($-$OH, $-NHCOCH_3$, and $-NH_2$). These reactive function entities allow the derivatization of the biopolymers if chemical tailoring would benefit sensor performance. Chitin and chitosan derivatives have been synthesized for various applications by covalent modification of the backbone that include sugar-modified, phosphorylated, quaternized, cyclodextrin-linked, thiolated, sulfated, azidated, and ferrocene-branched (Jayakumar et al. 2006; Mourya and Inamdar 2009).

Wu et al. prepared thin film of chitosan by electrodeposition on an electrode by cathodic hydrogen evolution from water electrolysis, which consumes protons and generates hydroxide ions at the interface between the negatively polarized electrode and the electrolyte. The local increase of the interfacial pH neutralizes the positive charges on the protonated groups in chitosan chains reaching the cathode through electrostatic attraction. Continuous electrophoretic delivery and concomitant removal of positive charges produce chitosan thin film formation on the sensor surface, as the polymer solution is heading to the point of precipitation ("sol–gel transition") (Wu et al. 2010).

Due to their behavior, chitosan-based Schiff bases (CSBs) are functioned as excellent pH stimuli sensors (Zhao et al. 2017; Zhang et al. 2017). Zhang et al. (2017) have studied the different voltage response of CSB (from chitosan and catechol) at different pH conditions. Based on the destruction-formation of imine bonds under different pH conditions, a CSB gel (derived from phenyl boronate-modified chitosan and dialdehyde polyethylene oxide) was tested as glucose-responsive material (Zhao et al. 2017). Different to this, the morphology of gold nanoparticle–CSB composite is tuned between spongy and wire morphologies at different pH values due to the destruction-formation behavior of imine bonds (Zhang et al. 2017). Fluorescent CSBs (prepared via the reaction between chitosan and highly conjugated aldehydes) and their reduced forms can detect the different chemical molecules (Jatunov et al. 2015).

CSB-modified glassy carbon electrode can act as the electrochemical sensor for the detection of hydrogen peroxide within 4 s. The aldehyde groups are initially introduced in chitosan through oxidation, and the oxidized chitosan is allowed to react with the amino groups of thionine for the synthesis of CSB (Feng et al. 2010). Chitosan modified with 2,5-dihydroxybenzaldehyde was tested as coatings on carbon electrode were tested as electrochemical sensor for the detection of lead

(II) ions (Kocak et al. 2012). The electrochemical sensor based on chitosan and salicylaldehyde can effectively detect the presence of tryptophan (Deng et al. 2011).

12.4 Conclusions and Future Perspective

Theranostics involves smart integrated system and materials that assures therapy, diagnosis, and monitoring of the treatment as a platform for personalized medicine. The performing theranostic systems offer opportunities to combine passive and active targeting, environmentally responsive drug release, or other therapeutic functions into a single biomedical platform. High-performance biomedical materials have been tested as theranostic nanosystems (superparamagnetic iron oxide nanoparticles (SPIONs), gold nanoparticles (AuNPs), quantum dots (QDs), carbon nanotubes (CNTs), mesoporous silica nanoparticles (MSNs), lipid-based nanoparticles platform, dendrimers), biological-sensitive hydrogels, 3D scaffolds, and micro- and nanowires with promising performances for medical applications.

Chitosan is a natural polymer extensively tested for theranostic platforms because of several advantages in biomedical applications, such as biocompatibility and controlled biodegradability, antimicrobial properties, and bioadhesive and bioresorbable nature. Its functionality and ability to form complexes with iron oxides and other inorganics and the processability in nanoparticles and nanocapsules made these materials very useful in theranostics, hyperthermia, and biosensing or cancer therapy. The polycationic nature based on protonated amino groups explains the chitosan biological properties: adhesion of cells and tissue, analgesic effect, and antioxidant effect. Compatibility of chitosan with physiological medium depends on the preparation method, deacetylation degree, molecular weight, crystallinity, solubility, and, eventually, the chemical modification with small or large molecules with specific properties. Various kinds of modification of chitosan have been investigated in recent years, and examples using acylation, alkylation, quaternarization, carboxymethylation, or functional derivative synthesis by reaction chemical modifications have been reported in the aim to increase the solubility in water, encapsulation of bioactives, cell penetrability, and blood interactions.

Pure and modified chitosans have been used in compositions for biomedical and pharmaceutical applications, such as drug delivery systems and 3D scaffolds for tissue engineering or 2D scaffolds, especially for wound healing purposes, stimuli-responsive hydrogels, micro- and nanoparticles, and bone implants. Huge progress in the application of chitosan in theranostic is based on surfaces' activation of chitosan-based nanoparticles or immobilization of cellular receptors, bioactives, or membrane penetrators. The future challenges include the strategies for balancing the scale of the carrier with the quantities of bioactive able to be included and that are clinically necessary. Other directions of investigations consider formulation as scaffolds, imprinted polymers, membranes, emulsions, and liposomes, which are biocompatible and nontoxic in human body and are able to respond to external stimuli.

Acknowledgment This work was supported by a grant of Grigore T. Popa University of Medicine and Pharmacy, no. 27499/2018.

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Grafted Chitosan Systems for Biomedical Applications

13

S. Dhanavel, Sheril Ann Mathew, and A. Stephen

Abstract

Chitosan is one of the most abundant biopolymers obtained from the deacetylation of chitin. It has received considerable attention in recent years owing to its various beneficial pharmacological properties such as anti-acid properties, antitumour, hypocholesterolemic action, wound-healing properties, antiulcer properties, spermicidal activity and haemostatic properties. Most recently it has been used as the matrix for the drug delivery, gene delivery and tissue engineering applications. The application of chitosan in pharmaceutical industry has been extensively explored. However, pure chitosan has its limitations in its processability and reactivity. Poor water solubility is the major problem for utilizing the material in biomedical applications. To overcome this limitation, chitosan surface has been altered using physical interactions (inorganic composites and polyelectrolyte complexes) and chemical modification. Recently chitosan has been grafted with other molecules to get some desired properties such as controlled drug delivery, targeting specific tissues and enhanced antimicrobial efficacy. Moreover, the formation of grafted chitosan increases reactive functional groups which make them superior material for the complexation with other metals, metal oxides or materials like graphene and carbon nanotube. The grafted chitosan-based composites enhance their usage in pharmaceuticals over bare chitosan. This chapter highlights the need of grafted chitosan, synthesis techniques to get desired properties and their applications in biomedical field.

Keywords

Grafted chitosan · Synthesis · Properties · Biomedical applications · Future aspects

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_13

13.1 Introduction

In pharmaceutics, over the past few decades, polymers are being employed in biomedical applications (Pillai and Panchagnula 2001) because they are biocompatible and ensure the bioavailability (presence of drug in the blood plasma/concentration of drug in the blood plasma) of the drug molecules (Harrison 2007). Also the release of the drug from the matrix can be controlled, i.e. there is controlled release of the therapeutics (Liechty et al. 2010; Oh 2017). This is highly advantageous since it avoids overdosing and repetitive dosing and keeps the drug from degradation.

Nanoparticles, micelles, hydrogels or matrices are the different drug delivery systems that are being researched (Huh et al. 2012). A biocompatible polymer and a therapeutic, bound together, constitute the system. Targeted delivery of specific release is achieved by functionalizing the system (Yu et al. 2016). The drug can be released at a specific organ or site which in turn will reduce the side effects of the chemicals and enhances the efficiency of the drug (Ritschel 1989; Glass et al. 1991; Kudgus et al. 2014; Levy 1973). In short, the pharmacokinetics of the drug changes when dispersed or covalently bonded with chitosan. To avoid side effects and toxicity, biodegradable and biocompatible polymers are used. Particularly natural polymers such as polysaccharides, polypeptides or phospholipids are used for these drug/polymer formulations. Chitosan is an excellent biopolymer that is extensively used because of its numerous favourable properties.

Chitosan is a polysaccharide (1,4-β-*N*-acetylglucosamine), obtained from alkaline deacetylation of chitin. It is one of the most abundant amino polysaccharides extracted from aquatic animals (shrimps, lobsters, crustaceans, arthropods, cephalopods, etc.), microorganisms (microalgae, yeast, etc.), insects (ants, spiders, scorpions, beetles, etc.), fungi, etc. The chemical configurations of cellulose, chitin and chitosan are depicted in Fig. 13.1. Chitosan has a similar structure of chitin and cellulose, but the difference is that the functional group at C-2 position, i.e. the

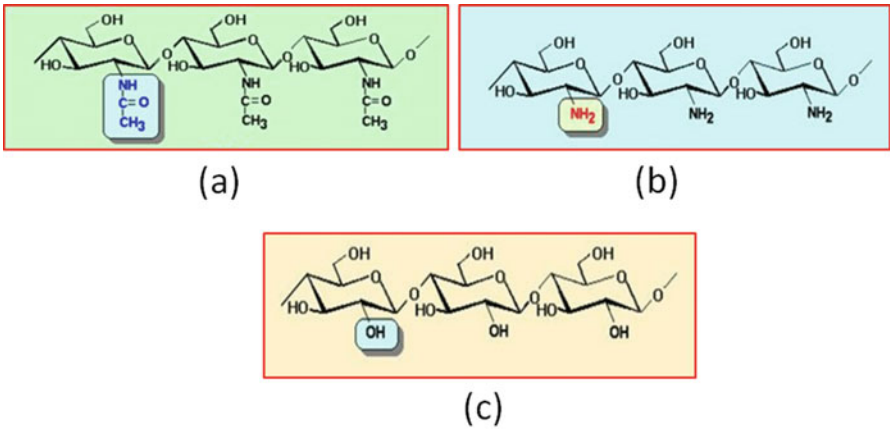

Fig. 13.1 Chemical structure of (**a**) chitin, (**b**) chitosan and (**c**) cellulose

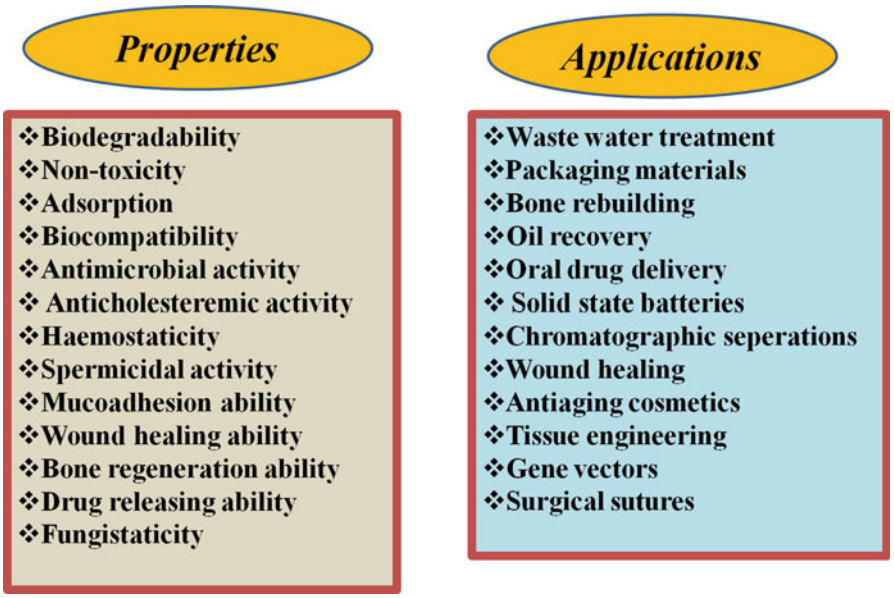

Fig. 13.2 Properties and applications of chitosan

cellulose and chitin, has hydroxyl and N-acetylamine group, respectively, whereas chitosan possesses amine group at the C-2 position in its structure. This derived chitin can be divided into two types: (i) low-density chitosan and (ii) high-density chitosan based on the density of the polymer chain or molecular weight (Elgadir et al. 2015; Kumari and Rath 2014; Ravi Kumar 2000).

The preparation of chitosan from chitin depends on various conditions such as time, concentration, temperature and deacetylation. These different conditions affect the chemical, physical and biological properties of chitosan. After the deacetylation treatments, the formation of the product from chitin can be confirmed from the Fourier transform infrared spectroscopy analysis. The presence of amide band I and amide band II at 1654 cm^{-1} and 1583 cm^{-1}, respectively, confirms the formation of deacetylated chitin. The higher intensity of band I and lower intensity of band II indicate the formation of NH_2 group which denotes the efficient deacetylation of chitin (Dhanavel et al. 2018; Liu et al. 2016a; Nivethaa et al. 2015). The degree of deacetylation determines the amount of amine groups present in chitosan, which has a direct influence on reactivity, pH, sensitivity and functionality. The presence of protonated amine groups makes chitosan more reactive and enables it to bind and chelate the toxic transition metal ions. This tremendous ability of chitosan makes them a useful template in pharmaceutical and medical applications (Foster et al. 2015; Jiang et al. 2017). Various properties and applications of chitosan are highlighted in Fig. 13.2.

Tissue and scaffold engineering, wound dressing, electrochemical sensing and drug delivery vehicle are a few major applications in biomedical field. Chitosan is

called a polycation since it has a positive charge in its structure, and this charge density can be manipulated by altering the degree of acetylation. Due to this an electrostatic force of attraction exists and this is favourable in forming complexes with negatively charged additives. This positive charge also enables chitosan to attach to the mucous layers of the body. This good mucoadhesive property is ideal for drug penetration. The antibacterial property of chitosan further avoids infection and is an added advantage (Singh and Ray 2000; Vunain et al. 2017; Wu et al. 2017).

The reason why chitosan becomes biocompatible is that it can be easily metabolized and excreted from the biological system after absorption or injection. Low-molecular-weight chitosan is usually eliminated by renal clearance. The high-molecular-weight chitosan which is synthesized from mushrooms has lower affinity for the enzyme and hence the rate of degradation is slower. The molecular weight and the degree of acetylation determine the rate of degradation (Cheung et al. 2015; Sangeeta et al. 2018).

Chitosan is often conjugated with metals, carbohydrates and polymers for the specific functionality. The physicochemical properties of chitosan which is a necessity for several applications can be modified through various chemical processes such as sulphonation, succinylation, azylation, nitration, methylation, hydroxylation, acylation, thiolation, quaternization and phosphorylation. Among these various approaches, grafting polymerization is a promising method to get the variety of molecular design. Grafting is the process of insertion of a favourable substance with another such that a continuous and unaltered connection is formed with the two different materials and an ideal property is achieved (Li et al. 2015; Tokuyasu 1998).

Chitosan is only soluble in acidic pH usually below 6.5. Its poor solubility in water is the major drawback of chitosan. The acidic pH enables the protonation of the primary amine which in turn leads to repulsion between the chains of exoskeleton and helps in making chitosan as soluble (Ali and Ahmed 2018).

Since this environment is not always favourable, many researchers resort to other means to attain a chitosan solution under any condition. There are two prominent ways, quaternization and grafting employed for the remedy of this problem. Quaternary chitosan is also known as N-(2-hydroxypropyl)-3-trimethylammonium chitosan (HTC). Here the primary amine is quaternized. This introduces permanent positively charged amine groups into the chitosan exoskeleton which facilitates solubility of chitosan over a wide range of pH. Glycidyltrimethylammonium chloride (GTMAC) and N,N,N-trimethyl chitosan chloride (TMC) are commonly used in the quaternization of chitosan. The quaternization maintained and improved the mucoadhesive properties of chitosan. Depending on the degree of quaternization, this chitosan derivative can act as an ideal candidate for gene delivery. It also improves the antimicrobial and antibacterial properties of chitosan (Cele et al. 2018; Hu et al. 2016; Tan et al. 2013).

Another excellent method used is the grafting of chitosan. The amino groups on deacetylated units and the hydroxyl groups on the C3 and C6 carbons on deacetylated/acetylated units are playing the role of active sites for grafting of chitosan with other polymers. The covalent bonding of a molecule onto the backbone of chitosan allows the formation of graft copolymerization. This grafting can be

initiated by various initiators such as ammonium and potassium persulphate, ceric ammonium nitrate, ferrous ammonium sulphate and Fenton's reagent (Don et al. 2006; Ismail et al. 2017; Xiao et al. 2010). A few examples of grafted chitosan are poly(vinyl alcohol)-g-chitosan, polyethylene glycol-grafted chitosan, polyacrylamide-g-chitosan, cyclodextrin-linked chitosan, protein-grafted chitosan, acid-grafted chitosan, polyethylene-oxide-grafted chitosan and others.

This chapter focuses on grafted chitosan developed for biomedical applications. In the first section, various methods of synthesis will be discussed. Further their properties and composites formed from them will be elaborated. Their various applications will also be discussed in detail.

13.2 Synthesis of Grafted Chitosan

In recent years the chemical modification of polysaccharides is focused because it is beneficial in various fields (He et al. 2017; Witono et al. 2012). Graft polymerization is an excellent modification that can be used to attach various functional groups to polysaccharides. A modification technique in which polymer monomers are covalently bonded to the backbone of a parent polymer is called grafting (Liu et al. 2015; Ma et al. 2016). The advantage of this process is that it retains the bulk properties of the parent polymer. Alteration of the surface properties will not interfere with the properties of the polymer. It is advantageous when compared to other techniques because grafted polymers are stable (Witono et al. 2014).

Types

1. Grafted chitosan using single monomer
2. Grafted chitosan using more than one monomer
   - "Grafting to" approach
   - "Grafting from" approach
   - "Grafting through" approach (Madill et al. 2017; Barbey et al. 2009; Chu and Sidorenko 2013; Uyama et al. 1998; Zhang et al. 2018)

- **Grafting to approach:**
  This method provides low grafting density. Here end-functionalized polymers covalently bond to a self-assembled monolayer or adsorbed functional polymers. This is shown in Fig. 13.3.
- **Grafting from approach:**
  The prerequisite for grafting from approach is a preformed macromolecule with distributed initiating functionality. In grafting from approach, the initiator is attached to the polymer surface. This is followed by in situ polymerization which is initiated from the surface. This method provides better grafting density.

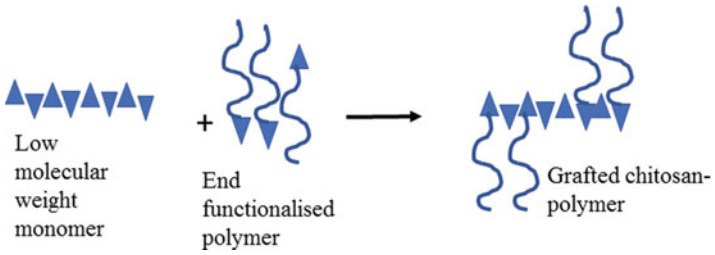

Fig. 13.3 Grafting "to" approach

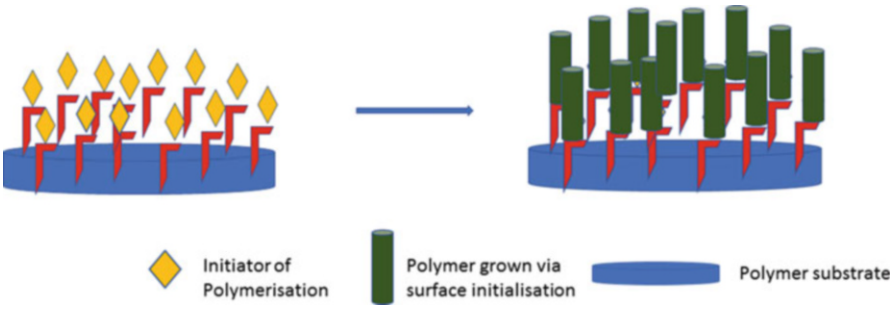

Fig. 13.4 Grafting "from" approach

Grafting "from" approach is depicted in Fig. 13.4. As shown in Fig. 13.4, on a polymer substrate, active sites or initiators of the polymerization process are present. When suitable conditions are provided, the active sites become sites of grafting and initiate the polymerization of a different monomer.

- **Grafting through approach:**
  This method ensures high stability, high grafting ability and homogeneity. Chemical anchors and propagations sites are present on the polymer to be grafted. These macroradicals act as active sites for the attachment/grafting of other polymers on the base. In Fig. 13.5, chitosan was first functionalized with glycidyl methacrylate (GMA) to form chitosan-g-GMA. Later poly(diethylaminoethyl methacrylate) (PDEAEMA) was grafted onto the chitosan to form chitosan-grafted PDEAMEA (CTS-g-GMA-PDEAMEA). This method of grafting a polymer onto the other is called grafting "through" process.

The three approaches mentioned above are types of synthesizing grafted polymer. Employing these techniques, various mechanisms can be utilised to ensure high-density grafting. The mechanisms used in the grafting of chitosan are briefly described below.

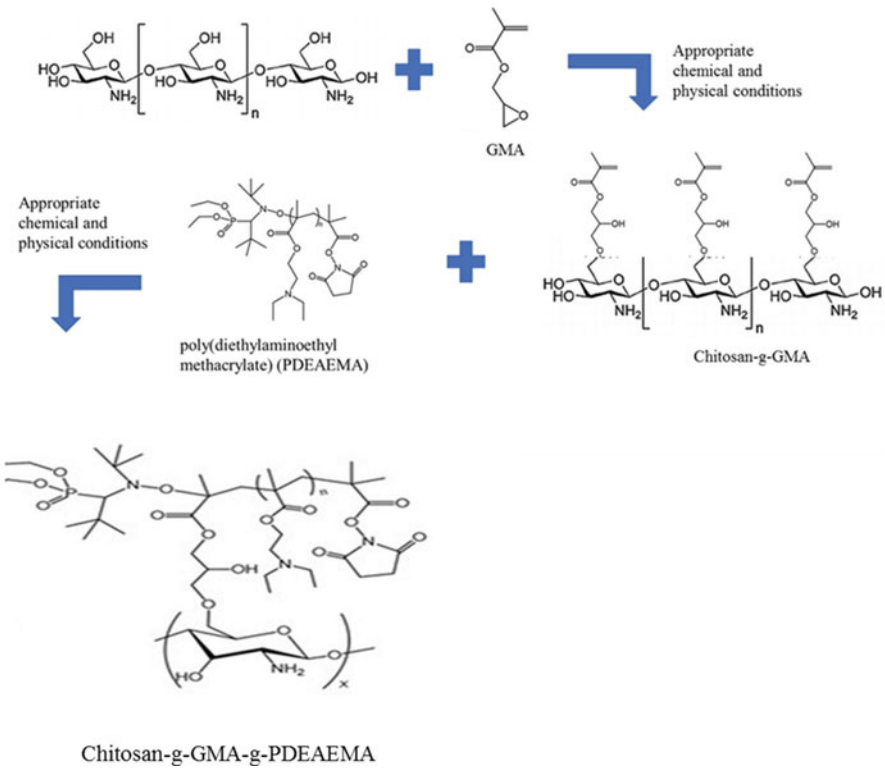

Fig. 13.5 Grafting "through" approach

13.2.1 Grafting by Schiff Base Formation and Reductive Amination

In this method, chitosan reacts with an aldehyde or a ketone, forms an intermediate and by the addition of sodium borohydride is converted to an N-alkyl or an N-aryl group. Functional groups containing a nitrogen-carbon double bond (C=N), where nitrogen forms a bond with aryl or alkyl groups, are known as Schiff bases. The condensation reaction of an aldehyde or ketone with a primary amine is commonly known as Schiff's base reaction (Dillow and Lowman 2002; Dufresne et al. 2013).

13.2.2 Grafting by Amide Formation

Carbodiimides such as dicyclohexylcarbodiimide (DCC), N,N′-diisopropylcarbodiimide (DIC) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) are used for grafting by amide formation. EDC is the most commonly used imide because of its high solubility in water. Carbodiimides have shown great potential for the attachment of molecules

onto the chitosan backbone, due to their allene functional groups (Jayakumar et al. 2005; Sarmento and das Neves 2012).

13.2.3 Grafting by Click Reactions

Kolb et al. (2001) introduced the concept of click chemistry (Kolb et al. 2001). This was done to simplify and increase robustness in chemical synthesis. If at room temperature two functional groups exclusively get attached to each other, then they are called click chemistry. These reactions are divided into four major groups:

Cycloadditions of unsaturated species, (ii) nucleophilic ring openings, (iii) non-aldol-type carbonyl chemistry and (iv) additions to carbon-carbon multiple bonds (Kulbokaite et al. 2009; Truong et al. 2014).

13.2.4 Grafting by Nucleophilic Substitution Reaction

The primary amine group is another reactive site in chitosan which is ideal for chemical modification (Harding and Sashiwa 2018; Yao et al. 2016). The electron pair acts as a proton acceptor and chitosan acts as a nucleophile. A monochlorotriazinyl derivative of β-CD was attached onto the chitosan backbone by Martel et al. The chloride atoms of the β-CD were substituted by the amino groups of chitosan (Martel et al. 2001).

13.2.5 Grafting by Photoinitiation

Electromagnetic radiation (ultraviolet or microwave) induces the formation of radicals. This is known as photoinitiated grafting. The technique can function either with or without a sensitizer such as methyl methacrylate (Periolatto et al. 2013). The sensitizer is used only when the polymer is not photosensitive. The sensitizer then transfers this energy to the polymer, causing dissociation of the molecule and formation of free radicals responsible for the grafting process (Jenkins and Hudson 2002). The main photosensitizers include benzoin ethyl ether, acrylated azo dyes, aromatic ketones (benzophenone and xanthone) and metal ions (Saber-Samandari et al. 2012).

13.2.6 Grafting Under Microwave Irradiation

Sharma and Rajesh (2017) reported a quick way to prepare β-cyclodextrin-grafted chitosan by a microwave-assisted method. This technique provides homogenous and

efficient grafted polymers which can be synthesized quickly. The elevated temperature is the initiator of these reactions. Chitosan with guanidine oligomers, chitosan-grafted-poly(acrylonitrile), chitosan-grafted-styrene, etc., have been successfully synthesized by this process (Kumar Sharma and Mishra 2010; Li et al. 2017; Singh et al. 2005).

13.2.7 Grafting by Cross-Linking Reaction

Cross-linking agents pave way for the reaction with the amine groups instead of the hydroxyl groups in chitosan. This reduces the adsorption capacity of the cross-linked chitosan and enhances the resistance of chitosan against acid and alkali medium. Chemical agents like formaldehyde and benzaldehyde are used to protect the amine group in chitosan until the cross-linking reaction is complete. Adsorption capacity and selectivity of modified chitosan can be increased by grafting functional groups on chitosan cross-linked. Masykur et al. and Gawish et al. give few examples of grafting chitosan via cross-linking reaction (Gawish et al. 2012; Masykur et al. 2016).

Graft polymerization of chitosan can also be initiated by gamma radiation and enzyme. It can therefore be seen that this is a useful technique for improving the properties of chitosan and broadening the range of its possible applications. Selection of the graft copolymerization method to be used can be based on the intended use of the hybrid polymer, and each of the different methods has variables that can influence the grafting process (Bhattacharya and Misra 2004; Minko 2008; Roy et al. 2005).

13.3 Properties of Grafted Chitosan

In biomedical applications, the main aim in grafting chitosan is to obtain a composite that is water soluble. While grafting chitosan with a hydrophilic material like vinyl acetate, the crystallinity of the polymer decreases on increasing the amount of grafting molecule. Also, there is a change in the glass temperature and decomposition temperature of chitosan with respect to grafting molecule. The grafting results in an enormous increase in the tensile strength of chitosan film in the dry state. In wet state it softens the polymer and increases the percentage of elongation. The swelling at different pH also varies considerably. In other terms, the grafting percentage is proportional to swelling behaviour. The swelling studies ensure that the hydrophilic/hydrophobic tuning can be achieved by properly adjusting the extent of hydrolysis of the chitosan-g-poly(vinylacetate) copolymers. The synthetic-natural hybrid copolymers having good mechanical properties and properly tuned hydrophilic/hydrophobic ratios are promising candidates which could be exploited for degradable, strong and pH-sensitive membrane applications. It also exhibited that the blood compatibility of grafted chitosan is greater than pure chitosan. The grafted polymers show much lesser cytotoxicity and higher biodegradability. It is observed that

chitosan films are transparent and flexible, but on increasing the grafting level, the films become brittle and dry as reported by Dinesh et al. The degree of swelling decreases for grafted chitosan due to the cross-linking (Liu et al. 2017; Radhakumary et al. 2007; Singh and Ray 1998).

13.4 Nanocomposites of Grafted Chitosan

Even though grafting chitosan improves its solubility, forming composites of grated chitosan with other materials can further enhance its application. Nanocomposites further help us functionalize the grafted polymer and improves its properties and stability.

Acrylic-acid-grafted biopolymer chitosan/TiO_2-based nanocomposite is used in water treatment by Sud et al. Visible light-induced photocatalytic degradation is studied. Chitosan was chemically functionalized with poly(diethylaminoethyl methacrylate) (PDEAEMA) to produce a CO_2-responsive material for adsorbing metals from wastewater streams (Madill et al. 2017). The CTS-g-GMA-PDEAEMA exhibited the highest adsorption capacity of all comparable materials as reported by Midall et al. Grafting of chitosan (CS) with graphene oxide (GO) sheets efficiently improved their desired properties for protein delivery purposes as reported by Emadi et al. (2017). Using GO-CS as a protein therapeutic nanocarrier can increase the half-life of proteins in a biological environment, reduce the frequency of administration, lower drug doses and optimize the costs related to protein therapy. Magnetic Fe_3O_4 nanoparticles were functionalized with chitosan-grafted poly(ethylene glycol) methyl ether (CTS-mPEG) for paclitaxel (PTX) delivery. About 86.9 $\pm$ 3.4% of drug-loading efficiency and sustained release up to 120 h were achieved when studied by Huong et al. (2016). Hsiao et al. studied hexanoyl-chitosan-PEG (CP6C) copolymer-coated, paclitaxel (PTX)-loaded and chlorotoxin (CTX)-conjugated iron oxide NP (CTX-PTX-NP) for targeted delivery of PTX to human glioblastoma (GBM) cells. High drug-loading efficiency (>30%), slow drug release in PBS and the CTX-conjugated NP were shown (Hsiao et al. 2015).

13.5 Antimicrobial Properties of Grafted Chitosan

The ability of a material to kill microorganisms or prevent their growth is called antimicrobial. Antimicrobial can refer to antiviral, antibacterial or antifungal. The main purpose of the antimicrobial therapeutics is to treat infection and also prevent it. Chitosan and chitosan composites have been used as carriers of therapeutics to deliver them to specific sites. It is necessary that these composites have to be antimicrobial since they are used for biomedical applications in biological systems (Chen et al. 2009; Kumar et al. 2018; Zhu et al. 2015).

Chitosan derivatives show higher antibacterial and antifungal properties when compared to pure chitosan. The results by Mohamed et al. proved that the derivatives showed higher inhibition than chitosan against all the tested bacteria (Badawy and

Rabea 2016). With increase in degree of substitution, the inhibitory effects against *E. carotovora*, *R. solanacearum*, *R. fascians* and *R. radiobacter* increase proportionally. The antifungal activity of chitosan and N-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives against *A. alternata*, *B. cinerea*, *Bd. theobromae*, *F. oxysporum*, *F. solani*, *P. digitatum*, *Ph. infestans* and *S. sclerotiorum* by using mycelia radial growth technique showed that a degree of substitution of 0.40 exerted the prominent antifungal activity.

Gallic acid-grafted chitosan (GA-g-chitosan) was capable of growth inhibition against *S. parauber* 12046 (Kim and Je 2015). The antibacterial activity of the GA-g-chitosan is also dependent on the bacterial species. Against foodborne and methicillin-resistant *S. aureus*, the GA-g-chitosan showed enhanced antibacterial activities with MIC values of 16–256 μg/mL compared to the unmodified chitosan. By ozone treatment, carboxyl groups of poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) were grafted to chitosan by Hu et al. (2003). Both PHBV-CS (chitosan) and PHBV-COS (chitooligosaccharide) showed an antibacterial activity against four clinically infectious bacteria.

An elaborate study using 13 different grafted composites with polyethylene terephthalate (PET) was studied by Benkocká et al. (2018). Improved antimicrobial activity of the prepared composites, especially against *S. epidermidis*, was seen in chitosan-grafted PET. The in vitro DPPH assay and MIC test confirmed that the essential oil component-grafted chitosan nanoparticles possessed both antioxidant and antibacterial activities. Chen et al. also reported that the oil component-grafted chitosan nanoparticles against *S. aureus* (gram-positive bacterium) and *E. coli* (gram-negative bacterium) are better than the unmodified chitosan nanoparticles (Chen et al. 2009). It is also less toxic than free essential oil components. Mohammad Hassan in his work on chitosan films graft copolymerized with poly (acryloyloxy)ethyltrimethylammonium chloride stated that antibacterial activity is achieved against bacterial and fungal infections (Hassan 2018).

13.6 Grafted Chitosan for Tissue Engineering and Regeneration

A suitable replacement of the damaged tissues and organs is one of the fastest growing biomedical fields which could offer a superior therapeutic strategy for the management of wound healing. Tissue engineering is an emerging discipline that involves natural medicine and engineering in the development of biological materials to replace or improve the function of diseased tissues. Surgical and traditional medical treatments impose side effects like tissue loss and organ necrosis on patients. Tissue engineering and regenerative medicine provide new way to cure diseases. The inherent biocompatibility, biodegradability and antibacterial properties make chitosan a popular choice for the tissue engineering and regeneration application (O'Brien 2011; Pandey et al. 2017).

Tissue engineering scaffolds are used to temporarily support the growth and guiding the growth. These scaffolds are two- or three-dimensional structures used

for the proliferation of tissues in definite direction. This scaffold constructs create an artificial environment suitable for cell migration, proliferation and migration along with the synthesis of extracellular matrix. There are various scaffolds such as hydrogels, fibres, foams and sponges that are reported for tissue engineering applications (Croisier and Jérôme 2013). Mostly the porous structures are preferred so as to allow better cell proliferation, removal of waste and oxygen supply. Moreover, higher porosity of the scaffolds provides physical support for extracellular matrix and also facilitates nutrient transport due to its porous connectivity (Loh and Choong 2013). Owing to the special properties including flexibility, softness, high water content and biocompatibility, hydrogels have gained popularity. There are various synthetic techniques such as electrospinning, solvent casting, freeze-drying and particulate leaching employed for the construction of such scaffolds. Recently various attempts have been made to use hydrogel as material for the engineering of muscle, fat, bone, cartilage and liver (Ahmed et al. 2018; Ahmed and Ikram 2016; Ribeiro et al. 2017; Rodríguez-Vázquez et al. 2015; Sultana et al. 2015). Figure 13.6 shows the various forms of scaffolds from different processes.

The ideal characteristics of scaffolds are as follows:

- Porous structure.
- Maintaining metabolic activity of cells.
- Delivery of nutrients.
- Biocompatible materials.
- Biodegradable materials.
- Promote cell adhesion and proliferation.

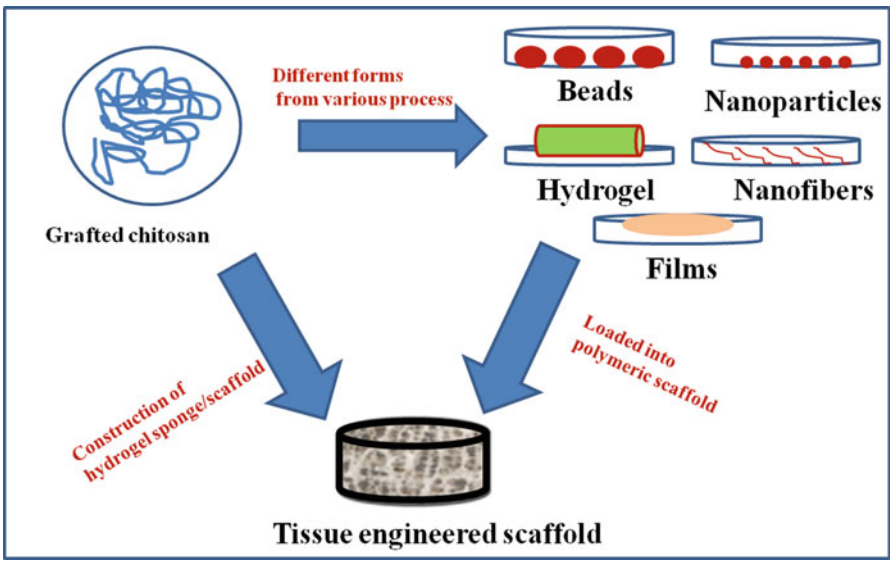

Fig. 13.6 Different forms of chitosan using various processes

- Facilitate diffusion of oxygen.
- Mimic natural environment.
- Ease of preparation.
- Non-toxic.

Chitosan-grafted polyurethane with functionalized multiwall carbon nanotubes via electrospinning method has been reported for the application of nerve tissue engineering by Sita Shrestha et al. The improved cytocompatibility and mechanical strength of this material, serving as natural hosting substrate to natural extracellular matrices (ECM) play a vital role in nerve tissue engineering (Shrestha et al. 2018). Chitosan-*grafted*-poly(acrylic acid-*co*-acrylamide)/hydroxyapatite nanocomposite for drug delivery and bone tissue engineering with good cytocompatibility without any cytotoxicity has been reported by Saber-Samandari and Saber-Samandari (2017). A quaternized chitosan (QCS), which was synthesized by grafting 2-[(acryloyloxy)ethyl]trimethylammonium chloride onto chitosan, exhibited enhanced antifungal activity as reported by Hsin-Lin Lee et al. This report also suggested that the tissue conditioners incorporated with antifungal agent serve as a barrier between the healthy tissues and infection site (Lee et al. 2018). Chitosan-grafted-aniline tetramer synthesized by electrospinning method was reported by Xiaojie Ma et al. for tissue engineering, and results demonstrated that the materials formed were nanofibrous electroactive scaffolds. Those scaffolds had good biocompatibility and possessed enhanced cell adhesion and proliferation of C2C12 cells (Ma et al. 2014). Electrospun fibres of chitosan-grafted-polycaprolactone/poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) blends possessed hydrophilicity, water absorption, biodegradability, good mechanical, non-toxicity to human dermal fibroblasts and good biocide action. Based on these, Ana M. Díez-Pascual et al. suggested this grafted chitosan scaffolds for tissue engineering (Díez-Pascual and Díez-Vicente 2016).

13.7 Grafted Chitosan in DNA and Gene Therapy

Gene therapy is the technique which uses genes to prevent disease. This technique involves the insertion of genetic material such as DNA, RNA and small interfering RNA (siRNA) into the somatic cells to treat the disorder instead of drugs and surgery. It has several approaches including replacement of a mutated gene, inactivating the mutated gene and insertion of a new gene to treat acquired gene associated diseases. The rectification of the abnormal gene sequence is a promising treatment option for a number of diseases. The optimization of the delivery vehicles is the main focus of this technique. The molecular carrier used to release the gene is called a "vector". These vectors are mostly viruses or plasmids (Han et al. 2000; Kasper and Mikos 2004). Nearly, 68% of the trails of gene therapy were investigated using viral vectors as a gene transfer material, due to their superiority of invading cells and inserting their genetic material. Lentivirus, adenovirus and retrovirus are the examples of the recombinant vectors. Although the efficacy of the viral vectors is

confirmed, it has been associated with some limitations such as the costly production, triggering of immunogenicity, limited uptake of DNA and toxicity. Similarly the delivery of naked therapeutic genes is not effective due to their non-specificity towards target cells, vulnerability to nuclease degradation and rapid clearance by the mononuclear phagocyte system. In addition to this high anionic charge density, hydrophilic nature and large size also hinder the usage of naked gene for intracellular delivery. Compared to the viral delivery systems, non-viral carriers offer many advantages such as ease of large production, safety with low immunogenicity and potential for repetitive administration. The non-viral vectors involve the use of plasmid DNA together with the physical methods such as electroporation magnetofection, microinjection, gene gun, hydrodynamic injection and sonoporation or chemical carriers such as peptides, cationic lipids or cationic polymers (Al-Dosari and Gao 2009; Nayerossadat et al. 2012).

The key features for being a good carrier for gene therapy are high DNA uptake, biocompatibility, the ability to protect DNA from nuclease degradation and simple synthesis. In addition to the physiological barriers, several anatomical barriers such as epithelial and endothelial cell linings and extracellular matrix limit the direct access of genes to the target cells (Jennings et al. 2016). The availability of DNA for gene transfection is limited because injecting DNA directly into certain tissues drains quickly into the lymphatics. In order to overcome the limitations and the considerations in safety aspects of gene therapy, non-viral polymers are employed as an alternating carrier due to their unrestricted gene loading capacity, ease of fabrication, low cost and low immunogenicity.

In this context, the polymers play as networks that capture a gene and further release it when they penetrate the cells. Recently various cationic polymers such as polyethylenimine (PEI), poly-L-lysine (PLL), polyamidoamine (PAMAM) dendrimer and chitosan have been utilized for the gene delivery (Dai et al. 2011). Even though PEI and PAMAM showed efficient performance, the cytotoxicity limits their usage in in vivo applications. Similarly the usage of PLL is restricted by its low gene transfection activity. In recent years, chitosan and its derivatives have gained increasing interest owing to their favourable physicochemical properties and their good safety profile. Chitosan derivatives possess various advantages such as low cytotoxicity, high cationic charge density and excellent biocompatibility. In an acidic environment, the amine groups of chitosan get protonated which promote the electrostatic interaction with negatively charged phosphate groups in DNA or pDNA. The addition of positively charged chitosan with negatively charged nucleic acid leads to formation of polyelectrostatic complex. It provides the protection for condensed nucleic acid from enzymatic degradation. The transfection efficiency of the polyplexes obtained from the pairing of chitosan and DNA strongly depends on the various parameters, including molecular weight, degree of deacetylation, pH of the transfection medium, serum concentration and stoichiometry of the polyplex (charge ratio of amine group of chitosan to phosphates of DNA) (Layek and Singh 2017).

Based on the previous results that pure chitosan is not effective for the gene delivery applications due to the poor solubility, low cellular uptake and strong interaction of pDNA impede its release (Saranya et al. 2011; Wong et al. 2018). To overcome these limitations, chitosan has been modified with either hydrophobic or hydrophilic molecules. The hydrophilic molecules on the chitosan matrix increase the solubility and serum opsonization (Layek and Singh 2017). This hydrophilicity of chitosan enhances the intracellular pDNA released from chitosan-pDNA complexes. Dextran, polymalic acid and polyethylene glycol are the few examples of the hydrophilic molecules. On the other hand, imparting the hydrophobicity to the chitosan would improve the cell adhesion and cell adsorption for the polyplexes. Alkyl group, cholesterol and polylactic acid are the few examples of hydrophobic groups. However, based on the pDNA and type of cells, a balance of hydrophobic and hydrophilic should be attained (Wong et al. 2018).

A methyl-methacrylate-modified chitosan conjugate synthesized through Michael addition reaction has been suggested as the gene delivery agent based on transfection efficiency studies in mammalian cancer cell lines. A highly porous nature was observed on linking chitosan covalently to methyl methacrylate (Jaiswal et al. 2019). Young-Min Kim et al. reported the polyethyleneimine (PEI)-grafted chitosan with RGD dendrimer peptide as a gene carrier for the transfection-enhancing material. The results revealed that polyplex could transfer genes into the cell via clathrin-mediated endocytosis and microtubule-dependent micropinocytosis (Kim et al. 2017). Xuan Liu reported the polyethyleneimine-grafted carboxymethyl chitosan for gene delivery and confirmed the presence of strong complexation capability with DNA to form nanoparticles and achieved less cytotoxicity and higher transfection efficiency compared with polyethyleneimine towards cancer cells (Liu et al. 2016b). Arginine-glycine-aspartate (RGD)/TAT (twin-arginine translocation)-functionalized chitosan-*graft*-PEI-PEG gene nanovector for multifunctional gene delivery nanovector was reported by Dongni Wu et al. and reported that nanovector was biocompatible with cells and showed excellent capability for DNA condensation (Wu et al. 2018).

Buddhadev Layek et al. designed and studied the performances of caproic acid-grafted chitosan cationic nanocomplexes for enhanced gene delivery. Caproic acid was selected as the hydrophobic modifier, and the influence of degree of substitution was examined in terms of particle size and physicochemical properties of the polymers. The results based on their demonstration revealed that the degree of caproic acid substitution greatly affected the polyplex stability, pDNA binding, transfection efficiency, polyplex unpacking and cellular uptake (Layek and Singh 2013). Hexanoic acid and polyethylene-glycol-grafted amphiphilic chitosan for enhanced gene delivery were studied by Layek et al. These hydrophilic and hydrophobic moieties were identified as monomethoxy poly(ethylene glycol) and hexanoic acid respectively. Monomethoxy poly(ethylene glycol) was selected because of its superior cell permeability, high water solubility, nonimmunogenicity, nonantigenicity and low cytotoxicity. Similarly, hexanoic acid was chosen for its biocompatibility and enhanced gene transfection efficiency (Layek et al. 2014)

13.8 Anticancer Activity and Release Mechanism of Drug and Protein

Cancer is a lethal disease that has uncontrolled cellular growth as well as the capacity of cellular invasion to different organs of the primary site. Compared with the normal cells, cancerous cells are metabolically active and destroy nearby cells by forming a lump called tumour. There are various factors such as solvent and pesticide exposure, limited physical activities, exposure of ionizing radiation, dietary fat intake and unhealthy lifestyle that can cause cancer (Khan et al. 2010; Kushi et al. 2012; Thomson et al. 2014). Reactive oxygen species including hydroxide radical ($OH^{\cdot}$), peroxides and superoxide radical anions ($O_2^{\cdot -}$) are cancer-causing agents by destroying an enzyme, a protein or even a complete cell. It exerts its effects via continuous chain reaction resulting in the release of more cellular oxidants that cause damage in DNA. It promotes the effects in various forms including decreased cell adhesion, changes in cell structure, promotion of new enzymes and finally death by invading other tissues. Several studies have been done to treat cancers and still it requires further investigation to find the appropriate medicine (Adhikari and Yadav 2018).

Natural biodegradable polymers such as chitosan and dextran attracted attention in the field of drug and protein delivery applications. Among them, chitosan has been focused for its biological properties such as anti-infection, antiulcer, antacid and wound-healing activities making them superior for drug delivery in terms of weakening or preventing drug-induced irradiation in the stomach (Ito et al. 2000). In addition to these properties, chitosan has anti-proliferation activity on tumour cells. Chitosan shows its effect via an inhibition of tumour-induced angiogenesis and tumour metastasis. Since it can be degraded enzymatically by lysozyme via the hydrolysis, chitosan is a versatile material for the delivery of anticancer drugs (Kimura 2008; Nwe et al. 2009). It has been found that the chitosan and its derivatives exert anticancer activity with low toxicity towards normal cells. This activity against tumour growth or uncontrolled cell growth depends on their degree of deacetylation and molecular weight. Based on the reports, soluble form of chitosan and low-molecular-weight chitosan shows remarkable biological activity and inhibits the growth of tumour (Kumirska et al. 2011). The anticancer activity of chitosan derivatives is associated with the antioxidant property due to the scavenging of cancer-causing radicals such as hydroxide radical, peroxides and superoxide radical anions (Adhikari and Yadav 2018; Chang et al. 2018).

The cationic nature of chitosan is dependent on pH and makes it a suitable material to interact with negatively charged molecules such as fatty acids, proteins and anionic polyelectrolytes. The notable scavenging activity of chitosan derivatives against different radical species comes via donating hydrogen atom and the mechanism proposed by Xie et al. for the scavenging activity (Huang et al. 2006; Kim 2018; Xie et al. 2001) as follows:

- Hydroxyl group of chitosan and its derivatives can react with hydroxyl radicals by H-abstraction reaction.
- Hydroxyl group present in the chitosan reacts with the free amino groups present in the polysaccharide to form stable macromolecule radicals.
- Protonated amine group (ammonium groups, NH_3^+) will be formed by adsorbing H^+ from the solution and NH_3^+ group can react with OH via addition reactions.

However, the drawback of chitosan for the utilization in anticancer purpose is its poor solubility and absorption under biological conditions. To overcome this problem, chitosan has been grafted with other molecules. Recently, tanshinone I grafted low molecular chitosan synthesized from Vilsmeier reaction and reductive amination reaction has been tested for anticancer activity by Dongdong Wang et al. They proposed that the anticancer activity was improved on increasing the substitution of tanshinone I. The experiments done by them demonstrate that the enhanced anticancer activity is through dissipation of mitochondrial membrane potential, inhibiting metastasis and inducing apoptosis (Wang et al. 2017). Adoption of chitosan for nutraceuticals delivery has been done by grafting it with gallic acid (Hu et al. 2015). Grafting of antioxidant agents such as gallic acid to the chitosan increases the physicochemical and biological properties of chitosan. While grafting gallic acid with chitosan, solubility of the chitosan increased and the degradation under neutral condition is prevented. In addition to this, the variation in anticancer activity is observed on changing the gallic acid grafting ratios. Grafting hydrophilic molecules onto chitosan backbone not only improve the solubility of chitosan but also improve the biocompatibility and stability under diluted conditions.

In this context, polyethylene glycol has been grafted with chitosan where critical micelle concentration was about 264.8 mg/mL in aqueous solution at pH~6.5, a relatively high value as compared to the literature, which was due to the low substitution degree of polyethylene glycol. And also in vitro release study of 5-FU from the PEG-grafted chitosan micelles was in a controlled-release manner, and the obtained results demonstrate that it could be a potential carrier for effective antitumour activity (Fu et al. 2014). To maintain the drug delivery system at the absorption site for a prolonged time is a significant factor for treatment of cancer and sustained systemic absorption. It is important to increase the mucoadhesive properties (attractive forces between a biological material and a mucous membrane) of chitosan which lead to remarkable improvements in therapeutic efficacy and avoid drug diffusion and reduction in side effects. Dihydrocaffeic acid was grafted with chitosan to improve the mussel adhesion and for pH-sensitive delivery of doxorubicin towards colon tumour cells (HCT116 cells) (Liang et al. 2019). On the other hand, the combined delivery of chemotherapeutic delivery and small interfering RNA (siRNA) using grafted biopolymer is a promising approach which could offer enhanced anticancer activity and low side effect. Cholesterol-grafted low-molecular-weight chitosan to deliver two therapeutic agents, curcumin and siRNA, to cancer cells simultaneously has been analysed by Omkara Swami Muddineti et al., and they concluded that both the agents were delivered in time-dependent manner via clathrin-dependent endocytosis mechanism (Muddineti et al. 2018). Kinetic release

behaviours of etoposide (VP16), an anticancer drug from beta-cyclodextrin-grafted chitosan, was pH- and thermosensitive. Based on these conclusions given by Jingwei Wang et al., drug delivery from the grafted chitosan is effective if they are pH sensitive and thermosensitive, hydrophilic and porous nature (Wang et al. 2019).

Similar to the drug delivery, some important factors affecting the delivery of proteins are molecular weight, deacetylation degree, the concentration of chitosan and initial concentration of protein and substitution degree of grafted molecule. Yongmei Xu et al. reported that chitosan with various deacetylation degrees from 75.5 to 92% and molecular weight from 10 to 210 kDa promotes encapsulation capacity and decreases the release rate of bovine serum albumin (Xu and Du 2003). The graft copolymer nanoparticles consisting of chitosan and the monomer *N*-dimethylaminoethyl methacrylate hydrochloride, methyl methacrylate (MMA) and *N*-trimethylaminoethyl methacrylate chloride for the delivery of insulin were proposed by Feng Qian et al., and they revealed that enhanced absorption, sustained release and improved bioavailability of insulin were observed on using graft copolymer nanoparticles (Qian et al. 2006). The drug and protein release kinetics are analysed using various mathematical models as follows:

$$\frac{M_t}{M_\infty} = K_0 t \tag{13.1}$$

$$\frac{M_t}{M_\infty} = K_{kp} t_n \tag{13.2}$$

$$[100 - (M_t/M_\infty)]^{1/3} = K_c t \tag{13.3}$$

$$\frac{M_t}{M_\infty} = 1 - \exp\ (-K_1 t) \tag{13.4}$$

$$\frac{M_t}{M_\infty} = K_H \times t^{1/2} \tag{13.5}$$

Here, percentage fractional drug release at time t is M_t/M_∞. $K_0 K_{kp}$, K_c, K_1 and K_H are the zero-order release constant, Korsmeyer-Peppas constant, Hixson-Crowell release constant, first-order release constant and Higuchi release constant, respectively. Zero-order kinetics denote that the drug release rate is constant over a period of time and highly suitable for the sustained drug delivery systems. First-order kinetics implies that the drug release rate depends on its concentration and it provides information regarding the dissolution process. Korsmeyer-Peppas model proposes the mechanism of drug/protein release from carrier of different geometry via release exponent (n), when $n = 0.5$ indicates the Higuchi kinetics and it describes the release of water soluble and low soluble drugs incorporated into solid matrixes. When $n < 0.5$, the device follows Fickian diffusion process (case I transport). If $0.5 < n > 0.85$, the release from the carrier follows the anomalous transport mechanism, which is due to the coupled effect of polymer relaxation/erosion and diffusion process. If $n = 0.85$, it indicates that the release from emulsion follows case

II transport mechanism and is swelling controlled. $n > 0.85$ represents the super case II transport mechanism, suggesting that relaxation of hydrophilic molecules is observed. Similarly, Hixson-Crowell kinetic describes that the drug release from the particle is proportional to the cubic root of its volume (Dhanavel et al. 2017; Nivethaa et al. 2015).

13.9 Biosensor Applications of Grafted Chitosan

A biosensor is an analytical device which is used for the analysis of biomaterial samples to gain an understanding of their function and bio-composition by converting a biochemical response into a quantifiable signal. It consists of three basic components, viz. (i) a signal processor, (ii) a transducer and (ii) a detector. Based on the physiochemical properties of the analyte and the diagnosis, the transducer can be optical, electrochemical, acoustic or calorimetric type. There are various detection principles such as voltammetric, amperometric, potentiometric and conductometric. Biosensors are much better in performance compared to any other currently available diagnostic device because of their sensitivity, selectivity, reproducibility, portability, stability and low cost (Vigneshvar et al. 2016). Biosensors can be broadly classified into various categories such as piezoelectric sensors, electrochemical sensors and optical sensors.

Various forms of nanomaterials and polymers were extensively utilized for employing electrochemical biosensing applications (Singh 2011). Among them, biopolymers such as chitosan were selected as a matrix for immobilization of the biological molecules because of its non-toxicity, biocompatibility, excellent membrane forming ability and high mechanical strength (Shukla et al. 2013). Chitosan has been widely used for the biosensing applications because it would provide a better performance in terms of stability and sensitivity and render its suitability in terms of immobilization of enzymes, antibodies, antigens, DNA and RNA. However, pure chitosan has its own limitations such as high viscoelastic value, specific solubility and modest electrical/ionic conductivity to use as a biosensor (Suginta et al. 2013). These limitations can be overcome by grafting chitosan with appropriate molecules. Recently grafted chitosan and their composites are utilized for the determination of biologically important analytes such as urea, glucose, dopamine, hydrogen peroxide and cholesterol.

Multiwalled carbon nanotubes (MWNTs) grafted chitosan has been prepared through phase separation method by Palanisamy Gomathi et al. to overcome the poor electrical conductivity and amperometry which were used to evaluate the electrochemical determination of glucose. The results exhibited high sensitivity (5.03 μA/mM) and lower response time (3 s) in a wide concentration range of 1–10 mM ($R^2 = 0.9988$) (Gomathi et al. 2011). Polyaniline-grafted-chitosan (PANI-g-CHIT) hybrid matrix by in situ chemical polymerization technique was employed as a electrochemical urea sensor and was found to be stable for 21 days with improved sensing properties to several reported sensors (Shekhar Kushwaha et al. 2018). Ali A. Ensafi et al. have reported the thiol group grafted chitosan

(S-CS)-modified carbon (carbon nanotubes or graphite) and hydrodynamic amperometry was used for the electrochemical sensing of glucose and H_2O_2. The linear range for glucose was from 0.5 to 1000 μmol L^{-1} with a detection limit of 0.05 μmol L^{-1} and linear range for H_2O_2 was from 0.1 to 1000 μmol L^{-1} with a detection limit of 0.025 μmol L^{-1}. The sensor showed good selectivity for glucose and H_2O_2 in the presence of dopamine, uric acid and ascorbic acid (Ensafi et al. 2014). The sensitivity of the biosensor can be enhanced by the improving the surface area which increases the immobilization capacity.

Porous-structured chitosan-grafted polyaniline cryogel for the fabrication of a sialic acid biosensor was explored by Amin Fatoni et al. The fabricated electrode exhibited excellent analytical performances with a wide linear range from 0.025 to 15.0 mM and a limit of detection of 18 M. This sensor did not demonstrate any response from common interfering compounds such as ascorbic acid, uric acid and pyruvate with high selectivity to detect sialic acid in the plasma sample (Fatoni et al. 2014). Xin Hua Xu el. have developed and analysed the polyaniline-grafted chitosan for amperometric glucose oxidase biosensor. The fabricated electrode exhibited a quicker response and a higher output current to the sensing of glucose in the normal and diabetic level (Xu et al. 2006).

13.10 Other Applications of Grafted Chitosan

Cardiovascular diseases is considered as one of the deadliest diseases in the world and increasing year by year. It has been reported that consumption of fish oil reduces the risk of many cardiovascular diseases because of high content of polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Vishnu et al. 2018). But fish oil is undesirable if oxidized which causes adverse effect rather than favourable effects. Encapsulation with appropriate materials could efficiently prevent undesirable oxidation of fish oils. In this regard, K. V. Vishnu et al. have reported the preparation of sardine oil loaded vanillic acid-grafted chitosan microparticles and analysed it against doxorubicin-induced cardiotoxicity. It has been proved that cardioprotection effect of fish oils is enhanced by encapsulation and grafting of chitosan with natural antioxidant agents (vanillic acid) (Vishnu et al. 2018). Microencapsulation of thiamine and pyridoxine with ferulic acid-grafted chitosan was reported by Niladri Sekhar Chatterjee et al. (2016). The results demonstrated that ferulic acid-grafted chitosan exerts potential anti-inflammatory activity, and it was found as novel dietary supplement or ingredient of functional food. Various studies reported demonstrate the mucoadhesive feature of chitosan due to their ability of producing interactions with mucin on the mucus layers via physical bond or non-covalent bonds such as chain entanglement, ionic interactions, hydrogen bonds, van der Waals forces as well as interpenetration and diffusion. Mucoadhesive properties of chitosan can be improved by grafting it with the thiolated polymers and acrylate functional groups. Nitjawan Sahatsapan reported that mucoadhesive properties can be improved by grafting chitosan with 6-maleimidohexanoic acid (Sahatsapan et al. 2018).

The allergy-related issue which is considered as a disorder of the immune system has increased dramatically within the last decades. It is an exaggerated response which occurs when exposed to normally harmless environmental substances such as insects, animal dander, foods, chemical agents, house dust mites and pollen. Thanh-Sang Vo et al. reported the grafting of gallic acid onto chitooligosaccharides enhances the inhibitory effect of chitooligosaccharides against allergic reactions in rat basophilic leukaemia (RBL-2H3) mast cells. The observed superior performance is due to the suppression of high-affinity IgE receptor (FcεRI) by gallic acid-grafted chitooligosaccharide. Histamine release suppression, cytokine generation and intracellular Ca^{2+} elevation were also observed. This study suggests that grafted chitosan can play as a potential candidate for the inhibitor of allergic reactions (Vo et al. 2012).

Diabetes mellitus is a common disorder in recent days which results in deficient insulin production (type 1) or combined resistance to insulin action and the insulin secretary response (type 2). The anti-diabetic activity of chitosan grafted with gallic acid via free radical mediated method was reported by Jun Liu et al. (2013). In vitro anti-diabetic potential is evidenced from the increased glucosidase and amylase inhibitory activity on increasing the grafting ratio. Chitosan is used for the neuroprotection by grafting it with protocatechuic acid (PCA) (Xu et al. 2018). Fluorescent bioactive corrole-grafted chitosan was prepared via chemical modification of chitosan with 5,10,15-tris-(pentafluorophenyl)corrole by Joana F. B. Barata et al. The results showed that grafting of chitosan with corrole did not affect its film forming ability and fluorescence property increases with the amount of grafted corrole units. It could be exploited as bioactive fluorescent films for bioimaging applications (Barata et al. 2016).

13.11 Summary and Conclusion

Biopolymer-based materials have been extensively studied in the past few years for their reduced side effects and improved efficiency in biomedical field. Among those investigated, chitosan a biocompatible and biodegradable polymer has drawn considerable attention in the view of reactive functional groups for easy surface modification. Under acidic conditions, the amine groups of chitosan get positively charged and they tend to adhere to the negative cell membrane. However, the drawback associated with the poor soluble nature at physiological pH hinders its usage in biomedical application. Thus, chemical modification of chitosan by forming a functionalization or composite with other active molecules through grafting, blending and cross-linking allows them with soluble nature at neutral and hydrophilicity. These chemical modifications explore new opportunities to expand the use of this polymer.

Based on the review of reports, it can be concluded that cross-linking is the multidimensional branching resulting from the polymerization of monomer. Grafting involves attachment of the molecules on the backbone of chitosan, whereas the blending results in the formation of distinct phases. The characteristics of grafted

chitosan were evaluated based on the various factors such as effect of pH, molecular weight, film forming ability, cytotoxicity, biodegradability, swelling behaviour, protein/drug adsorption and release kinetics, contact angle and mechanical properties such as elastic modulus and tensile strength. It is necessary to compare the properties of bare chitosan with the grafted chitosan to analyse the effect of respective active molecule. The properties of chitosan vary based on the synthetic technique also. Grafting of chitosan reported with various approaches such as radical-induced grafting method, grafting under microwave irradiation and Schiff base reaction has been discussed elaborately.

Based on the physical, chemical, mechanical and biological properties, grafted chitosan has been extensively applied in various fields such as antimicrobial (antibacterial, antifungal, antiviral), tissue engineering and regeneration, DNA and gene therapy, anticancer activity, drug/protein delivery and biosensor applications. The choice of the active molecules should be determined according to the desired property of the modified chitosan. In this regard, various grafted chitosan such as poly(vinyl alcohol)-g-chitosan, polyethylene glycol-g-chitosan, polyacrylamide-g-chitosan, cyclodextrin-linked chitosan, protein-g-chitosan and acid-g-chitosan and their properties were discussed. As a result, the type of active molecule used for the grafting, their ratio with chitosan and synthetic route have a direct impact on the morphology of the hybrid chitosan which poses their effect on different properties required for various respective applications.

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Chitosan-Based Systems for Controlled Delivery of Antimicrobial Peptides for Biomedical Application

14

Viorica Patrulea, Islem Younes, Olivier Jordan, and Gerrit Borchard

Abstract

Nowadays, topical microbial infections and antimicrobial resistance are global public health challenges. Despite the fact that many different antibiotics have been discovered during the "golden era," they cannot withstand due to increase in antimicrobial resistance. Antimicrobial polymers have gained attention due to their unique properties. Chitosan, a biocompatible and biodegradable polymer, has been used as an antimicrobial agent in different biomedical applications. As well, antimicrobial peptides (AMPs) are potent candidates to kill the microorganisms; however, their high toxicity and hemolytic activity hinder their clinical use. In this chapter, the use of chitosan as a matrix or as a carrier for antimicrobial peptides is addressed, with an emphasis on topical application. The first section provides an overview of the present challenges related to topical microbial infections and the antimicrobial resistance. Second, chitosan and its antimicrobial mechanism against both Gram-positive and Gram-negative bacteria, as well as fungi, are summed up. In the third section, antimicrobial peptides as efficient antimicrobial agents and their in vitro, in vivo, and current clinical applications are presented. In particular, binding AMPs to chitosan either by covalent conjugation or using chitosan as a carrier is investigated in the last section.

Keywords

Chitosan · Antibacterial · Antifungal · Antimicrobial peptide

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_14

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
CFU	Colony-forming unit
CMTMC	Carboxymethyl-trimethyl chitosan
DA	Degree of acetylation
DMCMC	*N*-(4-*N*,*N*-Dimethylaminocinnamyl) chitosan
DQ	Degree of quaternization
DS	Degree of substitution
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ESKAPE	*Enterococcus* spp., *S. aureus*, *Klebsiella* spp., *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HC	Hemolytic activity
HC_{50}	Hemolytic activity to cause 50% lysis of RBCs
hLF	Human lactoferin fragment
HMW	High molecular weight
LMW	Low molecular weight
MTGase	Microbial transglutaminase
MPyMeC	Methylated *N*-(4-pyridylmethyl) chitosan chloride
MW	Molecular weight
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
NHS	N-Hydroxysulfosuccinimide
NP	Nanoparticle
O-QCTS-DEBn	*O*-Quaternized-*N*,*N*-diethyl-*N*-benzyl ammonium chitosan
O-QCTSS	*O*-quaternized-*N*-benzylidene-chitosan
γ-PGA	Poly-γ-glutamic acid
PLGA	Poly-(lactic-co-glycolic acid)
Ppm	Parts per million
RBC	Red blood cells
TBDMS	*Tert*-butyldimethylsilyl
TMC	Trimethyl chitosan
TPP	Sodium tripolyphosphate
USP	United States Pharmacopeia

14.1 Introduction

In recent years, the use of natural compounds with active biological properties has attracted much interest in the field of biomedicine. A good example is the use of chitosan, a biopolymer derived from the ubiquitous chitin by deacetylation that has been often tested in pharmaceutical and biomedical applications due to its properties such as biocompatibility, biodegradability, and low or absent toxicity toward mammalian cells. Chitosan was found to have various biological activities, including antimicrobial activities against a wide range of microorganisms such as bacteria, yeast, and fungi. Given the increase in microbial resistance to conventional antibiotics, this property has received considerable interest.

The first section of this chapter provides an overview of the present challenges related to the treatment of microbial infections, with a special emphasis on topical infections and antimicrobial resistance (AMR). This section will highlight the urgent need of developing new drugs against multidrug-resistant bacterial populations and film-forming microorganisms.

In the second section, chitosan will be characterized as an antimicrobial agent. Benefiting from its biodegradability, low toxicity, as well as its immunostimulating, antibacterial, antifungal, antioxidant, antitumor, and wound healing properties, chitosan is widely used as an antimicrobial agent. Chitosan is a generally recognized as safe (GRAS) biomaterial and has monographies in European and United States Pharmacopeias supporting applications in pharmaceutical and biomedical fields (Dornish et al. 2012). However, as chitosan is available at differing qualities, factors that influence its antimicrobial activity and compliance with Pharmacopeias need to be addressed (e.g., solubility, content of heavy metals, amount of allergens, presence of impurities and endotoxins). A comprehensive knowledge of the exact mechanism of antimicrobial activity and the applicability of chitosan as antimicrobial agent will be presented. This section points out the importance of chitosan synthesis from chitin by a homogeneous rather than a heterogeneous deacetylation. Other important parameters for chitosan's antimicrobial activity are its degree of acetylation (DA), molecular weight (MW), solubility, chemical modification, concentration, pH, charge density, and type of microorganism. All these characteristics will influence both in vitro and in vivo activities of chitosan against Gram-positive and -negative bacteria, or fungi. The antimicrobial mechanisms of chitosan that lead to bacteria killing and/or inhibition are also described.

The third section focuses on antimicrobial peptides (AMPs) as antimicrobial agents pointing out the need to develop this new type of drugs. Their classification and mechanism of action will be provided to highlight the potency of AMPs against different microorganisms and, importantly, against most challenging bacteria or so-called "ESKAPE" microorganisms that developed resistance against most available antibiotics. This part will review the in vitro and in vivo applications of AMPs, emphasizing the most important parameters mainly the hemolytic activity (HC), the minimal inhibitory concentration (MIC), which shows the ability of AMPs to inhibit bacterial growth, and the minimal bactericidal/fungicidal concentration (MBC/MFC), which determines the bactericidal/fungicidal property of the material.

In this part will be discussed AMPs that have been approved by the US Food and Drug Administration (FDA) or are currently undergoing clinical studies in the context of topical infections.

As chitosan offers a range of possibilities of chemical modifications, the fourth section provides new chemical strategies to design chitosan derivatives for enhanced antibacterial activity and reduced toxicity. Another strategy to preserve or even enhance the activity of AMPs is their covalent coupling to chitosan, eventually using different cross-linkers and spacers. A further promising strategy is to use chitosan as a delivery carrier for AMPs in the form of nanoparticles (NPs), membranes, hydrogels, or films. All of these strategies may increase the half-life of the AMPs, stabilize them, and reduce their toxicity and hemolytic activity toward human cells.

Finally, general conclusions and perspectives on the antimicrobial application of chitosan, its derivatives, and chitosan-AMPs in the biomedical field are considered.

14.2 Challenges of Topical Microbial Infections

The increasing challenge to health care due to antimicrobial resistance and the subsequent absence of access to effective antimicrobials is of worldwide concern. Rapid emergence of drug-resistant microbes coupled with a decreasing rate of novel antibiotics entering into the clinical pipeline has created an alarming situation worldwide (Willyard 2017). This resistance complicates the treatment of infections and is associated with increased morbidity and mortality. Recent reports suggest that antimicrobial resistance kills about 700,000 people each year worldwide (Willyard 2017). Moreover, the paucity in the development of new antibiotics in over two decades adds to the urgency of preserving the antimicrobial efficacy of currently available drugs. Despite this urgent need, merely about 40 antibiotic candidates were in clinical development for the US market in September 2016, compared to hundreds of drugs for cancer treatment (Willyard 2017).

The establishment of isolated bacteria having transmissible resistance to carbapenems and colistin, considered as last-resort drugs used only when all other treatments have failed for Gram-negative bacterial infections, emphasizes this major public health threat (Allcock et al. 2017). For the first time, the World Health Organization (WHO) has released a list of the drug-resistant bacteria that pose the greatest threat to human health and for which new antibiotics are urgently needed (Willyard 2017). The list ranks 12 bacteria and is topped by carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and Enterobacteriaceae. The ranking also includes other multidrug-resistant pathogens, mainly vancomycin-resistant *Enterococcus faecium*, methicillin-vancomycin-resistant *Staphylococcus aureus,* clarithromycin-resistant *Helicobacter pylori,* cephalosporin-fluoroquinolone-resistant *Neisseria gonorrhoeae,* ampicillin-resistant *Haemophilus influenzae,* penicillin-non-susceptible *Streptococcus pneumoniae,* as well as fluoroquinolone-resistant *Campylobacter* spp., Salmonellae, and *Shigella* spp.

The use of large amounts of antibiotics to treat infections has created unusual conditions for the mobilization of resistance elements in bacterial populations and their capture by previously antibiotic-sensitive pathogens. Over time, these conditions have enabled the appearance of drug-resistant microorganisms.

Several mechanisms by which microorganisms can develop resistance to antibiotics have been described and include the production of antibiotic-inactivating enzymes, the alteration of target sites, metabolic pathways, outer membrane permeability, and efflux pumps (Blair et al. 2015). One or more of these mechanisms may be adopted by the resistant microorganisms to more than one class of antimicrobial agent. Genetic modifications are essential for microbial evolution and may arise by a variety of mechanisms, including point mutations, rearrangements of large segments of DNA from one location of a chromosome or plasmid to another, or acquisition of foreign DNA from other bacteria by horizontal transfer of mobile genetic elements. A single mutation that confers resistance to a bacterium in a population can enable survival of that organism where all other susceptible ones are killed. The resistant microorganism then continues to replicate and becomes the dominant variant (Walsh 2000).

In addition, biofilm formation has further complicated the treatment of infections with conventional antibiotics. Microorganisms can form biofilms on abiotic surfaces (hospital walls, medical devices, implants, etc.) as well as on biotic surfaces (surgical sites, wounds, lungs, urinary tract, cardiac tissues, bones, etc.) often leading to chronic infections (Konai et al. 2018). Efficient antimicrobials are of high importance to reduce microbial contamination of the inanimate environment in order to avoid hospital-acquired infections. The primary focus is to cover antimicrobial coatings and surfaces for use outside of the human body, rather than those designed for use within the body.

Moreover, patients with chronic non-healing wounds are immunocompromised allowing the formation of a biofilm over the wound, which results in serious infections. Actually, the frequency of chronic wound infections due to multidrug-resistant bacteria is progressively increasing and leads to impaired wound healing or even to mortality (Abdel-Sayed et al. 2016; Gonzalez et al. 2018). One of the three most frequent bacteria responsible for these complications is biofilm-forming *P. aeruginosa*, well protected from the host defense and developing a strong resistance despite changes in antibiotic use (Hsieh and Amin 2016).

As an alternative or a complement to antibiotic therapy, various classes of polymers have been developed as promising antimicrobial agents and have shown potent activity against both drug-sensitive and drug-resistant microorganisms.

14.3 Chitosan as an Antimicrobial Agent

Chitosan is obtained by deacetylation of chitin, the first polysaccharide discovered back in 1811 and the second most abundant natural polymer next to cellulose (Patrulea et al. 2015b). In the native state, chitin occurs as ordered crystalline microfibrils that form structural components in the exoskeleton of arthropods or in

the cell walls of fungi and yeast. So far, the main commercial sources of chitin are crab and shrimp shells (Younes and Rinaudo 2015). Chitin in crustaceans is closely associated with proteins, inorganic compounds, lipids, and pigments. They all have to be effectively removed to achieve the high purity of chitin needed especially for biomedical and pharmaceutical applications. In industrial processing, chitin is extracted by acid treatment to dissolve the calcium carbonate followed by alkaline solution to dissolve proteins. A decolorization step could be added in order to remove pigments and obtain a colorless pure chitin (Younes and Rinaudo 2015). All those treatments must be adapted to the type of chitin source, owing to differences in the ultrastructure of the initial material, to produce first a high-quality pure chitin and then chitosan.

Since a major source of chitosan is ocean shellfish, the material may contain heavy metals such as mercury and lead. According to the general chapter <232> of the United States Pharmacopeia (USP), chitosan should be free of arsenic, cadmium, lead, and mercury (USP 2013). The presence of other heavy metals may be tolerated depending on the administration route and should be limited according to their inherent toxicities. Otherwise, hypersensitive reactions to seafood are one of the most common food allergies. Determination of the amount of co-purifying allergens is an essential characterization step in establishing the safety profile of the chitosan-based material for its use as a pharmaceutical excipient (USP 2013).

Moreover, bacteria, yeast, and mold are also impurities that can arise in processed chitosan either due to the presence of microorganisms in the starting material or by contamination during preparation. Detection and specification of pathogenic bacteria should also be considered (Dornish et al. 2012). The presence of Gram-negative bacteria may also contribute to the presence of bacterial endotoxins. Endotoxins are highly toxic to mammals, particularly humans. The endotoxin level in chitosan will ultimately be critical to its use in biomedical applications (Dornish et al. 2012). As these residual impurities may have an impact on the polymer immunogenic behavior, limited amounts are defined by the USP. A monograph relating to chitosan was first introduced into the 29th edition of the USP 34-NF (USP 2010). The pharmacopeial requirements to ensure the quality of chitosan are defined as follows, determined on 1.0 g sample: loss on drying $\leq$5%, insolubles $\leq$1.0%, heavy metals $\leq$10 ppm, iron $\leq$10 ppm, protein $\leq$0.2%, aerobic microbials $\leq 10^3$*colony-forming unit* (cfu), molds and yeasts $\leq 10^2$cfu, as well as demonstration of absence of *P. aeruginosa* and *S. aureus*.

As reported in a previous review (Younes and Rinaudo 2015), from a chemical point of view, either acids or bases could be used to deacetylate chitin in order to obtain chitosan. However, glycosidic bonds are very susceptible to acid; therefore, alkali deacetylation is used more frequently. Moreover, it is noteworthy that in order to obtain chitosan, the *N*-deacetylation of chitin is performed either heterogeneously or homogeneously. Commonly, in the heterogeneous method, chitin is treated with a hot concentrated NaOH solution during a few hours, and chitosan is produced as a partially insoluble residue. These conditions give an irregular distribution of *N*-acetyl-D-glucosamine and D-glucosamine residues with some blockwise acetyl group distribution along polymeric chains, resulting in chitosan that shows a high

tendency of aggregation. According to the homogeneous method, alkali chitin is prepared after dispersion of chitin in concentrated NaOH at 25 °C for many hours or even many days, followed by dissolution in crushed ice at around 0 °C. This process produces chitosan with acetyl groups uniformly distributed along the chains with low aggregation rate and good solubility (Younes and Rinaudo 2015).

For commercial production, chitosan is manufactured on a large scale by alkaline heterogeneous *N*-deacetylation of chitin. This process results in different polymers obtained after chitin deacetylation to varying degrees. During deacetylation, acetyl groups are removed, but also depolymerization reaction occurs, indicated by changes in MW of chitosan. Thus, the pattern of deacetylation has a significant effect on the degree of aggregation and the solubility of chitosan resulting in variable behavior toward its properties even for samples of the same average DA and MW.

The wide range of chitosan sources and extraction processes leads to great differences in the purity and the physicochemical properties of chitosan, especially DA, MW, and acetyl group distribution, which as a result have an important effect on the biological properties of chitosan. Consequently, physicochemical properties of chitosan are important parameters to consider when studying its biological properties. Further understanding of the parameters influencing desired biological activity may allow researchers to identify the best combination for a particular application (Younes and Rinaudo 2015).

Despite having a great number of outstanding properties, some specially desired properties for a particular application can be incorporated by further modification of the chitosan backbone resulting in the synthesis of chitosan derivatives. The presence of certain functionalities like $–NH_2$ and –OH groups in the chitosan molecules provides the basis for interaction with other polymers and biological molecules (Vunain et al. 2017).

Chitosan and its derivatives have been widely investigated for their antimicrobial properties. They have several advantages over other disinfectants, as they possess a higher antimicrobial activity, a broader spectrum of activity, a higher killing rate, and lower toxicity toward mammalian cells (Younes et al. 2014). There are many reports discussing antimicrobial activity of chitosan at different conditions with conflicting results. In fact, it has been reported that the antimicrobial activity of chitosan is influenced by a number of factors such as the type of chitosan, average degree of polymerization and average acetylation degree, the solvent, the pH, and the microorganism involved (Younes et al. 2014).

14.3.1 In Vitro and In Vivo Antimicrobial Activity of Chitosan

Chitosan has been extensively investigated both in vivo and in vitro for its antimicrobial activity against a wide range of bacteria, fungi, and yeast with a higher activity toward Gram-positive than Gram-negative bacteria, which is still controversial in different reports. The introduction of chitosan as an antimicrobial agent dates from 1980s to 1990s and has attracted great attention in the medical, food, and environmental fields. Usually, previous reports discussing its antimicrobial activity

present chitosan as a bactericidal/fungicidal (when microorganisms are killed) or bacteriostatic/fungistatic (when microbial growth is stopped but microorganisms are not killed) agent (Goy et al. 2016). Very often, there is no clear border between these two activities. The exact mechanism of action remains not fully understood, and previous studies suggest that chitosan could be either bactericidal/fungicidal or bacteriostatic/fungistatic depending on the specific microorganism and also the characteristics of the polymer (Younes et al. 2014). Generally, a molecule is considered as -static and not -cidal, when the MBC (minimum bactericidal concentration, or the lowest concentration where a material is able to kill the microorganism) is at least 4 times higher than the MIC (minimum inhibitory concentration) (Steigbigel and Steigbigel 2018). MIC is determined as the lowest concentration of a drug to inhibit microbial growth by serial microdilution in multiwell plates, investigating multiple concentrations and different microorganism strains. MBC is determined by subculturing the microorganism in the MIC assay on agar plates or on liquid media and is defined at the concentration that kills 99.9% of the initial microorganisms. Because MIC is influenced by the initial concentration of the inoculum (initial concentration of the microorganism), it is not possible to discriminate between -static or -cidal potency. However, to choose the final antibacterial drug candidate, it is important to check for its hemolytic activity against red blood cells (RBC). Other parameters like LC_{50} (lethal concentration to reduce bacteria to 50%) or EC_{50} (half-maximal effective concentrations) can be determined by measuring the inhibition zone diameters. AlamarBlue and live/dead assays can be used as well to show bacterial viability based on inoculum of 10^4–10^7 cfu/mL; however, there are significant differences between different assays and those differences are not mentioned in the studies (Steigbigel and Steigbigel 2018).

However, the efficiency of an antibacterial biopolymer is measured by its ability to kill the bacteria without harming human cells. Therefore, the polymer should have the lowest MBC value and highest biocompatibility (lowest cytotoxicity). Table 14.1 includes the most important characteristics of chitosan, which highlights its in vitro antimicrobial property against Gram-positive and -negative bacteria and also against fungi. Unfortunately, most of the studies do not include a toxicity or biocompatibility evaluation.

Commercially available chitosan is synthesized by heterogeneous method, which produces a wide range of MW distribution of chitosan, thus explaining the occasional variation in MICs between reports. However, according to in vitro studies, chitosan is bactericidal and not only bacteriostatic, as shown in various reports mentioned in Table 14.1.

Table 14.2 summarizes the in vivo application of chitosan as antimicrobial agent with the main focus on topical infections.

Chitosan has been prescribed in clinics mostly as wound dressing for minor burns or as hemostatic agent, including HemCon™ bandage, ChitoFlex wound dressing, and Celox™ (Dornish et al. 2012; Raafat and Sahl 2009) or in different formulations, such as toothpaste (Chitodent®), mouthwash solutions, and chewing gums. To the best of our knowledge, until now, there are no available antibacterial dressings in clinical application yet.

Table 14.1 Antimicrobial activity of chitosan in vitro

Microorganism strain(s)	Chitosan MW (kDa)	Chitosan DA (%)	MIC (μg/mL)	MBC (μg/mL)	References
Gram-positive bacteria					
S. pneumoniae	322	14.45	100	NR	Shanmugam et al. (2016)
S. aureus			80	NR	
Streptococcus sp.			60	NR	
S. aureus ATCC 25923	220	15	8	32	Kong et al. (2010)
Bacillus cereus	80	NR	62.5–125	62.5–125	Tamara et al. (2018)
	200	NR	62.5–125	62.5–125	
	500	NR	62.5–125	62.5–125	
	1,500	NR	62.5–125	62.5–125	
S. aureus ATCC 25923	220	15	8	32	Qi et al. (2004)
Gram-negative bacteria					
Klebsiella pneumoniae	322	14.45	100	NR	Shanmugam et al. (2016)
P. aeruginosa			60	NR	
Salmonella sp.			80	NR	
Vibrio alginolyticus			80	NR	
Vibrio parahaemolyticus			100	NR	
Vibrio cholerae			60	NR	
Escherichia coli			80	NR	
Proteus vulgaris			50	NR	
E. coli ATCC 25922	220	15	8	64	Kong et al. (2010)
Salmonella choleraesuis ATCC 50020			16	32	
Salmonella typhimurium ATCC 50013			16	64	
E. coli	80	NR	125	250	Tamara et al. (2018)
	200	NR	62.5–125	125	
	500	NR	125	125–250	
	1500	NR	125–250	125–250	
E. coli K88	220	15	8	64	Qi et al. (2004)
E. coli ATCC 25922			8	64	
S. choleraesuis ATCC 50020			16	32	
S. typhimurium ATCC 50013			16	64	

(continued)

Table 14.1 (continued)

Microorganism strain(s)	Chitosan MW (kDa)	Chitosan DA (%)	MIC (μg/mL)	MBC (μg/mL)	References
Fungi					
Candida krusei ATC 6258	70	< 25	1 (pH 4)	NR	Alburquenque et al. (2010)
			32 (pH 7)		
Candida albicans ATC 64568			>128 (pH 4, 7)		
Candida tropicalis Rex MY1012			0.12 (pH 4)		
			2 (pH 7)		
Saccharomyces cerevisiae ATCC 9763			>128 (pH 4; 7)		
Candida lusitaniae Rex Cl 2819			4 (pH 4)		
			16 (pH 7)		
Candida parapsilosis ATCC 22019			0.5 (pH 4)		
			1 (pH 7)		

NR not reported

14.3.2 Antibacterial Mechanism of Action of Chitosan

The ambiguity in chitosan antibacterial response arises from factors such as (*i*) sensitivity to microorganism strains (Gram-positive or -negative bacteria, fungi or yeast) and (*ii*) chitosan physicochemical properties like charge density, MW, DA, water solubility, hydrophobicity/hydrophilicity, chelating ability, and solid/liquid state (Goy et al. 2009; Kong et al. 2010; Ma et al. 2017). Based on the aforementioned factors, three hypothesized mechanisms of action have been proposed:

(a) The most widely accepted mechanism is based on the electrostatic interaction between positive charges from amino groups present in chitosan and the negative charges on the microbial surface (Ma et al. 2017). This interaction is affecting the cell surface and detaching the cell wall from the cell membrane, thus causing leakage of the intracellular components that finally leads to cell necrosis. Thus, a higher positive charge leads to stronger antibacterial effect. Moreover, high DA, lower MW, and lower pH of chitosan lead to an enhanced antibacterial activity (Younes and Rinaudo 2015). In this case, the most important factors are the lipopolysaccharides present in Gram-negative bacteria and teichoic acid in Gram-positive bacteria, which bind to chitosan leading to membrane destabilization and death (Fig. 14.1) (Ma et al. 2017). Raafat et al. showed that chitosan is more effective against Gram-positive than -negative bacteria through electrostatic interaction with negatively charged teichoic acid (Raafat et al. 2008).

Table 14.2 In vivo antimicrobial activity of chitosan

Microorganism strain(s) found	Tested animals	Chitosan characteristics	Results	References
S. aureus ATCC 25923 injected into the percutaneous exit site of the implants	Rabbit	200–400 kDa, 10% DA	Chitosan-based sleeves prevented bacterial infection	Shao et al. (2018)
Pathogens in mammary gland	Cattle	LMW and HMW at 5% concentration	Accelerated mammary gland involution and activated immune response	Lanctôt et al. (2017)
V. vulnificus inoculated	Mice	1 and 10 kDa, 5–10% DA	1 kDa had weak inhibition effect	Lee et al. (2009)
			10 kDa (1–10 mg/mL) inhibited completely the growth of bacteria and prolonged the survival period of infected mice	
Microflora (*Pseudomonas* and Vibrionaceae most predominant)	Oysters	5.0 mg/mL, 18–20% DA	Extend the shelf-life of oysters	Cao et al. (2009)
Pathogens	Shrimp muscle	338 kDa, 29% DA	Inhibited microbial growth during 24 days of storage	Chouljenko et al. (2017)
Growth control of the intestinal bacteria	Hamsters	50% DA	Less activity with 50% DA, than 30% and 5% DA. Per total chitosan had little activity	Tsai and Hwang (2004)
		30% DA		
		5% DA		

A similar antifungal mechanism was observed in the fungal membrane of *C. fimbriata* (Xing et al. 2018), *C. albicans* (Mousavi et al. 2018), *Aspergillus* sp., *Rhizopus, Penicillium,* and *A. nigers* (Mohammadi Amirabad et al. 2018).

(b) The second mechanism involves chitosan binding to microbial DNA (Fig. 14.2). The ability of chitosan to bind DNA is of a major interest, especially for gene delivery (Borchard 2001). It is reported that chitosan, particularly of LMW, can penetrate into the cell nuclei and bind to DNA leading to the inhibition of mRNA and DNA transcription (Younes and Rinaudo 2015). Although this might be a plausible mechanism, it is not clear if this is contributing to the antibacterial activity of chitosan. For instance, Raafat et al. mentioned that the probability of it occurring is rather low, because chitosan is rather disrupting the outer bacterial membrane than acting as a penetrating substance (Raafat et al. 2008).

(c) The third mechanism reported by some authors is based on chitosan's ability to chelate Me^{2+} metallic ions such as Ni^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, and Cu^{2+} (Goy et al. 2009) (Fig. 14.3). Some researchers mentioned that the formation of complexes with bivalent metals would deprive bacteria of nutrients, thus leading to their death. However, Raafat et al. have shown that metal chelation is not

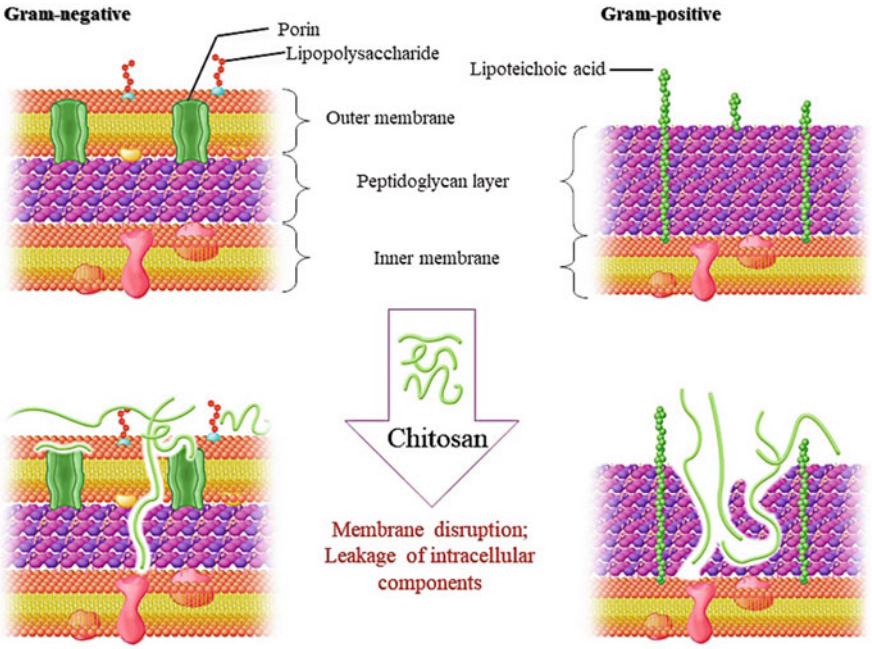

Fig. 14.1 First hypothesis for chitosan membrane disruption and the following cascade events in the presence of chitosan

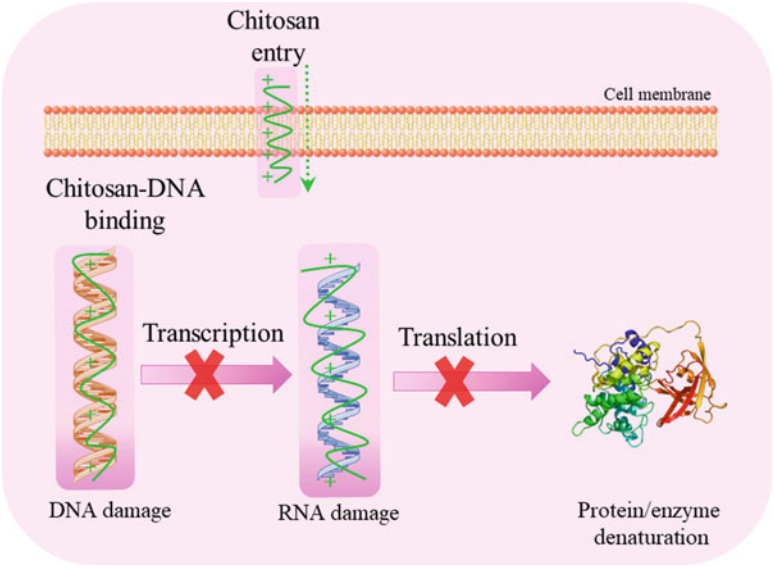

Fig. 14.2 Second hypothesis of chitosan-DNA binding mechanism

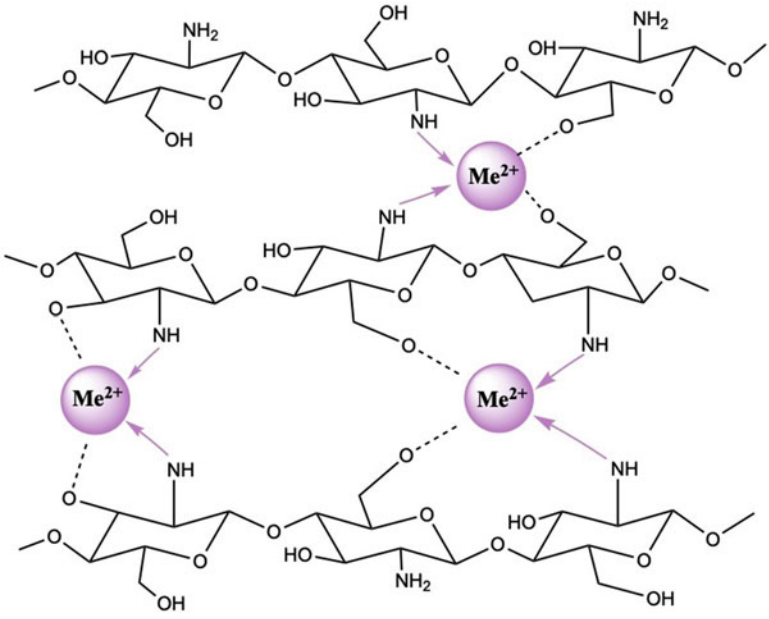

Fig. 14.3 Third hypothesis on chitosan chelation with bivalent metals

necessarily of importance for the antibacterial effect; on the contrary, it rather impedes the antibacterial activity of chitosan (Raafat et al. 2008).

The antimicrobial activity of chitosan, therefore, depends on different factors such as the microbial strain and chitosan's physicochemical properties, which may lead to different cytotoxic mechanisms.

14.4 Antimicrobial Peptides (AMPs) as Antimicrobial Agents

14.4.1 Classification of AMPs

The AMR to new antibiotics has led to finding new types of drugs to limit resistance-inducing mechanisms in microbes. Dubos and Gause, the fathers of the AMPs, first isolated the tyrothricin compound in 1939, independently from each other in their respective labs. They extracted an antimicrobial agent from *Bacillus* strain, which proved to be effective against pneumococci infection in mice (Brazhnikova 1987; Dubos 1939; Gall and Konashev 2001). Later Hotchkiss and Dubos found that the extract comprises two different molecules: 20% gramicidin or "the gentle protector" and 80% tyrocidine. Tyrocidine AMP (lysine based) was shown to be effective against both Gram-positive and -negative bacteria, but was very toxic toward RBCs (Dubos and Hotchkiss 1941). Gramicidin, the first natural antibiotic, was intensively

used during the Second World War for the treatment of topical infections of wounds and ulcers, mainly against Gram-positive bacteria (Van Epps 2006).

The first animal-originated AMP is defensin, which was isolated in 1956 from New Zealand Red rabbit leukocytes (Hirsch 1956), followed by a 24-amino acid bombinin isolated from frog skin (Kiss and Michl 1962) and lactoferin from cow milk (Groves 1960). AMPs appear to be promising alternatives to conventional antibiotics for the treatment of infections (Ostorhazi et al. 2018). AMPs or so-called host defense peptides are generally composed of short sequences of D-amino acids (<50 residues for most amino acids and <200 residues for human antimicrobial proteins), are part of the innate system for protecting against bacterial infection, and show little drug resistance (He et al. 2018; Wang et al. 2016; Zhang et al. 2017). Inspired by natural AMPs, synthetic or semi-synthetic analogues have been developed to overcome before-mentioned issues. Much effort is dedicated to develop synthetic AMPs, which have higher antimicrobial activity compared to natural analogues and provide low risks of toxicity toward host cells.

An online antibacterial peptide database (APD3) lists more than 2619 AMPs originating from all species. Only 112 were found as human host defense peptides out of which 100 have shown antibacterial activity (Andersson et al. 2016; Wang et al. 2016). They can be classified depending on the charge, length, and sequence of amino acids and have both amphiphilic and cationic properties, e.g., human AMPs have a charge range of −3 to +20 (Andersson et al. 2016). The most prominent AMPs are linear cationic molecules of α-helical conformation (Sahariah and Másson 2017). After AMPs have been discovered in polymorphonuclear leukocytes, their use has drawn attention of many researchers in the area of antimicrobial therapy, including β-defensins (Bensch et al. 1995) and cathelicidins (Gallo et al. 1997).

Based on their 3D structure, four different families of AMPs are found in APD3; however, this classification needs to be revised, as the structure of more than 2200 AMPs is not fully determined yet (Yeaman and Yount 2003).

(a) α-Family is based on AMPs with a α- helical structure, *e.g.*, human cathelicidin LL-37 (Kuroda et al. 2015), cryptidin-4, mangainin (Zasloff 1987), and protegrin (Fahrner et al. 1996).
(b) β-Family is comprised of AMPs with at least two β-sheets and two to four disulfide bridges, *e.g.*, human β-defensins and rhesus myeloid α-defensin-4 (RMAD4) (Schmidt et al. 2012), drosomycin (Fehlbaum et al. 1994), and protegrin (Fahrner et al. 1996) and plectasin (Mygind et al. 2005).
(c) Loop family contains single bond (either disulfide, amide or isopeptide) *e.g.*, thanatin.
(d) Extended or non-αβ-family, which is not comprised of neither α-helical nor β-sheets, *e.g.*, indolicidin tritrpticin (Khandelia and Kaznessis 2007).

However, natural AMPs have some limitations, like high manufacture costs, poor proteolytic stability and difficulties when formulating (e.g., peptide co-precipitation with proteins found in plasma), toxicity, and poor in vivo efficacy (Thaker et al. 2012). Therefore, to date, they are limited in the translation into clinical trials as listed in Table 14.3 (Costa et al. 2014; Zhang et al. 2017).

Table 14.3 In vitro and in vivo antibacterial activities of AMPs, including their hypothesized mechanism of action

Name of AMPs	MIC/MBC (μg/mL) and tested strains	Mode of action and hemolysis (HC_{50})	References
In vitro results			
Gramicidin S	8–16/8–16 in *E. faecalis* strains	Cell morphology alteration, membrane penetration	Berditsch et al. (2016)
Insect-derived melittin (CP26)	2.1/NR in *Mycobacterium tuberculosis*	Cell membrane disruption by electrostatic interaction of positive and negative charges and intracellular targeting	Rivas-Santiago et al. (2013)
RIWVIWRR-NH_2 (E2)	2.6/NR in *M. tuberculosis*		
RRWRIVVIRVRR-NH_2 (E6)	3.2/NR in *M. tuberculosis*		
Human cathelicidin LL-37	4.5/NR in *M. tuberculosis*		
CP26	0.5/NR in *P. aeruginosa*	Absence of hemolysis	
E2	4.7/NR in *P. aeruginosa*		
E6	2.5/NR in *P. aeruginosa*		
LL-37	9.0/NR in *P. aeruginosa*		
LL-37	2400/NR in *Mycobacterium smegmatis*	Membrane disruption and pore formation, $HC_{50} = 4\%$	Chingaté et al. (2015)
LLAP	600/NR in *M. smegmatis*	Membrane disruption and pore formation, $HC_{50} = 1.1\%$	
$M(LLKK)_2M$	62.5/NR in *M. tuberculosis* CSU87	Pore formation, membrane lytic, $HC_{50} > 1000$ μg/mL	Khara et al. (2014)
	62.5/NR in *M. smegmatis*		
	125/NR in *M. tuberculosis* H37Rv		
	15.6/NR in *Mycobacterium bovis BCG*		
Cathelicidin LL-37	14/28 in *S. aureus* ATCC 29213	Specific intracellular targeting, DNA alteration, and proteolytic degradation	Leszczynska et al. (2013)
	14/14 in *S. pneumoniae*		
	28/56 in *H. influenza*		
	224/448 in *P. aeruginosa* Xen 5		
	28/56 in *Neisseria meningitidis* (B)		
	28/112 in *N. meningitidis* (C)		

(continued)

Table 14.3 (continued)

Name of AMPs	MIC/MBC (μg/mL) and tested strains	Mode of action and hemolysis (HC_{50})	References
Ceragenin CSA-13	0.7/1.4 in *S. aureus* ATCC 29213	Hemolysis NR	
	0.35/0.7 in *S. pneumoniae*		
	0.35/0.7 in *H. influenza*		
	5.6/11.2 in *P. aeruginosa Xen 5*		
	0.7/0.7 in *N. meningitidis* (B)		
	0.7/1.4 in *N. meningitidis* (C)		
Ceragenin CSA-90	0.7/2.8 in *S. aureus ATCC 29213*		
	0.7/1.4 in *S. pneumoniae*		
	0.7/1.4 in *H. influenza*		
	22.4/22.4 in *P. aeruginosa Xen 5*		
	1.4/1.4 in *N. meningitidis* (B)		
	1.4/1.4 in *N. meningitidis* (C)		
Spermine-conjugated dexamethasone derivative D2S	2.1/2.1 in *S. aureus ATCC 29213*		
	0.55/1.1 in *S. pneumoniae*		
	1.1/1.1 in *H. influenza*		
	8.1/8.1 in *P. aeruginosa Xen 5*		
	2.1/8.12 in *N. meningitidis* (B)		
	2.1/4.05 in *N. meningitidis* (C)		
Polymyxin B	7.8/NR in *M. smegmatis*	Pore formation, inducing leakage of intracellular components, HC NR	Gupta et al. (2015)
LL-37	31.3/NR in *M. smegmatis*		
Cobra-derived cathelicidin NA-CATH	250/NR in *M. smegmatis*		
Mouse cathelicidin CRAMP	15.6/NR in *M. smegmatis*		

Magainin 2 monomer MG2a	31.25/NR in *E. coli* K12	Electrostatic interaction and intrinsic lipid curvature, HC NR	Leber et al. (2018)
Magainin 2 dimer MG2a-MG2a	3.9/NR in *E. coli* K12		
Bovine lactoferin L10	1-8/NR in *E. coli strains*	Membrane-disruption and pore formation	Mishra et al. (2013)
	2-8/NR in *K. pneumoniae strains*	No hemolysis at 800 μg/mL	
	2-4/NR in *A. baumannii strains*		
	NA-100/NR in *C. albicans strains*		
	12.5-100/NR in *C. tropicalis*		
King cobra cathelicidin OH-CATH30	2-128/NR in *Acinetobacter* sp., *Pseudomonas* sp., *Klebsiella* sp., and *Salmonella* sp.	Hemolysis at >250 μg/mL	Zhao et al. (2018)
D-OH-CATH30 (D-analogue of OH-CATH30)			
Melittin	Very effective against biofilm formation of *Borrelia* spp. at >100 μg/mL	Lysis of human erythrocytes, necrotic activity against gastrointestinal and vaginal epithelial cells, severe allergic reactions	Socarras et al. (2017)
RN7-IN8 hybrid	Morphological alterations in *S. pneumoniae* by disrupting the integrity of the cell wall	No reported toxicity	Jindal et al. (2017)
Nisin	Spermicidal effect on spermatozoa from rat (50 μg/mL), rabbit (200 μg/mL), and monkey and human (300–400 μg/mL) within 20 s	No toxicity or hemolysis reported	Aranha et al. (2004)
In vivo results			
Micafungin (FK463)	Efficient against *Candida* sp. at <0.125 μg/mL in rats, dogs, mice and rabbits	No reported dose toxicity	Niwa et al. (2004)
Innate defense regulator (IDR1)	No direct antimicrobial activity, but showed protection in mice from *S. aureus, Enterococcus,* and *Salmonella* spp.	No reported toxicity	Haney and Hancock (2013)

(continued)

Table 14.3 (continued)

Name of AMPs	MIC/MBC (μg/mL) and tested strains	Mode of action and hemolysis (HC_{50})	References
LL-37	Effectively protecting against Gram-positive and -negative bacteria and *Candida* sp.	Prophylaxis for intra-abdominal sepsis in rats	Torossian et al. (2007)
RN7-IN8 hybrid based on indolicidin and ranalexin	Improved survival rate of pneumococcal bacteremia infected mice at 20 mg/kg; 50% of the infected mice survived up to 7 days postinfection	No sign of toxicity reported at 20 mg/kg	Jindal et al. (2017)
Oligo-acyl-lysyl $C_{12}K\text{-}2\beta_{12}$	Reduction of *H. pylori* infection from the stomach at 1 day and 1 week postinfection in gerbil model	No hemolysis reported	Makobongo et al. (2012)
Nisin	Potent in rats as a vaginal contraceptive agent	Safe reported	Aranha et al. (2004)
Nisin Z	Efficient vaccine against staphylococcal mastitis in lactating cows	Safe reported	Guan et al. (2017)

NR not reported, *NA* not active

14.4.2 Mechanisms of Action of AMP

Even though the mechanism of action has not been fully elucidated, most researchers agree on the electrostatic attraction mechanism between positive charges of AMPs and negative charges of the bacterial cell surface, which leads to membrane disruption and, in turn, cell death (Chai et al. 2014; Costa et al. 2014). This mechanism is also known as Shai-Matsuzaki-Huang (SMH) model.

There are other hypothesized mechanisms of action to block the cellular process like pore formation, membrane thinning, and toroid pore/wormhole formation resulting in membrane disintegration, while carpet-like model leads to micelle formation and AMP translocation across the cell membrane (Ageitos et al. 2017; Li et al. 2017; Lind et al. 2015; Zhang et al. 2017). The antibacterial effects of AMPs can result as well from their preferential affinity to associate with different target microorganisms or can be due to their interference with cellular metabolic processes (Yeaman and Yount 2003). For example, nisin secreted from *Lactococcus lactis* is one of the most well-characterized AMPs and is active in the nanomolar range for the treatment of skin infections caused by methicillin-resistant *S. aureus*. It is believed that nisin has a specific affinity to bind to the peptidoglycans of Gram-positive rather than Gram-negative bacteria (Yeaman and Yount 2003; Zhu et al. 2017). Indolicidin, for example, acts slightly differently as it does not lyse bacterial cells, but it does inhibit DNA synthesis (Ghosh and Haldar 2015).

In addition, it was shown that antibacterial activity of AMPs could be modulated as well by shifting the pH or temperature. For instance, hepcidin-25 and hepcidin-20 (human liver-derived peptides) activity is enhanced in an acidic environment (Batoni et al. 2016).

14.4.3 Resistance to AMPs

AMR is a global concern due to increased resistance to existing antibiotics. It is estimated that the rate of mortality will increase by the end of 2050 and more than 10 million people will die annually due to continued rise in AMR if no solutions will be provided (Ragheb et al. 2018). The amount of antibiotics discovered during the years is low, while bacterial resistance is increasing, leading to an increase of chronic infections (Lewis 2013). At one point, even big pharmaceutical companies like Roche, Bayer, Pfizer, Lilly, and Abbott have stopped their research projects on antibiotics due to high AMR (Ghosh and Haldar 2015). As shown in Fig. 14.4, the "golden era" of antibiotics started with penicillin discovery in 1928, which had been used in clinics only in 1938, and shortly after that in 1945, the microorganisms already developed antibiotic resistance (Lewis 2013). However, history shows rise in AMR happens regardless of the nature or potency of the drug. In most instances, high AMR is due to the misuse or the overuse of antibiotics, which stimulates the bacteria to develop new mechanisms to resist antibiotics (Qiao et al. 2018).

The most challenging multidrug-resistant bacteria are the so-called "ESKAPE" microorganisms, abbreviation standing for *Enterococcus* spp., *S. aureus*, *Klebsiella*

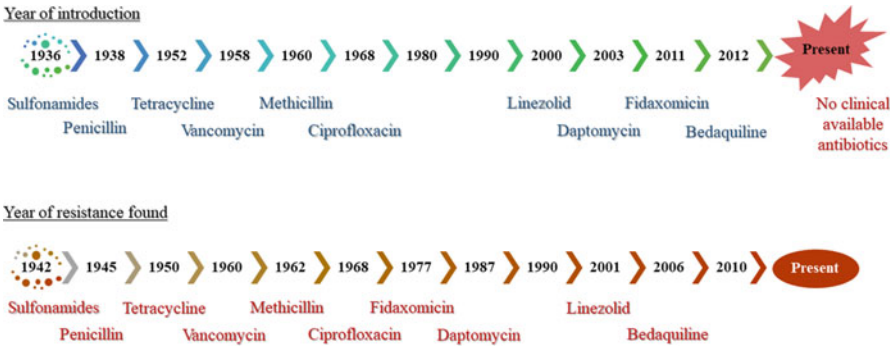

Fig. 14.4 Timeline of antibiotics introduction on the market versus their resistance acquisition. (Adapted from Lewis 2013)

spp., *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp., among which Gram-negative *A. baumannii* is resistant to all current antibiotics. *A. baumannii* is responsible for nosocomial infections in pulmonary and bloodstream infections (Xie et al. 2018). Therefore, it is a critical time to find alternatives to conventional antibiotics to treat and kill these deadly bacteria.

AMPs as therapeutic drugs offer specific advantages over existing antibiotics as shown over the years when bacteria quickly developed resistance against antibiotics. Compared to antibiotics, which target the synthesis of bacterial cell wall, production of proteins, and DNA replication, AMPs act directly on the bacterial cell membrane, thus bypassing the whole mechanism of AMR. Moreover, AMPs immunomodulate the innate system to fight against bacterial infections. Despite the aforementioned advantages, microorganisms find their way to develop resistance (Ghosh and Haldar 2015).

Mechanisms of resistance to AMPs are divided into two different categories (Yeaman and Yount 2003):

(a) *Constitutive resistance* or passive mechanism refers to properties that are inherited by the microorganism and confer resistance to killing even in the absence of exposure to AMPs. For example, *Pseudomonas* strains show an exceptional in vitro resistance to AMPs due to their stable structure, or due to the formation of a biofilm (Ghai and Ghai 2018). This kind of mechanism relies on electrostatic shielding, difference in membrane charge during bacterial growth and formation of a biofilm.

(b) *Inducible resistance* or adaptive mechanism includes strategies that trigger the response after AMP exposure or from a certain cell stress applied, which generally results in a greatest chance of microorganism survival and resistance to the host AMPs. Such strategies include substitution (Lewis et al. 2009), modification (Gunn 2001; Pagès et al. 2008), and acylation (Guo et al. 1998; Roy et al. 2009) of the bacterial membrane, impacting its permeability. Other effective mechanisms include proteolytic degradation and export of AMPs

through efflux pumps and steric hindrance by O-antigen of lipopolysaccharides (LPS). Noteworthy, linear AMPs like LL-37 are more prone to proteolytic degradation than other AMPs with a nonlinear structure, which include disulfide bonds (Joo et al. 2016).

AMPs' multiple ways of action and lack of specificity reduce the chances for microbial resistance. However, despite the efforts to control biofilm formation, resistance to AMPs or maybe increased tolerance to AMP hinders their therapeutic use for the treatment of infectious diseases. Moreover, the exact molecular targeting mechanism of the AMPs needs to be further explored.

14.4.4 AMPs: Activity Versus Toxicity Balance

In view of AMP clinical use, one should consider their mechanisms of action, stability, and the balance between their activity and toxicity (Sahariah et al. 2015b). Toxicity of AMPs includes membrane toxicity, mammalian cell toxicity, and systemic toxicity. We can define selectivity toward bacterial cells as the ratio of HC_{50}/MIC, where HC_{50} is the concentration needed to cause 50% lysis of RBCs and MIC is minimal concentration to inhibit the growth of the microorganism (Sahariah et al. 2015a). Usually, AMPs show high affinity toward bacterial cell membranes, thus resulting in higher bacterial inhibition (which means low MIC) and less toxicity to RBC (Table 14.3). Emphasis was put on topical bacteria responsible for infections.

Therefore, potent AMPs have to be selected based on their MIC and MBC, taking into account the crucial factor of HC_{50} which indicates the toxicity profile of the AMPs toward mammalian cells.

14.4.5 Clinical Applications of AMPs

As mentioned before, selection of AMPs for treatment of infectious diseases starts with an in vitro/in silico screening to determine their MIC and HC_{50}. In silico developed methods refer to chemo-informatic method of quantitative structure-activity relationship (QSAR) to predict the antimicrobial activity of a virtual library of thousands of AMPs. The advantage of such in silico method relies on the potential to increase the number of candidates with antimicrobial activity. This methodology proved to have 94% accuracy and is capable of predicting which peptides will be active in vitro (Haney and Hancock 2013). Still, there are discrepancies between the in vitro and in vivo results of the AMPs. The reason might be due to the in vivo environment, which is different from the in vitro conditions, and some microorganisms may develop different affinities to one or another environment (Steckbeck et al. 2014). Another explanation might be that AMPs can modulate the immune response in vivo by enhancing immunity and suppressing inflammation rather than killing the microorganism. Moreover, AMPs that show low in vitro MIC

and/or MBC may fail to show in vivo efficacy due to their fast proteolytic degradation (low stability in plasma), another important limiting factor. Systemic administration of these AMPs, like IV injections, will be ineffective due to the fast degradation by proteases and/or enzymes followed by their rapid clearance. Limited in vivo activity may also be related to AMP's high hydrophobicity and high conformational flexibility, leading to nonspecific or poor distribution when interacting with different receptors (Vlieghe et al. 2010). Therefore, a very poor correlation between in vitro and in vivo results hampers AMPs development toward clinical trials.

Currently, only a few AMPs, which hold potential as promoters of wound healing, reached clinical trials and some of them have been approved by FDA, as reported in Table 14.4. The majority of AMPs are limited to topical applications due to their systemic toxicity.

Although few AMPs are already approved, there are other challenges when designing a successful AMP, like peptide cross-linking, truncation, and addition of other amino acids. Another approach would be to use nanotechnology, encapsulating AMPs to safely deliver them to the desired site, while maintaining their long-term bioactivity.

Moreover, AMP's activity depends on their purity degree in the good manufacturing practices (GMP) manufacturing process (Vergote et al. 2009), which is mostly related to deletion and insertion of amino acids, oxidation/reduction of amino acids, undesired peptide counterions (e.g., trifluoroacetate), inefficient Fmoc-deprotection (leading to racemization of amino acids), unrelated reagents and solvents (during the purification step), aggregation (covalent and non-covalent interaction between the peptides), and contamination with other non-active peptides. Peptide degradation was as well reported leading to β-elimination, diketopiperazine, pyroglutamate, and succinimide (observed mainly during AMP storage) (D'Hondt et al. 2014).

Usually, AMPs are synthesized by solid-phase peptide synthesis (SPPS) and this process is now automated and less costly and can ensure GMP quality of the products even after many steps of the synthesis. For example, to synthesize enfuvirtide, an anti-HIV 36-amino acid peptide, it takes 6–8 months and 106 synthesis steps to obtain 3.7 tons per year (Bray 2003).

14.5 AMP-Chitosan Combination and Potential Synergies as Carriers

14.5.1 Chemical Modification of Chitosan Polymer

Chitosan, as discussed above, has antibacterial properties only at low pH when amino groups of chitosan are protonated and because chitosan is soluble at pH lower than its pk_a ($pK_a = 6.5$). Therefore, to overcome these solubility issues and enhance antimicrobial activity, chitosan can be modified/functionalized/derivatized, choosing the right strategy and target.

Table 14.4 Peptide drugs currently approved for clinical trials in the context of topical infections

AMP name/ trade name	Treatment approved	Side effects	Clinical status	References
Polymyxin B	Eye infections, but gradually withdrawn from clinical practice	Nephrotoxic effects	Used from 1950s to 1970s.	Rabanal and Cajal (2017)
Polymyxin E (colistin)	Infected wounds and sometimes in cystic fibrosis		Now used only as a last resort	
Cilofungin (anti-*Candida*)	*Candida* infections	High toxicity (solvent required for administration)	Discarded	Boto et al. (2018)
Iseganan derived from pig protegrin (IB-367)	Oral mucositis, ventilator-associated pneumonia	Low bacterial strain selection rate, taste disturbance	Phase III, discontinued due to health risks	Elad et al. (2012)
Novarifyn® (NP432)	Potent against multidrug-resistant Gram-negative and -positive microorganisms	Not reported	Preclinical trials	Ciumac et al. (2019)
Nisin	Control over *H. pylori* in the stomach and colon	Free from side effects	Phase I, GRAS approved	Aranha et al. (2004)
Human lactoferin fragment hLF1-11	Bacterial and fungal infections	Aggregation trend, weak antibiotic activity	Phase II, company suspended trials	Hamill et al. (2008) and Haney et al. (2017)
	Prophylaxis in hematopoietic stem cell transplantation		Phase I, company suspended trials	
Brilacidin® (defensin mimetic)	Acute skin and soft tissue infections, e.g., oral mucositis	Not reported	Phase II	Mensa et al. (2014)
Innate defense regulators (IDR1)	No direct bacterial recognition, but via toll-like receptors	Lacks direct antimicrobial activity	Phase II	Kumar et al. (2018)
IMX942 (IDR1 analogue)	IV injections against hospital-acquired infections			
OP-145	Chronic middle ear infection	Safety reported	Phase II	Riool et al. (2017)
$(CKPV)_2CZEN$-002	Vulvovaginal *Candida* sp.	Safety reported	>Phase II	
Human LL-37	Leg ulcer	Safety reported	>Phase II	Gronberg et al. (2014)
Novexatin (NP213)	Fungal nail infection	Safety reported	>Phase II	Ciumac et al. (2019)

(continued)

Table 14.4 (continued)

AMP name/ trade name	Treatment approved	Side effects	Clinical status	References
Omiganan (ox indolicin) (MX-226)	Prevention of catheter infection and acne, rosacea	Not reported	Phase II/III	Molchanova et al. (2017)
P-113 (histatin 5 derivative)	Oral mouth rinse solution for anti-*Candida* infections	Safety reported	>Phase II	Cheng et al. (2018)
Pexiganan (Locilex®) (mangainin analogue)	Diabetic foot ulcers	No evidence of higher performance than antibiotic	Phase III	Ciumac et al. (2019), Lipsky et al. (2008) and Uçkay et al. (2018)
Gramicidin isolated from *B. brevis* (cyclic β-sheet decapeptide)	Ophthalmic drops, wound and throat infections, and genital ulcers	Strong hemolytic activity	2005 approved	Wenzel et al. (2018)
Micafungin	Fungal infections	Infusion reactions, potential carcinogenicity ([a])	2005 approved	FDA (2018) and Kane and Muzevich (2016)
Anidulafungin	Fungal infections	Nausea or vomiting, diarrhea, headache	2006 approved	Verma et al. (2016)
Telavancin (Vibativ®)	Complicated skin and skin structure infections (CSSSIs), nosocomial pneumonia	Nausea, vomiting, taste disturbance	2009 approved	Schroeder et al. (2017)
Dalbavancin	Acute bacterial skin and skin structure infections (ABSSSIs)	Gastrointestinal distress, pruritus, and hypersensitivity reactions	2014 approved	Zorzi et al. (2017)
Oritavancin	CSSSIs and ABSSSIs	Nausea, hypersensitivity reactions, and osteomyelitis	2014 approved	Rosenthal et al. (2018)
Caspofungin (Candidas®)	Only for anti-*Candida* infections	Poor oral bioavailability, but efficient upon IV administration	2017 approved	Rodríguez-Cerdeira et al. (2019)

Clinical status was updated from FDA list of approved drug products (FDA 2018)
[a]No statistical significance
IV intravenous

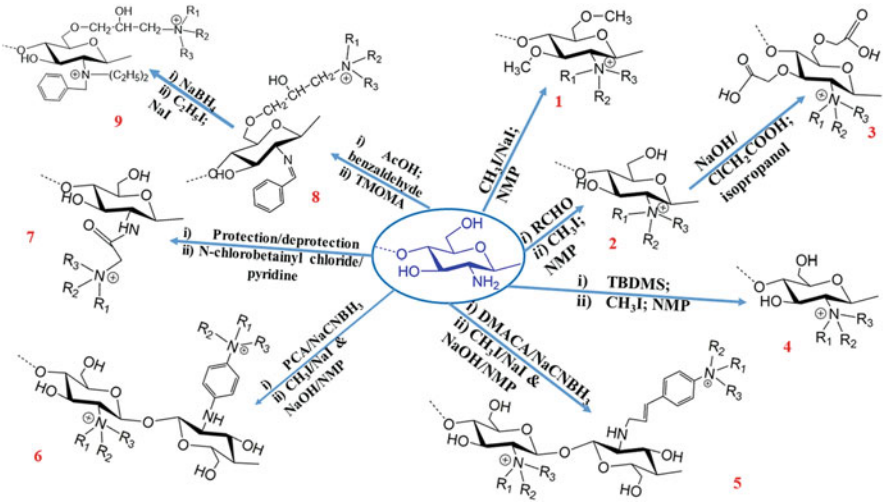

Fig. 14.5 Synthetic scheme for quaternary chitosan derivatives with antibacterial activity. $R_{1,2,3}$ = alkyl DMACA = 4-dimethylamino cinnamaldehyde; PCA = 4-pyridinecarboxaldehyde; TMOMA = N,N,N-trimethyl-1- (oxiran-2-yl)methanaminium

As an example, it was shown that alkylation/derivatization (Fig. 14.5) is enhancing the antibacterial activity; however, maintaining an acceptable toxicity against RBCs is a big challenge for this type of compounds (Oyervides-Muñoz et al. 2017). Chitosan has three functional groups, one amine group at C-2 and two hydroxyl groups at C-3 and C-6, and all of them can be subjected to derivatization. More specifically, N-selective derivatization can be performed by chitosan methylation via both conventional and improved (two-step) reactions to achieve permanent, positively charged trimethyl chitosan (TMC). However, conventional method or direct chitosan methylation with CH_3I/NaI leads to random *N*- and *O*-methylation (**1**) (Fig. 14.5) which, depending on the goal of the synthesis (e.g., further synthesis steps or further peptide grafting), could negatively influence the fate of the product (e.g., decreased solubility, chain scission). Reductive alkylation or two-step chitosan derivatization is preferred as it is *N*-regioselective and allows a control over the degree of substitution (DS). This reaction is performed in aqueous (**2**) (e.g., formic acid and/or formaldehyde) (Patrulea et al. 2015a) or organic (e.g., *tert*-butyldimethylsilyl) (TBDMS) (**4**) (Sahariah et al. 2015a) media through formation of a Schiff base intermediate and leads to mono- or di-alkyl derivatives avoiding non-desired *O*-methylation. Further quaternization of *N*-alkyl and *N*,*N*-dialkyl units results in highly substituted *N*,*N*,*N*-trimethyl chitosan (TMC) (**2**) (Patrulea et al. 2016). Carboxyalkylation of TMC is another step that could enhance antibacterial activity by carboxymethylating TMC with mono-chloroacetate, which leads to *O*-carboxymethylation (CMTMC) (**3**) of hydroxyl functional groups available in TMC. It is expected that the antibacterial activity increases from *N*,*O*-carboxymethyl

chitosan (N,O-CMC) > chitosan > O-carboxymethyl chitosan (O-CMC); however conflicting results were obtained in an in vitro study of Xu et al (2010).

Generally, it is hypothesized that chitosan derivatives have antibacterial activity due to their interaction between the polycation moiety (positively charged) and bacterial cell membrane by formation of a complex, or by disturbing gene expression inside the cell. Still, it is not clear yet whether *N*-quaternized or methylated amino groups are responsible for the antibacterial activity, e.g., for *S. aureus* (Martins et al. 2014; Rúnarsson et al. 2007; Xu et al. 2010). Sahariah and Masson have shown that the antibacterial effect decreases with the increased length of the spacer and increases with the increase of the DS at amino site of chitosan derivatives (Sahariah and Másson 2017).

Some reports clearly show that chitosan derivatives have stronger antibacterial activity than native chitosan. For instance, Jia et al. show that *N*-propyl-*N*,*N*-dimethyl chitosan has a 20-fold increased bacterial activity against *E. coli* ATCC 25925 compared to chitosan (4% DA; MW of 214, 19, and 7.8 kDa) (Jia et al. 2001). Similarly, *N*,*N*,*N*-trimethyl chitosan (TMC) (**2**) was reported for its higher antibacterial activity against *E. coli* and *S. aureus* compared to chitosan. TMC exhibited strong antibacterial activity at pH 7.2, while at pH 5.5 the antibacterial activity of TMC decreased with the increase of degree of quaternization (DQ). Higher MICs of 0.0125; 0.025 and 0.05 μg/mL were reported against *E. coli* compared to MICs of 0.00625; 0.0125 and 0.025 μg/mL against *S. aureus* corresponding to DQ of 41.2; 61.2 and 94.7%, respectively. However, CMTMC (**3**) had no antibacterial effect regardless of the degree of carboxymethylation (Xu et al. 2010).

MIC of quaternary chitosan derivatives against both Gram-positive and -negative bacteria (*S. aureus, E. faecalis*, and *E. coli, P. aeruginosa*, respectively) was shown to depend on the length of the alkyl chain and variation of the DS, while HC was very low. The activity of raw chitosan under neutral conditions was in the 1024–2048 μg/mL range against *S. aureus* and *E. coli*, while the quaternary derivatives had lower MIC, thus higher antibacterial activity. Moreover, quaternary derivatives (**4**) proved to possess bactericide properties and to be efficient even against *P. aeruginosa*, a multidrug-resistant bacteria (Sahariah et al. 2015a). Sajomsang et al. reported that antibacterial activity of quaternized chitosan, TMC (**4**), *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan (DMCMC) (**5**), and methylated *N*-(4-pyridylmethyl) chitosan chloride (MPyMeC) (**6**) depends on both MW and DS of the derivatives. In case of TMC (**4**), antibacterial activity against *S. aureus* (MIC decreases from 500 μg/mL to 125 μg/mL) and against *E. coli* (MIC decreases from 1000 μg/mL to 250 μg/mL) increases when increasing the DQ from 28 to 64 and decreasing the MW from 205 to 121 kDa. For DMCMC (**5**) and MPyMeC (**6**), the antibacterial activity was changed, regardless of the increase in DQ.

Noteworthy, a key parameter when designing an antibacterial chitosan derivative is the optimal positioning of the positive charge in relation to the polymer backbone and/or the resonance effect of the moiety (Sajomsang et al. 2009). For instance, positioning positive charges on the $–NH_2$ site, such as for chitosan N-betainate derivatives (**7**), showed higher antimicrobial activity against *E. coli* than against *S. aureus* at both pH 5.5 and 7.2 for all DS evaluated (0.05–0.9). Better activity was

found at lowest DS (0.05) at pH 5.5, with MICs above 128 and 512 μg/mL for *E. coli* and *S. aureus*, respectively. For *S. aureus,* bactericidal effect was observed at low DS (range: 0.05–0.6) and at pH 5.5, while for *E. coli,* the effect was observed at highest DS (Holappa et al. 2006). Introduction of more than one quaternary group into the chitosan structure showed high efficacy of 99% and 96% against *S. aureus* and *E. coli*, respectively. The antimicrobial activity of chitosan derivatives was higher in case of *O*-quaternized-*N*,*N*-diethyl-*N*-benzyl ammonium chitosan (O-QCTS-DEBn) (**9**) compared to *O*-quaternized-*N*-benzylidene-chitosan (O-QCTSS) (**8**), due to the two positively charged sites of the former (**9**). The activity of (O-QCTSS) reached 100% efficacy against *S. aureus* and close to 100% against *E. coli* when increasing the concentration to 3% (Fu et al. 2011). However, the toxicity against RBCs has not been reported, which precludes the evaluation of the whole profile of these promising candidates.

Aminoethyl chitosan showed MW-dependent antimicrobial activity against *E. coli* (MIC = 64 ug/mL) when dropping the MW to 1.4 kDa (DA = 20%), while derivatives with MW > 27 kDa showed MIC = 16 μg/mL at neutral pH. Moreover, the mechanism of action of aminoethyl chitosan derivatives was found to involve membrane disruption (Meng et al. 2012).

Therefore, chitosan quaternary derivatives are promising candidates against infection; however, more attention should be paid to their potential toxicity.

14.5.2 Chemical Coupling of Chitosan with AMPs

Chitosan has been chemically coupled with different amino acids and peptides. EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysulfosuccinimide) reaction is used as a routine chemistry by activating carboxyl groups from amino acids or peptides, subsequently coupled with chitosan (Sahariah and Másson 2017). On the other hand, AMPs can be favorable drug candidates to be coupled with chitosan or its derivatives for an enhanced hemocompatibility and/or antibacterial activity. As drug candidates, AMPs have the advantages over proteins or antibodies of higher stability, lower production costs, lower immunogenicity, higher efficiency and selectivity, higher biological activity, and smaller size, which allows them to penetrate cell membranes and reach to targeted tissues (Vlieghe et al. 2010). However, their coupling may lead to a dramatic decreased activity (Costa et al. 2014). Therefore, different strategies have to be addressed to preserve the activity of these AMPs coupled with chitosan, such as the addition of a spacer, or the choice of adequate coupling chemistry. The main methods for chitosan-AMP coupling, their chemistry, antibacterial activity (compared to the parent peptide at equivalent concentration), and reported toxicity or hemolysis are shortly depicted in Fig. 14.6.

14.5.2.1 Chitosan-Anoplin

Despite many efforts to enhance the antimicrobial efficiency of some AMPs, like anoplin originating from the venom of wasps, the high hemolytic activity (HC_{50} = 512 μg/mL) has prevented the use of such peptides. However, for anoplin,

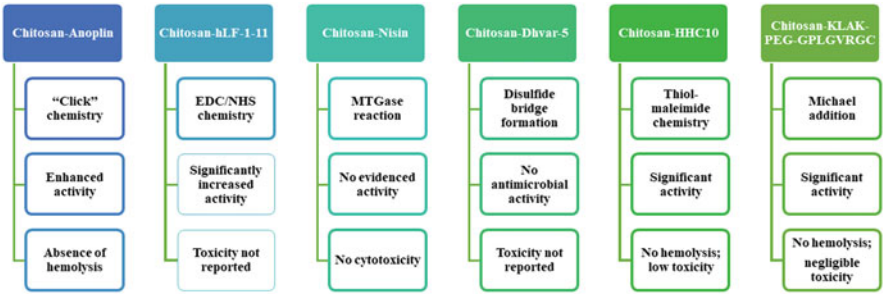

Fig. 14.6 Chitosan-AMP coupling chemical strategies and performances

covalent grafting to chitosan via copper-catalyzed alkyne-azide click chemistry showed not only to enhance the activity but as well abolished hemolysis. The activity of chitosan-anoplin conjugates against *S. aureus* and *E. coli* increased proportionally with the DS, the conjugate being particularly efficient against *E. coli* (MIC = 4 μg/mL of conjugate, equivalent to 1.9 μg/mL of peptide at DS of 0.15). By contrast, low activity was reported against *E. faecalis* even at higher DS (0.23) with a reported MIC of 1024 μg/mL of the conjugate, equivalent to 591 μg/mL of the peptide. No hemolysis potential was found for the conjugate up to a concentration of 2048 μg/mL, compared to HC_{50} of 512 μg/mL of anoplin alone (Sahariah et al. 2015b).

14.5.2.2 Chitosan-hLF-1-11

Covalent immobilization of thiol groups from hLF1-11 peptide with a thiol-derivatized chitosan film leads to significant increase in *S. aureus* adhesion (Costa et al. 2014). Moreover, they proved that covalent immobilization maintained antibacterial activity, especially when a spacer was added between chitosan and AMP. Chitosan films were prepared on gold surfaces and later functionalized with thiol groups. Chitosan-thiol functionalization was performed under EDC/NHS reaction with addition of N-acetyl cysteine. Subsequently, hLF1-11 was added to the thiolated chitosan either directly or by introducing a spacer for a better presentation of the AMP.

14.5.2.3 Chitosan-Nisin

A hydroxypropyl-chitosan derivative was coupled with nisin through a microbial transglutaminase (MTGase) as a reaction catalyst (Zhu et al. 2017). This conjugate was shown to be efficient against both *S. aureus* and *E. coli* at pH 6, while at pH 7 the same compound was efficient only against *S. aureus*. Unfortunately, provided qualitative data (yes/no) does not allow to prove whether this new compound has antibacterial activity or not. Parameters like MIC and/or MBC or even viable count estimation of bacterial number before and after microorganism contact with chitosan derivatives are very important to assess the biological response of a material.

Moreover, this study lacks information about RBC hemolysis and adequate controls, such as an antibiotic or another AMP.

14.5.2.4 Chitosan-Dhvar-5

Dhvar-5 peptide (histatin 5 active domain) was coupled with different peptide C- and N-termini to chitosan films. Chitosan was first functionalized with thiol groups through EDC/NHS chemistry; then Dhvar-5 was immobilized on chitosan through disulfide bridge formation between thiol groups from terminal cysteine of the AMP and sulfhydryl groups of the thiolated chitosan. Results showed that C-terminally immobilized Dhvar-5 lead to unwanted bacterial adherence after 24 h, while coupling at its N-terminus the peptide showed lower bacterial adhesion. However, viability of bacteria did not depend on the terminus coupling; therefore, the final product could be considered as an anti-adherent rather than an antimicrobial against *S. aureus*. Furthermore, the addition of a spacer leads to better results when longer spacers were used (Costa et al. 2015).

14.5.2.5 Chitosan-HHC10

Cysteine-terminated HHC10 (KRWWKWIRW) AMP was coupled with chitosan via thiol-maleimide "click" chemistry through modification of C-2 (N-functionalization) or C-6 (O-functionalization) of chitosan with maleimide-functionalized linkers. In vitro results showed that N-functionalized chitosan (DS = 0.18) was less active and less toxic against mouse fibroblasts than O-functionalized chitosan (DS = 0.23). MIC of O-functionalized chitosan was lower (higher activity) against *S. aureus* and *S. epidermis* (MIC = 15 and 4 μg/mL of the conjugate, respectively) in comparison with raw AMP (MIC = 32 and 8 μg/mL toward *S. aureus* and *S. epidermis*).

Moreover, HHC10-chitosan conjugates exhibited higher selectivity toward bacteria, lower hemolysis (HC_{50} > 4096 μg/mL for N-functionalized and >8192 μg/mL for O-functionalized chitosan), and lower toxicity (>60% viability after 24 h of exposure) than the native AMP (HC_{50} = 1024 μg/mL) (Pranantyo et al. 2018).

14.5.2.6 Chitosan-KLAK-PEG-GPLGVRGC

Qi et al. reported the "on-site transformation" based on chitosan backbone and two peptides, CGGG $(KLAKLAK)_2$, so-called KLAK peptide as AMP and poly(ethylene glycol) (PEG)-tethered enzyme-cleavable peptide (GPLGVRGC) or PEG-non-cleavable peptide (GPMGMRGC). Synthesized compound could self-assemble in water into pegylated NPs (34 nm). When exposing NPs to the site of the infection, both peptides were cleaved by gelatinase and gradually converted into fibrous structures, which helped AMP exposure to the bacteria. The activity of these "smart" assemblies was highly efficient against *S. aureus*. Interestingly, it was found in vivo that self-assemblies improve the accumulation and retention of the NPs at the infection site when using enzyme-cleavable peptides than usual AMP (Qi et al. 2017).

14.5.3 Chitosan as Carrier for AMPs

An important strategy when delivering AMPs of interest is the use of a carrier such as NPs, lipid self-assemblies, or hydrogels to potentially improve stability, toxicity, and half-life. Incorporation of AMPs into an NP system can stabilize the AMP both in vitro and in vivo. Moreover, the quantity of AMP can be increased to reach the site of action, and the prolongation of the residence time in situ could enhance the pharmacological effect and help to overcome physiological barriers thanks to their small size (D'Angelo et al. 2015).

14.5.3.1 Chitosan-AMP-Based NPs

Chitosan-protamine NPs were prepared by ionic gelation method, where chitosan was mixed with protamine, followed by cross-linking with sodium tripolyphosphate (TPP) – a method also referred to as coacervation or self-assembly. Ionic cross-linking of protamine to chitosan leads to an enhanced antimicrobial activity with an MIC of 31.25 μg/mL against *E. coli*, but lower antimicrobial activity with MIC values higher than 250 μg/mL against *B. cereus*. These data proved that hybrid chitosan-AMP NPs can reach a higher antimicrobial activity than chitosan NPs alone (Tamara et al. 2018).

Nisin was loaded into chitosan and in a poly-γ-glutamic acid (γ-PGA)/chitosan NP system by self-assembly/coacervation method. The chitosan coating enhanced the stability of NPs, the entrapment efficiency with a slow release of nisin at pH 6 and 8 and with a higher antibacterial activity against *E. coli* and *L. monocytogenes* (higher inhibition window, better controlled release, and longer antimicrobial effect (up to 15 days)). Unfortunately, the report lacks consistency and it is not clear whether the antibacterial activity was against *S. aureus* or *L. monocytogenes* (Wu et al. 2016).

Chitosan-colistin NPs (300 nm) were prepared by co-spray-drying of PLGA-colistin NPs using emulsion-solvent diffusion method as dry powder, as an inhalation treatment for lung infection. The efficiency of AMP entrapment was 63% and more than 70% of initial chitosan-colistin NPs could easily penetrate an artificial mucus layer during the first 6 h. Encapsulation into chitosan facilitated the transport of colistin since colistin alone could not penetrate the mucus. In vitro assays showed that colistin entrapped into NPs could successfully eliminate *P. aeruginosa* biofilm within 72 h at 7.5 and 15 μg/mL (D'Angelo et al. 2015).

Piras et al. could obtain AMP-chitosan-based NPs by ionic gelation method. Peptide LLPIVGNLLKSLL-amide (called TB) was added to chitosan, and upon addition of TPP, NPs were formed. Chitosan proved to be an efficient carrier by improving the bactericidal properties of TB against *S. epidermis* strains and by significantly reducing the toxicity against mammalian cells (Piras et al. 2015). Another RBRBR peptide was encapsulated into chitosan (51.33% encapsulation efficiency) by a similar methodology. Incorporated AMP into NPs (121 nm) enhanced the activity of the peptide against *S. aureus* and significantly reduced hemolysis and cytotoxicity (Almaaytah et al. 2017).

14.5.3.2 Chitosan-AMP-Based Membrane

Potential chitosan-gelatin membrane Pac-525-loaded PLGA microspheres were prepared by combining layer-by-layer electrospinning and electrospraying methods. AMP-loaded membranes were efficient against *S. aureus* and *E. coli* over 1 week exposure and absence of toxicity against mesenchymal stem cells (He et al. 2018).

Overall, these few studies involving chitosan nanocarriers suggest that chitosan may act as an antimicrobial activity enhancer (Tamara et al. 2018) or limit toxicity (Piras et al. 2015). In some instances, chitosan NPs act as mucosal permeation enhancers (D'Angelo et al. 2015). These self-assemblies are promising candidates for further studies against bacterial development.

14.6 Conclusions and Perspectives

Recent progresses in the field of biomaterials have led to a renewed interest in designing new drugs for treating different infectious diseases, not only topical infections. On one hand, chitosan has the potency to be used as antimicrobial agent in many pharmaceutical and biomedical formulations. On the other hand, AMPs are promising candidates and can attack bacteria through different mechanisms of action. The synergy of different drugs, such as AMPs and biopolymers, can be seen as a promising strategy in overcoming current multidrug-resistance issues. In this context, the feasibility of using chitosan as a matrix or as a carrier for AMPs to improve their activity through their synergistic effect would fit the need of agents against ESKAPE and other bacteria. Interestingly, conjugation of AMPs onto chitosan and/or its derivatives may not only enhance antimicrobial activity and half-life of the AMPs but may also reduce the toxicity and hemolytic activity, considered as critical points when developing antimicrobial biomaterials.

Concerns about microbial infection are rising even outside our planet. Scientists recently confirmed the existence of five different strains of multidrug-resistant *Enterobacter* on the International Space Station (Singh et al. 2018). Whether or not chitosan and its derivatives, potentially conjugated to AMPs, would be active against these strains under extraterrestrial conditions remains to be shown.

In this chapter, we have highlighted recent advances in the synthesis and applications of chitosan-AMP conjugates, which are synthesized by binding AMPs to chitosan and/or to its derivatives either by covalent coupling or by using chitosan as a carrier for AMPs. Covalent coupling or ionic cross-linking was shown to preserve the functions of both components and may create new properties otherwise absent. The foreseen antimicrobial pharmaceutical products could include NPs, microparticles, as well as macroscopic hydrogels and films.

Even though there still seems to be a long way to reach clinical applications of AMP-chitosan conjugates materials, they are an important strategy in addressing bacterial drug resistance. Still, several important challenges should be addressed

before entering into clinical trials, especially biocompatibility, immunomodulatory responses, in vivo studies including stability, circulation, accumulation, and ADMET (absorption, distribution, metabolism, excretion, and toxicity) issues of the new molecules. The antimicrobial mechanism of AMP-chitosan conjugates should also be investigated very clearly, which is of high importance for their translation into clinical applications. MIC and MBC of the polymers and AMPs are the most important parameters that should be considered for further studies.

Overall, due to the emergence of antibiotic resistance and the fact that many AMP-based drugs are in clinical trials, the next decade will reveal the benefits of these novel conjugates and lead to their commercialization. We believe that the current research topic could pave the ground to develop new approaches in tackling current and forthcoming antimicrobial resistance health threats.

Acknowledgments We would like to acknowledge Aleksandar Jovanovic for his help in drawing Fig. 14.1.

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Antibacterial Activity of Chitosan-Based Systems

15

Hüsnügül Yilmaz Atay

Abstract

Chitosan and its derivatives can be called environmental purification functional materials as they can effectively control the growth and reproduction of hazardous bacteria and also control toxic pollutants. From the basic science to the latest developments and innovations, starting with the history of the material, this chapter presents a facile way to understand the antibacterial activity of the chitosan, together with other materials, to the reader. This chapter also summarizes the general developments in the study of antimicrobial applications. In the light of the current situation of the research and the progress in the related fields, this chapter discusses the differences among influencing factors in detail and compares the antimicrobial activity between different physical states of chitosan. Also, this chaper discusses the more recent processes and applications.

Keywords

Chitosan · Antibacterial materials · Antibacterial mechanism · Affecting factors

15.1 Introduction

Antibacterial and antimicrobial agents and its disinfected systems are becoming important day by day. They have been studied for possible use in a variety of healthcare environments, industries, laboratories, and even houses (Ali et al. 2015) (Hosseinnejad and Jafari 2016). Most importantly, the use of these materials is to sterilize medical environments and equipments in order to prevent thousands of deaths due to hospital-acquired infections, such as linens and clothing where bacteria

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S. Jana, S. Jana (eds.), *Functional Chitosan*,
https://doi.org/10.1007/978-981-15-0263-7_15

could grow and infect the human body. Therefore, a suitable environment should be provided in order not to let infectious diseases spread from fungi or viruses (Yılmaz Atay and Çelik 2017).

According to the Centers for Disease Control and Prevention (CDC), each year in the United States, 48 million people get sick, 128,000 are hospitalized, and 3000 die due to foodborne diseases. Therefore, ensuring microbiological safety of the products, while maintaining their nutritional and organoleptic properties, is still a priority nowadays (Severino et al. 2014). Another option is the use of antimicrobial packaging to provide an increased margin of safety and quality. This packaging may prevent the growth of microorganisms on the product's surface and, hence, lead to an extension of its shelf life (Cha and Chinnan 2004). Therefore, it can be said that antibacterial and antimicrobial systems are highly important not only in the hospital and healthcare environments but also for laboratory, home, marine and some industrial applications (Hosseinnejad and Jafari 2016; Lin et al. 2015).

15.2 Antibacterial Materials

Antibacterial materials are being developed to prevent harmful bacteria and viruses from spreading (Chang et al. 2015). These materials can effectively control the growth and reproduction of hazardous bacteria and also control toxic pollutants (Claesson and Ninham 1992). They contain an antimicrobial agent that inhibits the ability of microorganisms to grow in the material. Such materials are becoming more widely investigated for possible use in various settings including clinics, industry and even the home. The most common and important use of antimicrobial coatings has been in the healthcare setting for sterilization of medical devices to prevent hospital-associated infections, which have accounted for almost 100,000 deaths in the United States. In addition to medical devices, linens and clothing can provide a suitable environment for many bacteria, fungi and viruses to grow when in contact with the human body which allows for the transmission of infectious disease (Chapter 26: Microbial Growth Control 2018).

Antibacterial materials could be classified into two groups: inorganic and organic materials. Inorganics are metals, metal oxides and metal phosphates (Tuncer 2007). Among the inorganic materials, metal oxides such as TiO2, ZnO, MgO and CaO are of particular interest as they are not only stable under harsh process conditions but also generally regarded as safe materials to human beings and animals (Teli and Kale 2011). Organics are phenols, halogenated compounds and quaternary ammonium salts; in recent years, studies about antibacterial materials have been focused on natural materials, such as chitosan (CTS) and chitin (Hosseinnejad and Jafari 2016).

Ren et al. (Chen and Chou 2005) characterized and investigated copper oxide (CuO) nanoparticles with respect to potential antimicrobial applications. They generated nanoscaled CuO by thermal plasma technology. CuO nanoparticles were effective in killing a range of bacterial pathogens involved in hospital-acquired infections. In addition, Cioffi et al. (Chien et al. 2015) manufactured polymer composites including copper nanoparticles with antifungal and bacteriostatic

properties. Polymer/metal nanocomposites are a viable choice, as a spinnable coating capable of releasing metal species to a broth of living organisms in a controlled manner is an extremely interesting material for a number of biotechnological applications. At their study, a polymer-based nanocomposite loading stabilized copper nanoparticles is proposed as a biostatic coating, and systematic correlations between material properties and biological effects are established. Researchers in Japan (Chung et al. 2004) have discovered a nickel-alloy coating with antibacterial properties, which is believed to be effective at reducing the SARS coronavirus. SARS is believed to be spread through close contact, such as coughing, sneezing or contact with faeces of patients. It can also be transmitted when people touch surfaces contaminated with the virus. The use of this antibacterial material has the potential to radically alter the quality and cleanliness of laboratory and pharmaceutical workplaces. In laboratory tests, the nickel-alloy coating developed by Kobe Steel reduced the growth of mouse hepatitis virus (MHV, or mouse coronavirus), which is a close relative of the SARS (severe acute respiratory syndrome) coronavirus (CoV). Both MHV and SARS CoV are in the same group of coronaviruses (Chung et al. 2004; Yilmaz Atay 2013).

In our previous study, silver-supported materials and titanium dioxide photocatalyst materials were investigated as inorganic environmental purification functional materials by applying an in vitro test. The bactericidal activity for these bacterial cells was estimated by zone of inhibition on the nutrient agar plates. Zone of inhibition is produced by the silver-loaded polymer coating against the bacteria representing its antibacterial effect. All Ag nanoparticle-reinforced polymer composites showed a good inhibition zone for *S. aureus* and *E. coli*. This antibacterial activity of silver is thought that it results from the interaction of silver and thiol groups in bacteria proteins (Yılmaz Atay et al. 2015).

Another material we work on this area is an active biomolecule: chitosan [poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose]. Recently, the research on this material has been increased dramatically because of its great potential for a wide range of applications. Due to its biodegradability, biocompatibility, antimicrobial activity, non-toxicity and versatile chemical and physical properties, chitosan has a significant role in food application area in view of recent outbreaks of contaminations associated with food products as well as growing concerns regarding the negative environmental impact of packaging materials currently in use. Chitosan-based polymeric materials can be formed into fibres, films, gels, sponges, beads or even nanoparticles (Chung et al. 2003; Yilmaz Atay 2013). In the following sections, the antibacterial properties and applications of chitosan will be analysed thoroughly.

15.2.1 Antibacterial Activity of Chitosan-Based System

One of the most investigated properties of chitosan is its antimicrobial effect embracing from biomedical to cosmetic and from food to agriculture applications. To make use of the antimicrobial activity of chitosan together with its peculiar features in order to produce self-preserving materials, many studies have been

conducted up until now. This has led to the design of a large range of products containing chitosan as beads, films, fibres, membranes and hydrogels that are intended for various uses (Perinelli et al. 2018).

15.2.2 History

Chitosan has been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving in vivo and in vitro interactions with chitosan in different forms (solutions, films and composites) (Kong et al. 2010). Ever since the broad-spectrum antibacterial activity of this material was first proposed by Allan and Hardwiger (Allan and Hardwiger 1979), along with great commercial potential, the antimicrobial property of chitosan and its derivatives have been attracting great attention from researchers. Investigation of the antimicrobial properties of chitosan has been a long journey of scientific exploration and technological development. The journey began two decades ago, with studies on the biological phenomena arising from foodborne and soilborne pathogenic fungi in the food and agriculture industries (Rabea et al. 2003). In light of their intimate relationship with human activities, bacteria rightly began to receive more attention in the search for efficacious antimicrobials. The studies at that time were typically carried out via chemical, biochemical, microbiological and medical assays of chitosan and its derivatives. In some cases, but rarely so, molecular and cell approaches were utilized. The outcomes obtained through this period suggested that antimicrobial activities of chitosan and its derivatives relied on numerous intrinsic and extrinsic factors, such as pH and molecular weight (Mw). Some basic hypotheses about underlying antimicrobial mechanisms were also proposed (Zivanovic et al. 2004). Based on the outcomes, various antimicrobial agents based on chitosan or its derivatives emerged. At the same time, since biocide-resistant bacteria and fungi, growing public health awareness of pathogenic microorganism raised demands for safe and efficacious agents that were less prone to stimulating development of resistance. In addition to tremendous advancements in molecular biological, pharmaceutical, cell biological technologies and detecting methods, nanotechnology emerged and began playing an extraordinary role, carrying the potential to extend antimicrobial treatment to the atomic level. The many approaches that have been used in studying antimicrobial activities of chitosan and its derivatives have given rise to various physical forms of chitosan in differing methods, from the original solution applied in agriculture to film structure in food sector and to ubiquitous pharmaceutical nanostructure materials (Kong et al. 2010).

15.2.3 Sources of Chitosan

Chitosan is a natural antimicrobial agent found in the shells of crustaceans, such as crab, shrimp, squid pen and crawfish (No et al. 2002). Recently, some studies have

pointed to the possibility of chitosan production from fungi. In one study, chitosan was extracted from cell wall of filamentous fungus, *R. oryzae*, by Jeihanipour et al. (Jeihanipour et al. 2007), and its antimicrobial properties were studied against *E. coli*, *K. pneumoniae* and *S. aureus* (Hosseinnejad and Jafari 2016).

15.2.4 Water Soluble

Although chitin and chitosan have been confirmed as attractive biomacromolecules with relevant antimicrobial properties, applications are somewhat limited due to both being water insoluble. Water-soluble chitosan derivatives can be obtained by the introduction of permanent positive charges in the polymer chains, resulting in a cationic polyelectrolyte characteristic independently of the pH of the aqueous medium. This can be accomplished, for instance, by the quaternization of the nitrogen atoms of the amino groups (Goy et al. 2009).

15.2.5 Derivatives of Chitosan

Due to its unique polycationic nature, chitosan and its derivatives have been recommended for applications in agriculture, food, biomedical, biotechnology and pharmaceutical fields. However, the antibacterial functions of chitosan are limited because amino groups on chitosan backbone can only function as relatively weak positive charge centres. To improve the antimicrobial activity of chitosan, it is reasonable to enhance the strength of positive charges on the chitosan molecules by endowing it with some more positively charged groups (Xiao et al. 2011). Therefore, in the past two decades, extensive investigations have been carried out to increase solubility of chitosan in water and broaden its applications by preparing functional derivatives of chitosan such as carboxymethyl chitosan and quaternized carboxymethyl chitosan, chitosan-N-arginine by reacting amino groups of chitosan with arginine, N-alkylated disaccharide chitosan, water-soluble maltose chitosan derivative and water-soluble quaternary. Chitosan derivatives are obtained by N-acylation with betaine and water-soluble oligochitosans (Hosseinnejad and Jafari 2016).

15.2.6 Degree of Deacetylation

Chitosan is produced commercially by deacetylation of chitin. In the process of deacetylation, acetyl groups from the molecular chain of chitin are removed to form amino groups. The degree of +, Mg-2 + 2 deacetylation, which determines the content of free amino groups in polysaccharides, can be employed to differentiate between chitin and chitosan. It is very well known that the degree of deacetylation is one of the most important chemical characteristics, which could influence the performance of chitosan in many applications (Hosseinnejad and Jafari 2016).

15.3 Mechanism of Antibacterial Activity

The exact mechanism of antibacterial activity is yet to be fully understood. It is known that chitosan's antimicrobial activity is influenced by a number of factors that act in an orderly and independent fashion. The most prevalent proposed antibacterial activity of chitosan is by binding to the negatively charged bacterial cell wall causing disruption of the cell, thus altering the membrane permeability, followed by attachment to DNA causing inhibition of DNA replication and subsequently cell death (Nagy et al. 2011). Another possible mechanism is that chitosan acts as a chelating agent that electively binds to trace metal elements causing toxin production and inhibiting microbial growth (Divya et al. 2017).

The polycationic structure of chitosan is a prerequisite for antibacterial activity. As environmental pH is below the pKa of chitosan and its derivatives, electrostatic interaction between the polycationic structure and the predominantly anionic components of the microorganisms' surface (such as Gram-negative lipopolysaccharide and cell surface proteins) plays a primary role in antibacterial activity (Kong et al. 2010).

The polycationic structure forms unnecessarily in acidic conditions, because the grafted groups of specific derivatives may change the pKa of chitosan and cause protonation at higher pH value (Yang et al. 2005). When the positive charge density of chitosan strengthens, the antibacterial property will increase consequently, as is the case with quaternized chitosan and chitosan metal complex (Xie et al. 2007). On the contrary, if the polycationic property of chitosan is deprived or reversed, the corresponding antibacterial capacity will be weakened or lost. Besides protonation, the number of amino groups linking to C-2 on chitosan backbones is important in electrostatic interaction. Large amount of amino groups are able to enhance the antibacterial activity. Accordingly, native chitosan with higher DD shows a stronger inhibitory effect than a molecule with a lower DD. Moreover, it has been reported that asparagine N-conjugated chitosan oligosaccharide that possesses two positively charged sites provides strong interaction with carboxyl-negative charges on the bacterial cell wall (Jeon et al. 2001). Another attempt to increase the amount of amino groups via substituting amino by formamidine obtained a guanidinylated chitosan, which showed better antibacterial activity than chitosan (Hu et al. 2007a, b).

HMw water-soluble chitosan and solid chitosan including larger size nanoparticles interact with cell surface instead and alter cell permeability resultingly (Leuba and Stossel 1985) or form an impermeable layer around the cell, thus blocking the transport of essential solutes into the cell. Experiments conducted with *E. coli* treated with CM and oleoyl-chitosan nanoparticles (OCNP) have revealed that the same microbial species can display significant differences in mode of action depending on the two different dimensions of chitosan particles (Xing et al. 2009).

As shown in Fig. 15.1, the cells located on the surface of chitosan microsphere showed various states: some were intact, some were leaking intracellular substances, and some had already ruptured leaving only the membrane. These results are consistent with the idea that CMs kill bacteria through an interfacial contacting

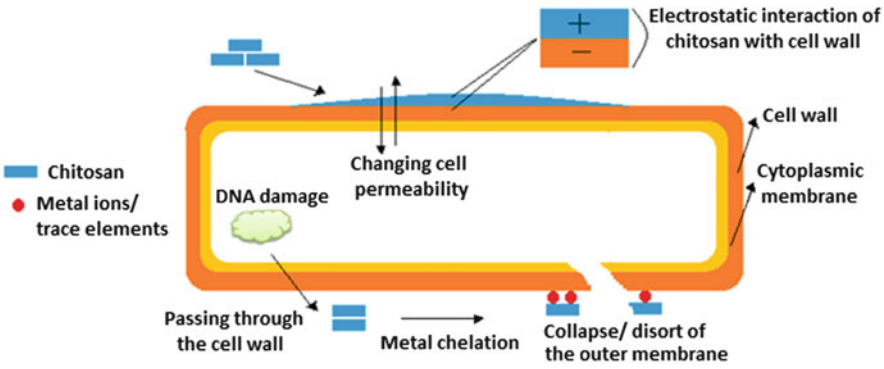


Fig. 15.1 Schematic representation of antimicrobial mechanisms of chitosan and its derivatives (Hosseinnejad and Jafari 2016)

inhibitory effect that occurs on the surface of the microspheres (Kong et al. 2008). Chitosan undergoes a surface-to-surface and local reaction mode, rather than a thorough contacting mode that occurs in liquid state (Hosseinnejad and Jafari 2016).

Similar to bacteria, the chitosan activity against fungus is assumed to be fungistatic rather than fungicidal with a potential to communicate regulatory changes in both the host and fungus. Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth. Most of the studies have been done on yeasts and moulds associated with food and plant spoilage. For these, in the presence of chitosan, several biological processes are activated in plant tissue, where chitinases are induced with action on biotrophic and necrotrophic mycoparasites, entomopathogenic fungi and vesicular arbuscular mycorrhizal fungi (Ghaouth et al. 1992; Goy et al. 2009).

15.4 Factors Affecting Antibacterial Property

Variations in chitosan's bactericidal efficacy arise from various factors. Several properties are reviewed in Fig. 15.2. They are explained in detail as follows:

15.4.1 Concentration of Chitosan

At lower concentrations, chitosan binds to the negatively charged cell surface, disturbs the cell membrane and causes death of the cell by inducing leakage of intracellular components, whereas, at higher concentrations, the protonated chitosan may coat the cell surface and prevent the leakage of intracellular components. In addition, the positively charged bacterial cells repel each other and prevent agglutination (Lim and Hudson 2004). An antimicrobial packaging material was prepared

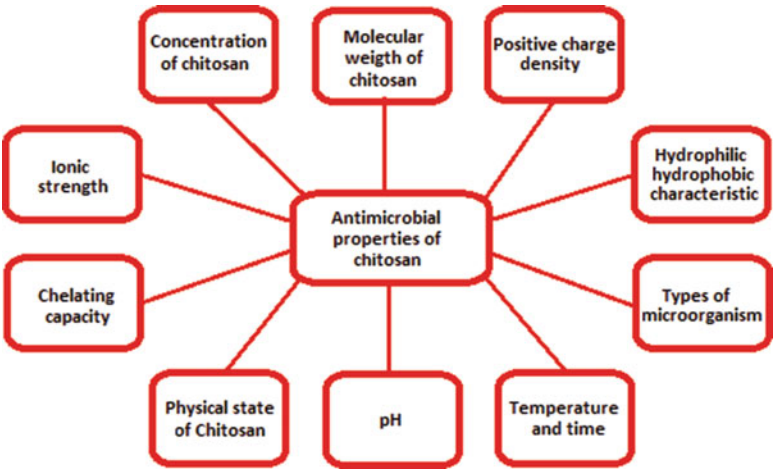


Fig. 15.2 Factors affecting antibacterial property of chitosan

by uniformly embedding 1, 3 and 5% chitosan (w/w) in low-density polyethylene (LDPE) matrix (Reesha et al. 2015). The antimicrobial assay against *E. coli* proved that LDPE/chitosan composite (LDPE/CS) films were highly efficient than virgin LDPE films. Virgin LDPE and 1%, 3% and 5% LDPE/CS films tested as packaging films for chill-stored tilapia showed that samples packed in LDPE films were rejected by seventh day, whereas fish packed in 1%, 3% and 5% LDPE/CS films remained acceptable up to 15 days. This study revealed that 3% LDPE/CS films had a better physical and antimicrobial property and enhanced the keeping quality of tilapia steaks during chilled storage when compared to the other films (Hosseinnejad and Jafari 2016).

15.4.2 Molecular Weight

Numerous studies on bactericidal activity of chitosan have generated equivocal results concerning correlation between bactericidal activity and chitosan Mw. Some studies reported increasing chitosan Mw leads to decreasing chitosan activity against *E. coli*, while in other studies high Mw (HMw) chitosan displayed greater activity than low Mw (LMw) chitosan. In addition, activities still were found to be equal against *E. coli* and *Bacillus subtilis* regardless of Mw (Tikhonov et al. 2006). Even though the limited available results on bactericidal activity of LMw chitosan were comparable depending on bacterial strains, conditions of biological testing and respective chitosan Mw, the results are not accordant with each other. For instance, 9.3 kDa chitosan inhibits growth of *E. coli*, while 2.2 kDa chitosan promotes its growth. Also, LMw chitosan (4.6 kDa) and its derivative showed better activity against bacteria, yeast and fungi (Kong et al. 2010).

15.4.3 Positive Charge Density

Tremendous literatures support the importance of polycationic structure in antimicrobial activity. A higher positive charge density leads to strong electrostatic interaction. Therein, the positive charge is associated with DD or degree of substitution (DS) of chitosan or its derivatives, which affect positive charge density. To some extent, chitosan microspheres with a high DD (97.5%) lead to higher positive charge density, which confers stronger antibacterial activity than moderate DD (83.7%) against *Staphylococcus aureus* at pH 5.5 (Kong et al. 2008). One study reported that a higher DD with more positive charge was especially successful in inhibiting the growth of *S. aureus*, suggesting that antibacterial activity of chitosan towards *S. aureus* was enhanced with increasing DD (Takahashia et al. 2008). Concerning chitosan derivatives, antimicrobial activity mostly depends on DS of the grafting groups. Investigation of the antibacterial activities of water-soluble N-alkylated disaccharide chitosan derivatives against *Escherichia coli* and *S. aureus* revealed that the antibacterial activity of chitosan derivatives is affected by the DS of disaccharides and the type of disaccharide present in the molecule (Yang et al. 2005). The same study suggested that, irrespective of the kind of disaccharide linked to the chitosan molecule, a DS of 30–40%, in general, produced the most pronounced antibacterial activity against *E. coli* and *S. aureus* and that both microorganisms were most susceptible to cellobiose chitosan derivative DS 30–40% and maltose chitosan derivative DS 30–40%, respectively, among the various chitosan derivatives examined (Kong et al. 2010).

15.4.4 Hydrophilic/Hydrophobic Characteristic

Irrespective of their form or quantity, antimicrobial agents typically require water for activity. Totally dry samples are virtually incapable of releasing their energy stored in chemical bonds to initiate interaction. Hydrophilicity and hydrophobicity are conceptions also based upon water ambience, upon which the manner of antimicrobial interaction of chitosan is determined. The hydrophilic characteristics of chitosan profoundly determine water solubility. The use of chitosan is limited by the compound's poor solubility in water (Dutta et al. 2004). Chemical modifications as an approach are efficient in improving the water solubility of chitosan and its derivatives and widening their applications (Xie et al. 2007). The creation of water-soluble chitosan and its derivatives has been a central goal of investigations of antimicrobial activity, which have included saccharization, alkylation, acylation, quaternization and metallization. As one example, quaternary ammonium chitosan can be prepared by introducing quaternary ammonium group on dissociative hydroxyl or amino group. For chitosan and its derivatives, the hydrophilic-lipophilic variation influences the antimicrobial properties. The hydrophobic characteristic of N-acylated chitosan can be favourable for the interaction of polymer molecule and bacterial cell, where the hydrophobicity of NHCS0.5 (N-hexanoyl chitosans, corresponding to a molar ratio of 0.5 compared with chitosan residue) is likely to

be a contributing factor for its enhanced inhibitory effect (Hu et al. 2007a, b). In another study, the presence of a long aliphatic chain facilitated the absorption and enhanced the effect of a substituted LMW chitosan, N−/2(3)-(dodec-2enyl) succinoyl/chitosans, onto cell walls via hydrophobic interaction with cell wall proteins (Kong et al. 2010).

15.4.5 Chelating Capacity

Chitosan possesses high chelating capacity for various metal ions (including Ni2+, Zn2+, Co2+, Fe2+, Mg2+ and Cu2+) in acid conditions, and it has been widely applied for the removal or recovery of metal ions in different industries (Kurita 1998). Metal ions that combine with cell wall molecules of microorganism are crucial for stability of the cell wall. Chitosan-mediated chelation of such metal ions has often been implicated as a possible mode of antimicrobial action (Rabea et al. 2003). Not only does chelation play a part in acid condition, it is also able to combine divalent metal ions in neutral condition. Additionally, via chelating capacity, chitosan metal complex is prepared and exerts strong antimicrobial activity (Kong et al. 2010).

15.4.6 pH

The antimicrobial activity of chitosan is pH dependent. Because chitosan is soluble in an acidic environment, and the molecule becomes polycationic as pH below the molecule's pKa (6.3–6.5) (Lim and Hudson 2004). It has been reported that chitosan displayed antibacterial activity only in an acid environment, as is not proven to be strictly correct. Chitosan definitely shows stronger inhibitory effect at lower pHs, with inhibitory activity weakening with increasing pH. The failure of chitosan to remain bactericidal at pH 7 may be due to the presence of a large majority of positively uncharged amino groups as well as poor solubility of chitosan (Sudarshan et al. 1992). However, chitosan and its derivatives completely lose their antimicrobial activities under neutral condition as reported by some workers which may not be totally correct. A novel approach of antibacterial research, chitosan microsphere (CM) in solid dispersing system, showed that CM sample with DD of 62.6% exerted inhibitory effect uniquely among the three DD (97.5, 83.5, 62.6%) under neutral condition (Kong et al. 2008). The CM samples in this experiment retained the properties of native chitosan without alteration. Another research observed that antibacterial activity of the N-alkylated chitosan derivatives (DS 30–40%) against *E. coli* increased as the pH increased from 5.0 and reached a maximum around the pH of 7.0–7.5 (Yang et al. 2005). These results also verify that positive charge on the amino groups is not the sole factor resulting in antimicrobial activities. However, little is known about the antimicrobial activity of chitosan under alkaline conditions (Kong et al. 2010).

15.4.7 Ionic Strength

Alternation of the ionic strength in a medium may disturb the inhibitory activity of chitosan, probably caused by two mechanisms. First, increase of metal ions, especially divalent ions, could attenuate the effective chelating capacity of chitosan. With the addition of 0.05 mol/L magnesium ions into a medium, the inhibitory ratio of chitosan samples decreased badly and resulted in abrogated antibacterial activity (Kong et al. 2008). In another study, 10 and 25 mM concentrations of divalent cations reduced the antibacterial activity of shrimp chitosan against *E. coli* in the order of Ba. Furthermore, the addition of Zn2+ and Ca2+ ions inhibited the antibacterial activity of chitosan most effectively compared with Ba2+, Ca2+, Mg2 + 2+ and Mg ions (Chung et al. 2005). Secondly, along with polycationic chitosan, existing cations in medium may interact competitively with the negative components dominating on the cell wall of bacterium, consequently weakening the antimicrobial activity. Addition of anion affected the antibacterial efficacy as well (Kong et al. 2010).

15.4.8 Physical State

Antimicrobial activity of chitosan is the result of series of reactions, rather than the cause of the reactions. The reactions take place between molecules of chitosan and cell wall. Morphology of molecules is responsible for the reactions effciency. Equally, physical state of chitosan, upon which the existing morphology of molecules depends, acts a decisive role in its antimicrobial activity. Nonetheless, scant focus has been paid to the influence of different physical state (Kong et al. 2010).

15.4.8.1 Antimicrobial Activity in Soluble State

Soluble chitosan existing as a disassociating form in solution has an extending conformation, which enables reaction with the counterparts to a sufficient degree and brings the potential to full play. This explains why soluble chitosan and its derivatives are more effective in inhibiting bacterial growth. According to the literatures (Chung et al. 2005; Xie et al. 2007), the minimal inhibitory concentration (MIC) of chitosan derivatives is significantly decreased against all tested bacteria than those of native chitosan. Meanwhile, owing to a sufficient touch with solution, soluble chitosan and its derivatives are readily affected by outer environmental factors as well as many intrinsic factors. In one study, chitosan derivatives (chitosan and maltose, glucose, fructose, glucosamine) produced through the Maillard reaction enhanced the solubility of the native chitosan. Among them, chitosan-glucosamine derivative appeared to be more effective than other chitosan or chitosan derivatives as a natural bactericidal agent (Chung et al. 2005). Quaternary ammonium chitosan is another major example to improve solubility of chitosan by introducing hydrophilic groups into molecule. After quaternization, derivatives exhibited better water solubility and stronger antibacterial activity as compared to chitosan (Xie et al. 2007; Kong et al. 2010).

15.4.8.2 Antimicrobial Activity in Solid State

Compared with soluble chitosan, rather than the extending conformation contact to solution, solid chitosan only gets into touch with solution through surface, such as fibres, membrane, hydrogels, microspheres and nanoparticles. Hydrogels can be formed by covalently cross-linking chitosan with itself. Recently, many attempts have been made to create chitosan particulate systems that could form dispersion in solution with considerable reactive surface area. The shift of physical state is sure to bring variation of its antimicrobial efficiency. Nanoparticles have less inhibition effect on *S. aureus* ATCC 29737 than the polymers in free soluble form since nanoparticles have less positive charge available to bind to the negative bacterial cell wall (Sadeghi et al. 2008). Conversely, another research reported that chitosan nanoparticles exhibit higher antibacterial activity than chitosan on account of the special character of the nanoparticles, likely the nanoparticle's larger surface area and higher affinity with bacterial cells, which yields a quantum-size effect (Kong et al. 2010).

15.4.9 Temperature and Time

For commercial applications, it would be practical to prepare chitosan solutions in bulk and to store them for further use. During storage, specific characteristics of chitosan, viscosity or Mw might be altered. Therefore, altered viscosity of a chitosan solution must be monitored since it may influence other functional properties of the solution. Stability of chitosan (Mw of 2025 and 1110 kDa) solutions and their antibacterial activity against Gram-positive (*Listeria monocytogenes* and *S. aureus*) and Gram-negative (*Salmonella enteritidis* and *E. coli*) bacteria were investigated at 4 °C and 25 °C after 15-week storage (No et al. 2006). Generally, chitosan solutions before storage showed higher antibacterial activity than those after 15-week storage. Chitosan solutions stored at 25 °C possessed parallel or weaker antibacterial activity compared with those at 4 °C. In one study, the susceptibility of *E. coli* to chitosan increased upon increasing temperature from 4 to 37 °C (Tsai and Su 1999), suggesting the low temperature stress was capable of changing the cell surface structure in a way that decreased the number of surface binding sites (or electronegativity) for chitosan derivatives (Kong et al. 2010).

15.4.10 Microbial Factors

15.4.10.1 Microbial Species

Although owning a broad spectrum of antimicrobial activity, chitosan exhibits differing inhibitory efficiency against different fungi, Gram-positive and Gram-negative bacteria. Chitosan exerts an antifungal effect by suppressing sporulation and spore germination (Hernandez-Lauzardo et al. 2008). In contrast, the mode of antibacterial activity is a complicating process that differs between Gram-positive and Gram-negative bacteria due to different cell surface characteristics. In several studies, stronger antibacterial activity was apparent against Gram-negative bacteria

than Gram-positive bacteria (Chung et al. 2004; No et al. 2002), while in another study Gram-positive bacteria were more susceptible, perhaps as a consequence of the Gram-negative outer membrane barrier. Still many workers demonstrated that there were no significant differences observed between the antibacterial activities and the bacterium. Various initial reaction materials and conditions contribute to the diverse consequences. Based on the available evidences, bacteria appear to be generally less sensitive to the antimicrobial action of chitosan than fungi. The antifungal activity of chitosan is greater at lower pH values (Roller and Covill 1999; Kong et al. 2010).

15.4.10.2 Part of Microorganism

Gram-negative bacteria possess an outer membrane (OM) that contains lipopolysaccharide (LPS), which provides the bacterium with a hydrophilic surface. The lipid components and the inner core of the LPS molecules contain anionic groups (phosphate, carboxyl), which contribute to the stability of the LPS layer through electrostatic interactions with divalent cations, demonstrated in Fig. 15.3 (Helander et al. 1997). Removal of these cations by chelating agents such as ethylenediaminetetraacetic acid results in destabilization of the OM through the release of LPS molecules. The OM serves as a penetration barrier against macromolecules and hydrophobic compounds; thus Gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs. Therefore, overcoming the OM is a prerequisite for any material to exert bactericidal activity towards Gram-negative bacteria (Kong et al. 2008).

The cell wall of Gram-positive bacteria comprises peptidoglycan (PG) and teichoic acid (TA) shown in Fig. 15.4. TA is an essential polyanionic polymer of the cell wall of Gram-positive bacteria, traversing the wall to contact with the PG layer. They can be either covalently linked to N-acetylmuramic acid of the peptidoglycan layer (wall teichoic acids) or anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid (lipoteichoic acids, LTA) (Raafat et al. 2008). Poly

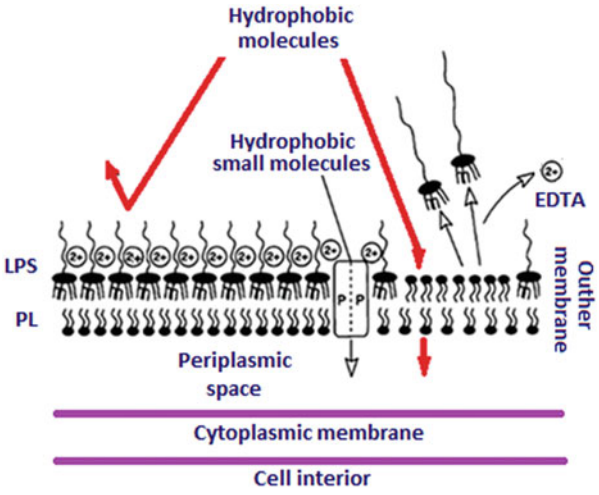


Fig. 15.3 Schematic view of the Gram-negative bacterial cell envelope. Data is based on Helander et al. (1997) and Kong et al. (2010)

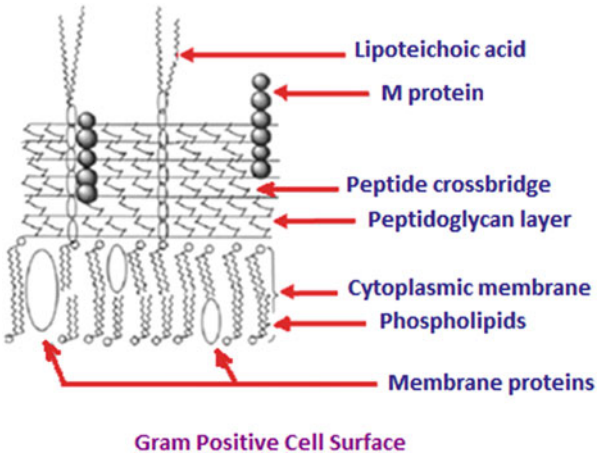


Fig. 15.4 Schematic view of Gram-positive bacterial cell wall (Kong et al. 2010)

(glycerol phosphate) anion groups make TA responsible for structural stability of cell wall. Besides, it is crucial for the function of various membrane-bound enzymes. Comparatively, TA's counterpart LPS acts similarly in the cell wall of Gram-negative bacteria (Kong et al. 2010).

15.4.10.3 Cell Age

For a given microbial species, age of the cell can influence antimicrobial efficiency. For example, *S. aureus* CCRC 12657 in late exponential phase are most susceptible to lactose chitosan derivative with no viability evident after 10 h of incubation. Meanwhile, a relatively less population reduction in viable cells of 3.75 and 3.96 log cfu/mL, respectively, was observed with cells in the mid-exponential and late stationary phases (Chen and Chou 2005).

It is suggested that the differences of cell surface electronic negativity vary with the phase of growth, which can lead to the differences in the susceptibility of cells towards chitosan. In contrast, *E. coli* O157:H7 in mid-exponential phase were the most susceptible, while stationary phase cells were the least susceptible to maltose chitosan derivative (Yang et al. 2007). The discrepancies were attributable to the different microorganisms examined, since the surface charge of microbial cells also varied with the microorganism (Kong et al. 2010).

15.5 Complexes of Chitosan with Certain Materials

In order to improve antimicrobial activity, complexes of chitosan with certain materials can be prepared. Incorporation of essential oils (EOs) in chitosan-based coatings has gained interest in the agricultural sciences owing to the bactericidal and fungicidal properties associated with these volatile compounds (Ali et al. 2015). Recently, different EOs, such as clove, carvacrol, oregano and lemongrass, have been successfully incorporated into chitosan showing strong antimicrobial activity

against a wide range of microorganisms. Also Ojagh et al. (Ojagh et al. 2010) showed that a unique compatibility can be achieved between chitosan and cinnamon EOs; their incorporation improved the antibacterial properties of chitosan. Films containing cinnamon EOs are useful for coating of highly perishable foods such as fish and poultry. In a further experiment by Gómez-Estaca et al. (2010), a complex of gelatin-chitosan film incorporating clove EOs was applied to fish during chilled storage. Results of this study revealed that clove film delayed or even prevented both the growth of microorganisms and the occurrence of total volatile nitrogen. Therefore film incorporating clove EOs could assure an extended shelf-life for chill-stored fish. Generally, the structure of chitosan/metal complexes depends on chitosan/metal ion molar ratio, type of metal ion, molecular weight and deacetylation of chitosan as well as the preparation conditions (Hosseinnejad and Jafari 2016).

15.5.1 Antimicrobial Activity of Chitosan Nanoparticles Loaded with Antibiotics or Other Microbicidal Substances

Chitosan was employed as nanocarrier for the delivery of both synthetic and natural substances in order to potentiate or modulate their antimicrobial activity. These substances depicted in Fig. 15.5 include antibiotics, antimicrobial peptides (AMP), natural compounds and proteins. In particular, chitosan nanoparticles were employed to improve the internalization of the antibiotics into cells infected by intracellular bacteria or to increase their efficacy against multiresistant microorganism. Zaki et al. demonstrated the cellular uptake of ceftriaxone sodium encapsulated in chitosan nanoparticles in Caco-2 and J774.2 (macrophages) cells and the higher intracellular antibacterial effect of these nanoparticles against *S. typhimurium* compared to the drug in solution (Zaki and Hafez 2012). A similar study was also performed using tetracycline-loaded O-carboxymethyl chitosan nanoparticles, and, in this case, the drug-loaded chitosan nanoparticles were found to enhance the efficacy of the antibiotics against intracellular infections caused by *S. aureus* (Maya et al. 2012). Jamil et al. evaluated the efficacy of cefazolin-loaded chitosan nanoparticles against multiresistant Gram-negative bacteria such as *E. coli*, *K. pneumoniae* and *P. aeruginosa*. The drug-loaded chitosan nanoparticles showed antibacterial activity against the three microorganisms, as determined by agar well discussion method and microdilution broth assay, greater than cefazolin in solution. Similarly, the efficacy of drug-loaded chitosan nanoparticles against antibiotic-resistant bacterial strains was also demonstrated for vancomycin against drug-resistant *S. aureus*. Recently, peptides and proteins with antimicrobial activity were encapsulated in chitosan nanoparticles. Among these molecules, lysozyme has received attention for its application as preservative in food products and pharmaceuticals (Wu et al. 2017), while the amphiphilic peptide temporin B has shown a strong and fast killing ability, especially against Gram-positive, multidrug-resistant nosocomial bacterial species (Mangoni et al. 2008).

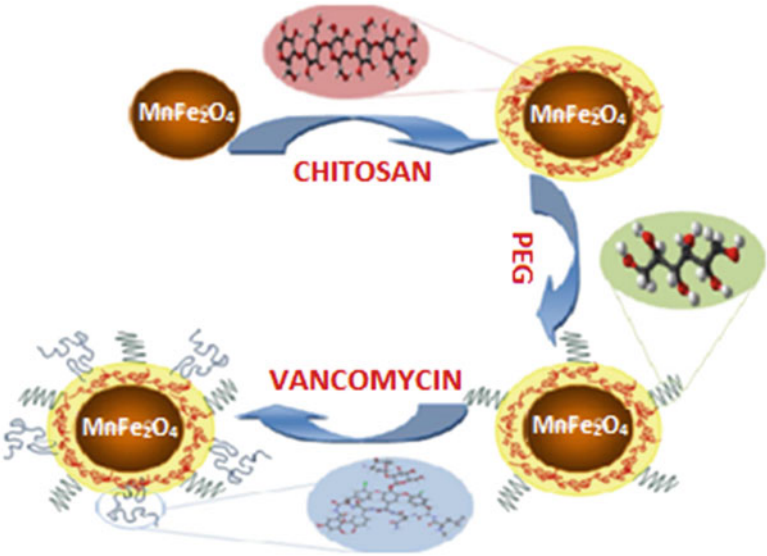


Fig. 15.5 Architecture of mixed chitosan/metals/antibiotic nanoparticles effective against Gram-negative bacteria. (Reprinted from Esmaeili and Ghobadianpour (2016) and Mangoni et al. (2008))

15.5.2 Antimicrobial Activity of Chitosan/Metal Nanocomposites

The combined antimicrobial effect of chitosan and metals was explored to prepare novel nanocomposite materials with improved microbicidal properties (Esmaeili and Ghobadianpour 2016). In particular, a broad spectrum of activities against both Gram-positive and Gram-negative bacteria were demonstrated for gold-, silver- or copper-loaded chitosan nanoparticles. Such nanoparticles were prepared by adding metal ion solutions into chitosan nanosuspension or by reducing a soluble salt of the metal in the presence of chitosan solutions (Rhim et al. 2006). The antibacterial activities by disk diffusion method were evaluated for nanoparticulate colloidal dispersion or in form of a thin film, in which the metal nanoparticles remained embedded inside the chitosan polymeric matrix. In all cases, a remarkable antimicrobial effect was observed for all the examined bacteria (*S. aureus*, *L. monocytogenes*, *E. coli* and *S. typhimurium*) with inhibition diameters ranging from 8 to 10 mm. Silver-based nanocomposites are the most frequently investigated metal/chitosan complexes. In order to investigate if the greater antibacterial effect of the silver-based chitosan composite material was exerted by the presence of silver as metallic nanoparticles or ions, Kumar-Krishnan et al. prepared chitosan films containing Ag nanoparticles or Ag + ions at different concentrations and tested them for their activity against *S. aureus* and *E. coli*. They found that the maximum bactericidal effect of the chitosan films was obtained with those containing 1% w/w of silver nanoparticles or 2% w/w of silver ions, concluding that Ag/chitosan nanoparticles have higher antibacterial effect than Ag + 2+ and Zn ions. The

maximum 2+ antibacterial effect was observed at a concentration close of that of the electrical percolation threshold (Kumar-Krishnan et al. 2015). It was also proposed that nanoparticles size could affect the antimicrobial effect. Indeed, smaller silver nanoparticles have a higher specific surface area and release Ag + ions at a faster rate (Perinelli et al. 2018).

15.5.3 Antimicrobial Activity of Chitosan Nanoparticles on Bacterial Biofilm

Biofilms are microbial communities embedded in a matrix of slimy extracellular polymers. Microorganisms in biofilms are significantly more resistant to antimicrobial agents. Natural biological molecules are currently being evaluated for their anti-biofilm activity in order to develop alternative preventive or therapeutic rationale. In this context, chitosan-streptomycin conjugates/gold nanoparticles were evaluated in terms of their anti-biofilm properties against Gram-negative *P. aeruginosa* and *S. typhimurium* and Gram-positive *Listeria monocytogenes* and *S. aureus* (Mu et al. 2016). In particular, the chitosan-streptomycin gold nanoparticles showed a disruption effect on biofilms formed by Gram-negative or Gram-positive bacteria and an inhibition effect on biofilm formation of Gram-negative bacteria. The conjugation of streptomycin to chitosan and gold nanoparticles facilitated its penetration into the biofilm matrix and improved the contact with the bacterial surface, thereby enhancing its bactericidal effect. Another application of chitosan nanoparticles is represented by photodynamic activation. Darabpour et al. investigated the effect of chitosan nanoparticles on the efficiency of methylene blue (MB)-mediated antimicrobial photodynamic inactivation (APDI) of *S. aureus* and *P. aeruginosa* biofilms. The authors observed that chitosan nanoparticles enhanced the efficacy of MB-APDI, causing the disruption of biofilm structure and subsequently a deeper and effective penetration of MB into *S. aureus* and *P. aeruginosa* biofilms (Darabpour et al. 2016). The use of chitosan nanoparticles has also been reported to combat biofilm formed by oral pathogens on tooth surfaces, which are associated with human caries, gingivitis and periodontitis. Chavez de Paz et al. investigated the antimicrobial activity of chitosan nanoparticles of different DA and MW on *S. mutans* biofilm. The low chitosan MW formulations (up to 150 kDa) disturbed the cell membrane integrity of *S. mutans* in a homogenous manner across the entire biofilm and the chitosan particles directly interacted with bacterial cell (> 95% of damaged cells) (Chávez de Paz et al. 2011) (Perinelli et al. 2018).

15.6 Applications of the Antimicrobial Activity of Chitosan-Based Nanosystems

Due to versatility, biocompatibility and biodegradability of chitosan, chitosan-based nanosystems have attracted a large interest in the last years especially for the formulation of mixed systems with improved properties. The antimicrobial activity

of chitosan has been exploited for a wide range of applications, ranging from agriculture to biomedical area (Perinelli et al. 2018). An ideal antimicrobial material should possess the following characteristics: (1) easily and inexpensively synthesized, (2) stable in long-term usage and storage at the temperature of its intended application, (3) not soluble in water for a water-disinfection application, (4) does not decompose to and/or emit toxic products, (5) should not be toxic or irritating to those who are handling it, (6) can be regenerated upon loss of activity, and (7) biocidal to a broad spectrum of pathogenic microorganisms in brief times of contact (Kenawy et al. 2007) (Kong et al. 2010). The following sections introduce the advances of chitosan-based nanomaterials in wound healing, textiles and food packaging fields.

15.6.1 Wound Healing

Skin wound treatment is an important research area. Poor wound management could lead to severe complications and loss of function. Wound healing is a complex process where numerous steps take place in order to re-establish the normal functionality of the skin. It includes an inflammatory, a proliferation and, finally, a remodelling phase. Many factors have to be considered when designing a wound material to provide an adequately moist environment and allow gas exchange in order to avoid dehydration and exudates accumulation. Moreover, wound-related infections represent a serious problem since bacteria can easily invade the tissues and proliferate, hampering the regeneration process. Hence, agents that are able to prevent infection and promote wound healing have been extensively explored. Chitosan has been widely applied as wound dressing due to its properties that include biocompatibility, biodegradability, haemostatic and antibacterial activities (Siafaka et al. 2016). As such, chitosan has been approved in commercial medical devices for topical applications in wound healing (e.g. HemCon bandages). Moreover, the gradual depolymerization of chitosan to N-acetyl glucosamine promotes fibroblast proliferation, thereby accelerating wound closure. In another study, a nanofibrous membrane made of chitosan and silk fibroin was fabricated. Its antibacterial activity against Gram-negative *Escherichia coli* was demonstrated to be directly dependent on the chitosan concentration in the composite nano-dressing, with a higher effect increasing chitosan proportion (Cai et al. 2010). A slightly different result was obtained by Sarhan and Azzazy that studied the combined antibacterial activity of honey, chitosan and polyvinyl alcohol electrospun nanofibrous wound dressing. These nanofibres showed a pronounced antibacterial effect against *S. aureus*, with an increased effect on chitosan concentration, while a weak antibacterial activity was observed against *E. coli* (Sarhan and Azzazy 2015). Recent studies reported the loading of different compounds such as antibiotics, antimicrobial agents and metal nanoparticles within the chitosan nanofibres in order to increase chitosan antibacterial activity and accelerate the wound-healing process (Perinelli et al. 2018).

15.6.2 Textile and Fabrics

Chitosan has been proposed to act as an antimicrobial agent in fabrics or textile in order to prevent microbial growth. In fact, textiles, especially those made of natural fibres such as proteins (silk) or cellulose (cotton), represent a favourable environment for the proliferation of different microorganisms including bacteria or fungi, due to extensive surface area, high porosity and ability to retain humidity. The research in the field of antimicrobial textiles has a great impact on many technological applications such as clothing, furnishing, filtering, medical devices, healthcare and hygienic products. With the increasing interest in the use of silk, collagen or cellulose for the production of membranes and supports for biomedical applications (as regenerative medicine), there is an enhanced demand for safe and biodegradable antimicrobial agents. Chitosan is a good candidate, with attractive characteristics in comparison to other commonly used organic antimicrobial agents (e.g. phenolic and formaldehyde derivatives), especially in terms of toxicological profile. The major drawbacks regarding the use of chitosan include its poor solubility in most of the solvents except acidic aqueous solution, the high viscosity of concentrated high MW chitosan solution for coating application and the thermal stability of chitosan. Nevertheless, some commercial textile products based on chitosan are available on market. Although the commercial products have reached the market more than 10 years ago, the research on the use of chitosan in textiles still has been flourishing. Different strategies have been applied in textiles and fabrics to improve the antimicrobial activity of chitosan. Microencapsulation is one of the most explored approaches (Ibrahim et al. 2017; Perinelli et al. 2018).

15.6.3 Food Packaging

Food industry is facing the problem of microbial contamination. Foodborne bacteria are responsible for many serious human infections, and they can also accelerate food spoilage with enormous economic losses. In this regard, food packaging represents a solution to prevent and retard bacterial invasion and proliferation. Different biopolymers, characterized by a good environmental profile, biodegradability and biocompatibility, have been screened in order to find alternatives to the conventional petroleum-derived materials for food packaging. Many research focused on the development of chitosan-based systems, as the intrinsic properties of chitosan could enhance the antimicrobial efficacy of the packaging. The film-forming properties of chitosan have led to the development of film packaging materials in combination with various natural polysaccharides such as starch, pectin and hydroxypropyl methylcellulose (HPMC). An example was given by Möller et al., who studied the antimicrobial activity of a chitosan-HPMC film against *Listeria monocytogenes*, demonstrating a complete growth inhibition. When chitosan-HPMC films were cross-linked with citric acid by the amino groups of chitosan, the antibacterial activity decreased drastically, thus demonstrating the critical role of the protonated amino groups of chitosan for the antimicrobial activity (Möller et al.

2004). Chitosan nanofibres have also been exploited as a packaging material by many research groups due to their numerous advantages such as biocompatibility, large surface area and good functional and antimicrobial properties. Nanocarrier systems have attracted an increasing attention in food packaging, being able to load different active compounds, including those with low solubility and stability. Chitosan nanoparticles have been intensively exploited for this purpose, and with their well-known antimicrobial properties, a potential combined effect with the loaded active compound could be achieved. As proof of concept study, Feyzioglu and Tornuk examined the antimicrobial activity of summer savoury essential oil-loaded chitosan nanoparticles against three different foodborne bacteria (*E. coli*, *L. monocytogenes* and *S. aureus*), showing the promising application in food packaging materials. Both chitosan nanoparticles and summer savoury essential oil-loaded chitosan nanoparticles displayed antibacterial activity, with the loaded nanoparticles demonstrating a higher effect (Feyzioglu and Tornuk 2016) (Perinelli et al. 2018).

15.6.4 Application in Medical Industry

In the area of healthcare and hygienic applications, biocidal polymers may be incorporated into fibres, membrane or hydrogel and used for contact disinfectants in many biomedical applications, including wound dressing, orthopaedic tissue engineering, drug delivery carrier and haemodialysis. Generally, an ideal wound dressing material must be capable of absorbing the exuded liquid from the wounded area and should permit water evaporation at a certain rate and allow no microbial transport (Yang and Lin 2004). As a key parameter regarding wound dressing, the antimicrobial property assessment is necessary for evaluating the eligibility and capability of the candidate. Polysaccharides, e.g. chitosan, owning hydrogel-forming properties have been considered to be advantageous in their application as a wound dressing materials (Chen et al. 2005). Chitosan-based materials have received much attention in this regard. Typically, there are four forms in which chitosan provides antimicrobial effect to wound dressing materials: fibre, membrane, sponge and hydrogel. The different approaches count on particular physicochemical characteristics of chitosan, which impart talent on specific displaying form. Majority of antimicrobial products perform their talent in fabric form. Micro- and nanofibre materials are suitable for preparing wound dressings. Among these, electrospinning is a favourable technique for producing continuous polymer fibres with diameters down to nanoscale range (Deitzel et al. 2001). Because of unique properties such as high surface-to-volume ratio, high porosity and diameters in the nanoscale, electrospun mats made from ultrafine polymer fibres have been drawing great attention. One study reported the cross-linked QCh/PVP (quaternized chitosan/polyvinylpyrrolidone) electrospun materials were efficient in inhibiting growth of Gram-positive bacteria and Gram-negative bacteria (Ignatova et al. 2007), while, in their previous work, the antibacterial activity of cross-linked electrospun QCh/PVA (polyvinyl alcohol) mats made of quaternized chitosan derivative against *S. aureus*

was observed to be bactericidal rather than bacteriostatic. PVP and PVA are both non-toxic, biocompatible and highly hydrophilic, possess good complexation properties and have good film-forming ability, which are crucial for wound-healing materials (Kong et al. 2010).

15.6.5 Antibacterial Coating

As it is explained above, chitosan has positively charged amino group which interacts with negatively charged microbial cell membranes. This leads to the leakage of proteinaceous and other intracellular constituents of the microorganisms. That may be one of the reasons of having antimicrobial feature of chitosan (Shahidi et al. 1999). Another reason is related to the moves of chitosan on the outer surface of bacteria. At a lower concentration (0.2 mg/mL), polycationic chitosan can be probably bound to the negatively charged bacterial surface for causing agglutination. The larger number of positive charges may impart a positive charge to the bacterial surfaces in order to keep them in suspension at a higher concentration (Dutta et al. 2009; Yılmaz Atay and Çelik 2017). In the UV absorption studies, it was detected that chitosan causes the considerable losses of proteinic material for *Pythium oaroecandrum* at pH 5.8 (H. Liu et al. 2004). Chitosan can bind tracing metals like a chelating agent and this can prevent the formation of toxic materials and the growth of microbes. By activating defensive processes in the host tissue, it can act as a water binding agent and also prevent various enzymes. Due to the penetration towards the nuclei of the microorganisms and the interference with the synthesis of mRNA and proteins, binding of chitosan with DNA and inhibition of mRNA synthesis take part (Sudarshan et al. 1992).

Some innate factors, such as structure of chitosan, its degree of polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates and the environmental conditions, can impress the antimicrobial activity of chitosan. *This will be discussed later in this chapter.* Coating materials including antimicrobial agents have been attractive areas for researchers due to preventing the growth of pathogenic bacteria. Chitosan has been still pointed out as an antimicrobial film or a forming agent because of its biodegradability, biocompatibility, cytotoxicity and antimicrobial activity (Dutta et al. 2009).

In contrast to neutral and alkaline conditions, acidic solutions lead to dye better grasping higher (Hasan et al. 2008). Yoshida et al. (1991) showed that at a lower pH, more protons are prosperous for protonation amino groups of chitosan molecules to form groups of –NH3+. Hence, it could be possible to see increasing electrostatic attractions between negatively charged dye anions and positively charged adsorption sites. Therefore, this will bring about an increase in dye adsorption. Chiou and Li (2003) presented similar explanations for the adsorption of RR 189 (reactive dye) on cross-linked chitosan beads. The adsorption was lower than acidic solution, for example, they can go down from pH 10.0 to 13.0. They expressed this action by the fact that chemical cross-linking reduces either the total number or the diameter of

the pores in chitosan beads. Thus, the transferring of the dye molecule was more difficult (Hasan et al. 2008; Yılmaz Atay and Çelik 2017).

In our study, an acrylic resin was converted to an antibacterial coating material by using chitosan. Different states of chitosan, solid state (powders) and colloid state, were inserted to the polymer matrix individually. For obtaining the powders, chitosan (poly-(D)-glucosamine, Sigma) was grounded in a grinding mill at 25 °C for 5 h in the air. The aim of the comminution is to obtain homogeneous distribution and to increase the effect of the particles by increasing contact points. Figure 15.6 shows SEM micrograph of pure chitosan.

Chitosan colloids were prepared as presented in Fig. 15.7. Different amounts of chitosan were dissolved in the acid solution. After mixing the solution thoroughly by using magnetic stirrer at 25 °C for 20 min, homogeneous chitosan colloids were obtained.

Acrylic composites were prepared by adding chitosan powders and colloids to the polymeric matrix. For the manufacture of the composites, acrylic resin (polymethyl acrylate) was used as a polymeric matrix supplied from DYO, Turkey. Chitosan powders and colloids were incorporated into the acrylic resin with different loading levels to assess the concentration dependence of material's antimicrobial effect. Glass substrates were coated with those polymeric composites. After the obtained

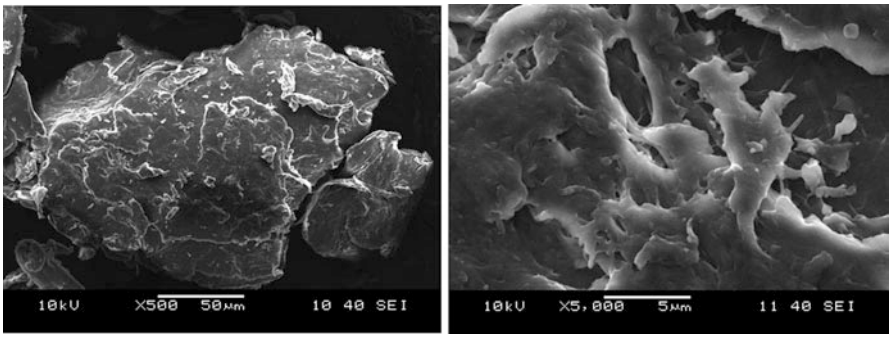


Fig. 15.6 SEM micrograph of pure chitosan

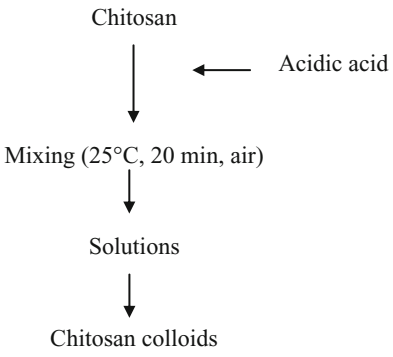


Fig. 15.7 Preparing method of chitosan colloids

Table 15.1 Description and sample codes of composite coatings

Sample codes	Chitosan	Chitosan percentage in the composites (%)
CH00	None	0
CHP1	Ground powder	1
CHP2	Ground powder	5
CHC1	Colloid	0.01
CHC2	Colloid	0.05
CHC3	Colloid	0.10

composite coatings were subsequently dried for 24 h at the room temperature in the air, no more curing process was performed. The sample codes and descriptions of coatings are indicated in Table 15.1.

SEM images of composite coatings are given in Fig. 15.8. It can be seen that chitosan particles look like sphere, not flake, and they were well blended with dye homogeneously. Smooth and rough areas could be seen in chitosan incorporated into the coatings. As characteristic property of chitosan, large crystals appeared. Rough surfaces and crystalline structures were raised as dominant features by increasing chitosan content. Concurrently, by increasing chitosan, the dissociation process occurred. In fact, the morphology of the coating samples was agreed well with this (Abdelrazek et al. 2010). Increasing with colloid amounts, formation of flocculation and cracks appeared.

Antimicrobial properties of the samples against *Staphylococcus aureus* were determined by inhibition zone test and percent decreasing test.

Inhibition Zone Test The agar disc diffusion method was employed for the determination of antimicrobial activities of chitosan-reinforced specimens with the size of 2.4 × 2.4 mm against *S. aureus* ATCC 6538P, a Gram-positive bacterium (NCCLS 1977). Briefly, test microorganisms were activated in Müller Hinton Broth (MHB) at 37 °C for 18 h, and a suspension of the test bacteria was spread on solid medium plates containing MHA. After 2 h, chitosan-reinforced polymer coatings were placed at the centre of inoculated plates and incubated at 37 °C for 24 h. At the end of the incubation period, the plates were inspected for growth on and under composite samples, as well as for the presence or absence of growth in a halo around the samples. The width of the halo was measured across the centre line of the sample, both horizontally and vertically. An average of these two values was then taken to give an estimate of the antimicrobial activity of the samples as shown in Eq. 15.1 (Fig. 15.9).

$$\text{Inhibition radius} : (r_1 + r_2)/2 \tag{15.1}$$

in which r_1 and r_2 are vertical and horizontal widths, respectively. Each experiment was repeated three times (NCCLS 1977).

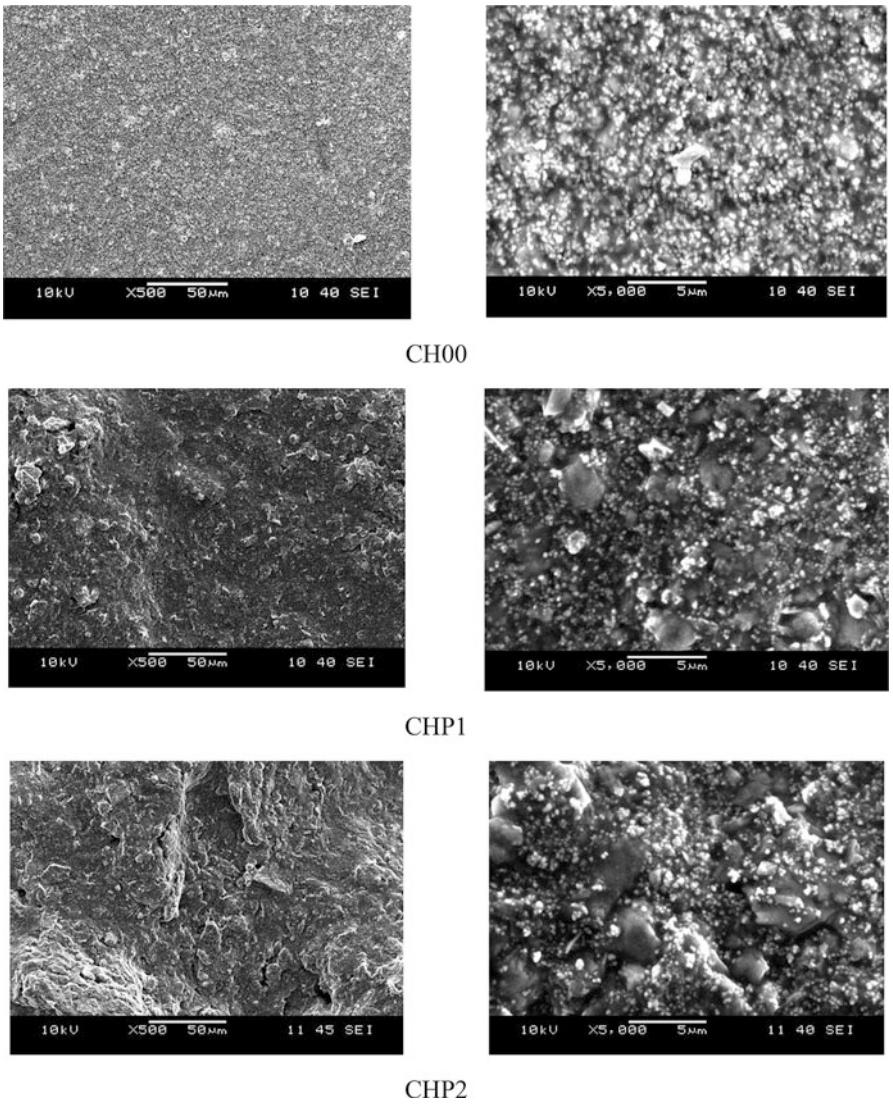
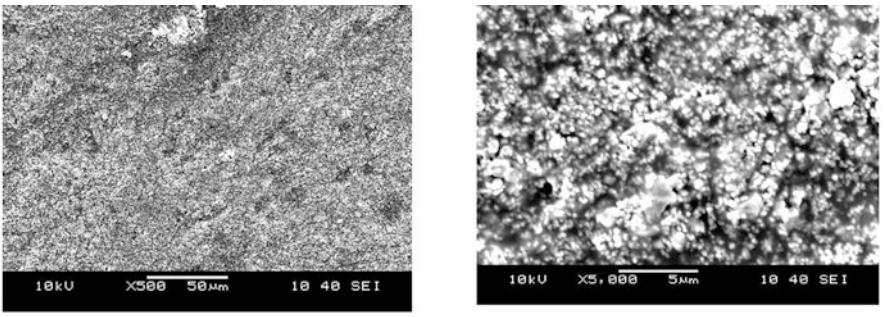
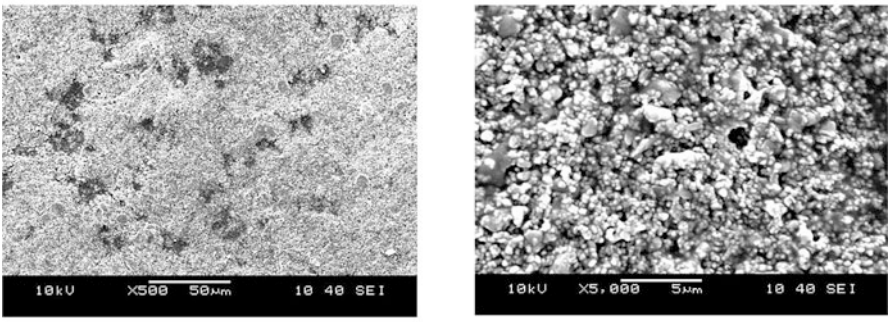


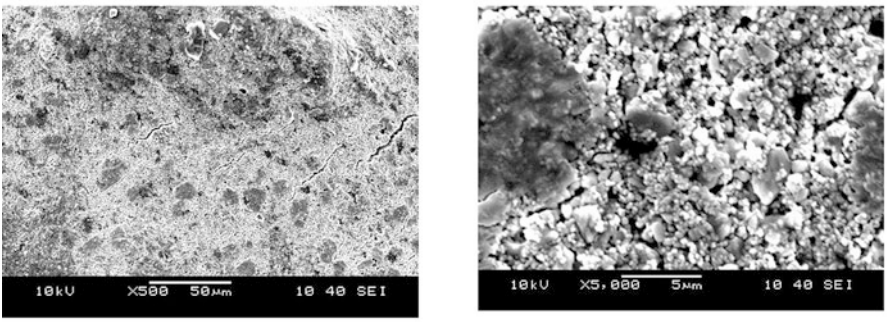
Fig. 15.8 SEM micrographs of chitosan-reinforced composites



CHC1



CHC2



CHC3

Fig. 15.8 (continued)

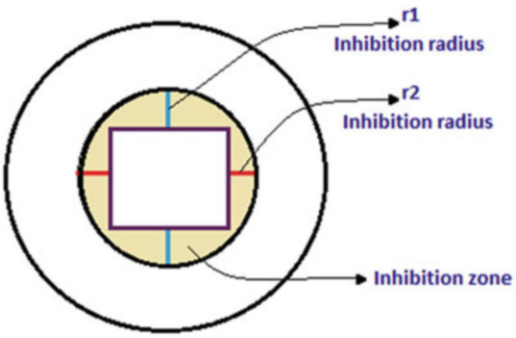


Fig. 15.9 Inhibition zone view (Yilmaz Atay et al. 2015)

Percent Decreasing Test A plate counter agar solid culture medium was poured into the plates that were subsequently incubated at 37 °C for 24 hours so that the v*ital* cells, eventually presented, could grow into colonies. The microorganisms' colonial presence was then evaluated, by counting the colony-forming units per Petri plate (CFU/mL). Used bacteria were *E. coli* (ATTC 11228). The difference between the number of the bacteria obtained at zeroth hour and the one obtained after 24 h will show the antibacterial performance (Eq. 15.2).

$$\% \text{ decrease} = \left[\frac{A - B}{A}\right] \times 100 \tag{15.2}$$

where A is the number of bacteria at zeroth hour and B is the number of bacteria after 24 h.

For the mentioned antibacterial tests, coated samples and agar discs are shown in Fig. 15.10 after the antibacterial tests. In the test sample, there is not any antibacterial activity. The inhibition zones around those specimens can be clearly seen in chitosan colloid-reinforced coatings. Increasing the loading level of the chitosan also increases the inhibition radius in those samples. Regarding the samples including chitosan powder, antimicrobial effects are seen on the surface of the coated region. It means the antimicrobial property works with direct contact of chitosan powders on the surface. To obtain the better results, particle size can be decreased to nanoscale if possible, as by this way the antibacterial effect of the particles can be increased due to increasing of contact surfaces. However, some researchers observed that nanoparticles can have less inhibition effect on *S. aureus* ATCC 29737 than in free soluble form polymers. The reason is that nanoparticles have less positive charge for binding to the negative bacterial cell wall (Sadeghi et al. 2008). On the contrary, in another research, it was recorded that due to the special character of the nanoparticles, the chitosan nanoparticles can present higher antibacterial activity against *S. aureus* (Qi et al. 2004). Similarly, larger surface area of the nanoparticle and affinity with bacterial cells, which yields a quantum-size effect, has influence in the antibacterial action (Kong et al. 2010).

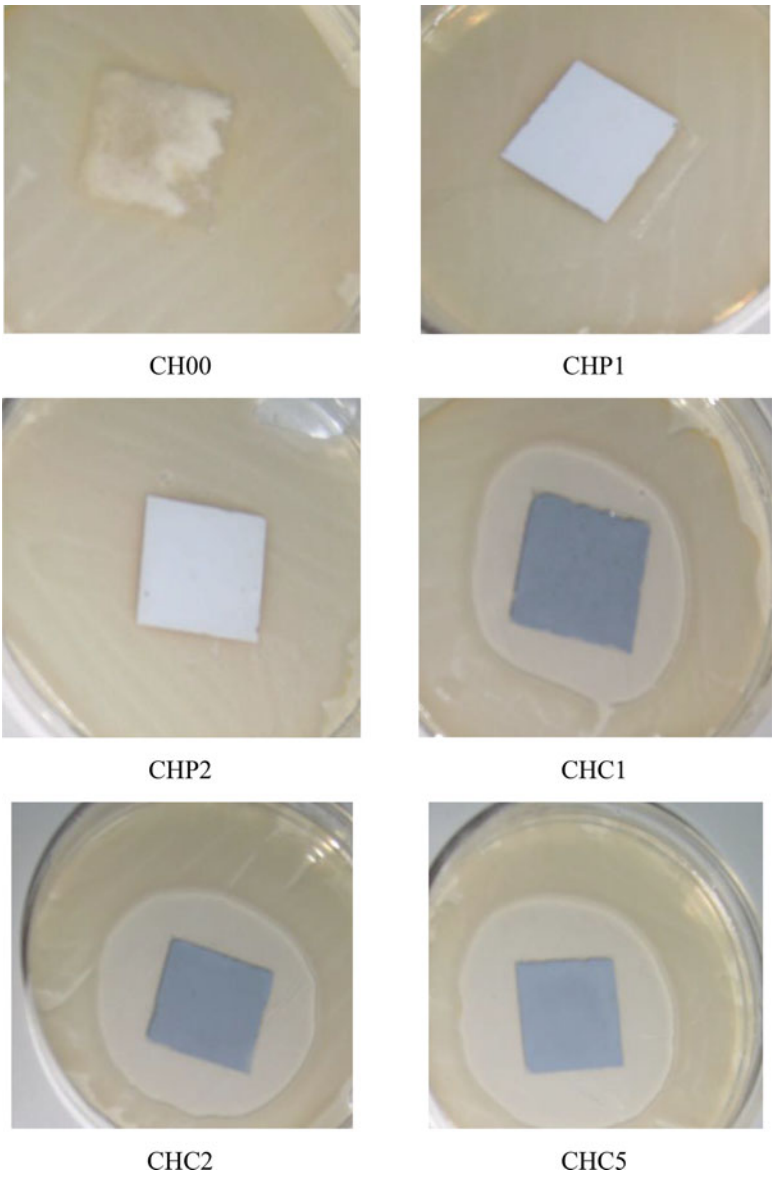


Fig. 15.10 Antibacterial test results: photographs of agar plates (Yılmaz Atay and Çelik 2017)

In addition, the prohibition activity of chitosan depends on different types of factors, such as solid surface characteristic and the morphology. Particle size, membrane and fibre thickness cause to occur different results. It was investigated the effect of particle size and shape of chitosan powder on *S. aureus*, and it was recorded that antibacterial activity was improved by decreasing the particle size. Conversely, the antibacterial property of chitosan

powders depends on the shape as well as specific surface area. The researchers showed that chitosan powders in the range of 74–500 μm looked like a flake or board, but they looked like spheres in the range of 37–63 μm (Kong et al. 2010; Phaechamud 2008; Yılmaz Atay and Çelik 2017).

Soluble chitosan and its derivatives are more efficient for preventing bacterial growth because soluble chitosan allows reaction with the counterparts to a sufficient degree by existing as a disassociating form in solution and an extending confirmation. Solid chitosan only gets into touch with solution through surface, such as fibres, membrane, hydrogels, microspheres and nanoparticles. However, by extending conformation contact to solution, hydrogels can be formed by covalently cross-linking chitosan with itself. Chitosan particulate systems can form dispersion in solution with the considerable reactive surface area (Kong et al. 2010).

Chitosan-reinforced composite samples were subjected to another type of an antibacterial test called "percent decreasing test". The initial number of bacteria was 2000, and they were counted again after 24 h. The results are shown in Table 15.2. It can be expressed that chitosan-reinforced coatings showed antibacterial property. The results support inhibition test results. As mentioned above, colloid chitosan demonstrated much better antimicrobial activity in the composite compared with powder chitosan-reinforced composites.

In this study, the antibacterial behaviour and effectiveness of solid and colloid chitosan in a polymer matrix were investigated. Chitosan can be considered as an effective antibacterial additive. Increasing loading level of the chitosan colloids in the polymer composites increased inhibition zone. Colloid chitosan demonstrated much better antimicrobial activity in the composites compared with powder chitosan-reinforced composites.

15.7 Conclusions and Future Perspectives

Considerable interest and attention have been focused on chitosan due to its potential application area and its unique advantages over the last decades. Investigations on its antimicrobial property are growing rapidly. Due to their multitude of application areas and people's environmental mindfulness, biodegradable, and non-toxic products from 'natural' sources are going to be more and more appealing for the replacement of synthetic compounds. Against other antibacterial materials, chitosan is a non-toxic, harmless and environmentally friendly vegetable material. Using this

Table 15.2 Decreasing test results of the chitosan-reinforced composite samples (Yılmaz Atay and Çelik 2017)

Code	Bacteria quantity after 24 h
CH00	1650
CHP1	1345
CHP2	1056
CHC1	0
CHC2	0
CHC3	0

material at the points of our life will be more healthful for people. Therefore, the investigations of this material for the antibacterial studies need to be improved which will incorporate a combination of disciplines involving chemistry, physics, informatics, nanotechnology and genetic engineering. This will be beneficial for the exploitation of new generation of antimicrobial agents and for the development of new biomedicine. On the other hand, future works can focus on the use of chitosan in the composites to avoid lower pH values, as soluble chitosan is used as a generally acidic environment. It will be beneficial to clarify the molecular circumstance of the underlying mechanisms and their involvement to the antimicrobial action of chitosan.

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